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Associations Among Beef Cattle Genotypes, Neospora Caninum Infection, and Reproductive Performance

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Associations Among Beef Cattle Genotypes, *Neospora caninum* Infection, and Reproductive Performance

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by

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Abstract

Reproductive performance is crucial for sustained financial success in the beef cattle industry. This dissertation includes a population study that quantified the incidence of *Neospora caninum* infections in the central region of the United States and tested its relationship with reproductive performance in beef cattle. Trial one of that study concluded that 6.9% of open, replacement heifers (n = 1306) tested seropositive. The second trial in that project found that 9.6% of the breeding age females (n = 500) tested were seropositive for *Neospora caninum*; and that state in which the cattle lived and age impacted ($P < 0.05$) infection rate. Breed composition, number of farm dogs on the ranch, and use of total mixed rations were not associated ($P > 0.1$) with seropositive tests. Fewer ($P < 0.05$) seropositive females were pregnant in Oklahoma, but overall infection rate was not associated ($P > 0.1$) with non-pregnant females. The second study, tested for a relationship between heat shock protein 70 (*Hsp*70) genotypes and reproductive characteristics. Blood samples were collected from beef heifers (n = 165) being developed for replacements and *Hsp*70 genotypes (A1125C, C895D, G1851A, G2033C) were determined using a commercial laboratory (Neogen Corporation; Lincoln, NE). There was an association ($P = 0.04$) between C895D genotype and the percentage of lymphocytes in circulation. Pelvic area, reproductive tract score, pregnancy rate, and white blood cell concentrations were affected by ($P < 0.05$) genotype at G2033C. Cows with heterozygous genotype for G2033C had a lower ($P = 0.02$) pregnancy rate; conversely, those same cows had larger ($P = 0.02$) PA. No associations were detected between A1125C or G1851A and the traits evaluated. While more research is needed in this area, these projects indicate that identifying *Neospora caninum* infected cattle and *Hsp*70 genotypes could be useful tools for selecting beef cattle.

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Introduction

Reproductive efficiency is one of a few ways to increase profit in beef cattle operations. Infertility and reproductive failure account for billions of dollars in losses each year (Prevatt et al., 2009). Stress is one of many factors that influence reproductive performance in beef cattle, and it can present itself in many forms including nutritional, physical, and environmental affects. Each of those stressors can inhibit normal cellular functions that are crucial for peak performance. Therefore, livestock producers spend precious time and money attempting to eliminate environmental stressors that may be detrimental to production characteristics. Two of the more difficult forms of stress to manage are heat and health stress. In the United States livestock industry, alone, economic losses due to heat stress were once estimated to surpass $2.4 billion annually (St-Pierre et al., 2003). Over half of that dollar figure was accounted for by the cattle industry (St-Pierre et al., 2003). Even though these figures are startling, they still do not account for other forms of stress that would likely make this dollar figure much more substantial. While it is possible to mitigate the effects of heat stress by offering shade and misters, most breeders who struggle with this issue choose to select more heat tolerant breeds and coat colors. In addition to heat stress, health stress can also have substantial impacts on reproductive performance. Even though some reproductive diseases can be managed through vaccines, not all common diseases have effective vaccines available. A good example of this would be *Neospora caninum*. In this instance, testing and culling may be the most efficient way to manage these issues. Because controlling the weather is yet to be conquered by mankind and diseases are difficult to completely eliminate, further research is needed to create management strategies and selection tools that will help producers select the proper animals that will reproduce in these circumstances.

Literature Review

Neospora Caninum

Neospora caninum is an infectious parasitic protozoon that can be detrimental to multiple animal species, including bovine animals (Dubey, 2003). It can lead to major economic losses in the livestock industry and appears to be a growing concern among cattle producers. In fact, it has been reported to be as high as a 90% infection rate in some herds (Dubey, 2003). It seems to be most widely recognized for its link to abortions because it directly impacts calf crops and net income (Gondim et al., 2004a). Even though abortions are a major concern there are also many indirect associations with economic losses such as decreased weight gains (Barling et al., 2001), decreased milk yield (Hernendez et al., 2001; Romero et al., 2005), weak or unhealthy offspring (Monney et al., 2011), and rebreeding and increased culling rates (Waldner et al., 1998). Because of the strong similarities it has with *Toxoplasma gondii,* it has previously been misdiagnosed. While studying canines in the 1980's, researchers discovered and described the new parasite as a coccidian and suggested the parasite was capable of causing major reproductive issues in canine animals (Bjerkas et al., 1984; Dubey et al., 1988). Soon after those discoveries, Dubey and his colleagues discovered the presence of *Neospora caninum* in canine tissue dating back to 1957 (Dubey et al., 1990), suggesting that the parasite has been an issue far before its initial discovery. At the time of the discovery, it was unclear as to what other species may be impacted by *Neospora caninum*, but since then, it has been reported in sheep, goats, cattle, buffalo, horses, deer, and many other non-livestock species including rodents, poultry, and primates (Dubey et al., 2007). Although it is not known to be zoonotic, controlled studies have shown that oral treatments of *Neospora caninum* infected monkeys (Barr et al., 1994), which raised concerns for human exposure; however, it has never been diagnosed in humans (Dubey,

2003). Not only is it important to fully understand exactly what species of animals can be susceptible to this disease, but also research has shown that the rate of infection can be highly geographical (Dubey et al., 2007). Evidence of the protozoa has been detected nearly all around the world, but aside from geographical influences, the incidence of infection also appears to be linked to many other factors.

Clinical Signs and Outcome of Infection

Clinical Signs in Cattle

Most producers do not realize they have a Neosporosis issue in their herd until they begin noticing unexplained abortions or a higher incidence of open cows during later pregnancy checks. Because many of the seropositive animals are asymptomatic and their initial breed up rates tend to be normal (Lopez-Gatius et al., 2005), they often go unnoticed. Producers who perform pregnancy detections earlier in gestation may notice they have an issue quicker because they realize they have a pregnancy loss problem rather than an issue of establishing pregnancies. Even then, it can be very difficult to diagnose because often times the fetus is never found in order to perform a Neospora test on. Even when *Neospora caninum* is properly diagnosed in the cow, it is still difficult to prove it is the true cause of the abortion because there are so many other diseases that may lead to abortions. For this reason, much of the research tends to be anecdotal. Even so, multiple studies have linked *Neospora caninum* to bovine abortions (Hassig and Gottstein, 2002; McAllister et al., 1996; Anderson et al., 1991) and the Merck Veterinary Manual along with Almeria and Lopez-Gatius (2015) have recognized it as one of the leading causes of abortions in bovine animals. Abortions caused by *Neospora caninum* generally occur around 5-6 months of gestation but range from three-month gestation to term (Dubey, 2003). The stage of gestation the female is in at the time of the first exposure to the infection is

important for the livelihood of the offspring (Williams et al., 2000). There is some disagreement on which stage the fetus is most vulnerable to being aborted. Williams et al. (2000) reported that abortions are more likely to occur if they are exposed prior to 30 week of gestation. Other research has supported this belief claiming that calves are more likely to survive if they encounter the infection once their immune system has developed (Pare et al., 1996; Wouda et al., 1998). In contrast, Lopez-Gatius et al. (2004b) reported that abortions occur at a higher rate after 90 days of gestation. To help clarify that *Neospora caninum* is linked to abortions, Anderson et al. (1991) analyzed 95 aborted fetuses over a four-and-a-half-year period and determined that the coccidian like protozoa reacted with Neospora antiserum in the brain of 88 fetuses. McAllister et al. (1996) reported an abortion rate upwards of 18% and also found mummified fetuses as a result of the infection. Another study performed in dairy cattle found that seropositive cattle had an abortion rate as high as 30.1% (Lopez-Gatius et al., 2005a). Just a short year prior to that their work concluded that abortion rates were 12.2 times more likely to abort than seronegative cows (Lopez-Gatius et al., 2004a). Domestically, abortion rates of seropositive cows that aborted in mid-late gestation, have been seen as high as 40% (Jenkins et al., 2000). There has been research that generated contrasting results compared to those previously discussed. In one dairy herd where seroconversion rates of seronegative cows reached 47% over a six-month period, they still did not see an increased abortion rate within the herd (Dijkstra et al., 2002). It could be that the abortion storm may occur in the year following. Nonetheless, if no additional abortions are detected the first year of seroconversion, it is easy to see how the seroconversion rate could go unnoticed. Contrasting results by Romero et al. (2005) reported that sero-status did not have a significant impact on reproductive performance. It is still unclear why some studies did not see the same abortion hikes as many of the others, but it is plausible to believe that there

are other factors that work in conjunction with the protozoa that may help determine the severity of symptoms. Although it has not been conclusively proven, there is some belief that season may have a role on abortion rates due to Neosporosis (Thurmond et al., 1995). In the previously mentioned study, there was variation among seasons, but it was not statistically significant. Perhaps the timing of the first exposure to the parasite in conjunction with the season matters. In other words, seasonal research performed on cows that are already seropositive may yield different results than research that is conducted on cows that are just converting to seropositive. Research has also explored the possibility of relative humidity effecting the severity of the symptoms. In what little data is available on this matter, Yaniz et al. (2010) suggested that as rainfall increased, as well as the number of days with relative humidity less than 60%, the abortion rate increased in parous cows infected with *Neospora caninum*. The same exposure to relative humidity less than 60% also increased abortion rates of infected first calf heifers (Yaniz et al., 2010). Wouda et al. (1999) agreed with the fact that relative humidity worked in favor of the parasite. They performed their study in the Netherlands during the summer months and discovered that abortion storms tended to occur when it was warm and humid. Dubey et al. (2007) offer several justifications to this observation by stating that humidity may be advantageous for sporulation and survival of the parasite, and also that it favors the growth of fungi. Fungal toxins present in the forages and feed sources may be detrimental to the immunocompetence of the animal (Dubey et al., 2007). It is interesting to entertain the possibility that factors such as season and climate could be a determining variable on how each animal responds to a *Neospora caninum* infection, but there needs to be more research focusing on this possibility.

Many other factors have been associated with the outcome of the infection. Feed quality appears to have some link to the severity of the infection. Poor quality and moldy feedstuffs seemed to be linked to epidemic abortions (Bartels et al., 1999), but it is unclear what mechanism actually hindered the cow's immunity (Dubey et al., 2007). For example, was it the toxins produced from mold, poor palatability leading to decreased consumption, or just the fact that the feed source was contaminated with an elevated oocyst load?

Bjorkman et al. (2000) explained that *Neospora caninum* and Bovine Viral Diarrhea (BVD) may have concurrent effects, but other studies have failed to generate statistical significance supporting this notion (Hassig and Gottstien, 2002; Stahl et al., 2006). They also compared *Coxiella burnetii, Chlamydia psittaci,* and *Leptospira* but were unable to conclusively link the concurrent effects.

The age of the dam and parity number may also influence the fetal survival rates in cows with Neosporosis. In herds experiencing epidemic abortion storms, the rate of abortion seemed to heighten as the number of parity increased (Wouda, 1998; Wouda et al., 1999a). Interestingly, the opposite occurred in herds experiencing endemic abortion storms (Thurmond and Hietala, 1997). This is likely due to the fact that some seropositive cows develop a latent infection and have the ability to hold the infection in check in the absence of a stressed immune system. Several studies have indicated that titer levels decrease with number of gestations, which supports that theory that cows can develop the ability to stave off the infection. Along with this, research has pointed out that naïve cattle are more likely to abort due to a *Neospora caninum* exposure than cows that are chronically infected (Williams et al., 2003).

Even though abortions garner most of the attention, there are multiple other signs that can insinuate that cattle producers may have a Neosporosis problem. If calves survive a full

gestation and are born alive, they can still be born with other defects. Barr et al. (1991) was one of the earliest to document Neospora-like encephalomyelitis in infected calves. In their manuscript, they stated that a three-day old calf had neurological damage with a porencephalic cyst in the cerebellum. In addition, that same calf had contracted tendons on the front legs, a misshaped skull, ulcerative ecophagitis, and abomastitis. According to Donahoe et al. (2015) other symptoms of congenitally infected calves include: proprioceptive deficits, hydrocephalus, and CNS lesions. Another inclination that herds may have a Neosporosis issue is if there is an elevated incidence of retained placenta. Studies are limited on this issue, but two publications have associated *Neospora caninum* induced abortions with retained placentas (Bartels et al., 1999; Hobson et al., 2005).

Clinical Signs in Other Ruminants

Several studies have shown that both sheep and goats can harbor a *Neospora caninum* infection (McAllister et al., 1996; Abo-Shehada and Abu-Halaweh, 2010; Diakou et al., 2013). In a mixed operation that had both sheep and goats, research has indicated a seropositive rate of 16.8% and 6.9%, respectively (Diakou et al., 2013). In clinically induced infections, sheep not only became seropositive, but they also had many of the same symptoms as cattle. McAllister et al. (1996) reported that if ewes were inoculated on day 65 of gestation then it resulted in an abortion. If they were inoculated on day 90 of gestation, then they either aborted or gave birth to weak lambs. If they were inoculated at 120 days of gestation, then they proceeded to give birth to clinically normal lambs. With the exception of a few studies performed domestically, the majority of the research available was performed in other countries.

Monetary Impacts

There are very few studies that have even attempted to put a number on financial losses due to *Neospora caninum* because it would be near impossible to truly evaluate all that this disease actually encompasses. With that said, one study conducted determined that in California alone, the loss estimations were approximately $35 million annually (Dubey, 1999). It is worth noting that this study only accounted for abortions and did not factor in other indirect losses associated with the infection. In another study on beef cattle located in Texas, an estimation was formulated, and expected losses ranged between $15-24 million annually (Kasari et al., 1999). Inevitably, much has changed in the industry since those two studies were performed, including production expenses and cattle prices, so the estimations may be drastically different now. A more recent study reviewed the economic global impact that *Neospora caninum* had on both the dairy and beef industry. The most startling figure was that global annual losses could be as much as $2.38 billion annually and $546.3 million of that was from the United States dairy industry alone (Reichel et al., 2013). Several studies on milk loss provide evidence that this number is quite realistic. One study stated that seropositive cows milked 3-4% less than seronegative cows which resulted in $128 less per lactation (Hernendez et al., 2001) and another reported a reduction of 84.7 liters per laction (Romero et al., 2005). Reichel et al. (2013) also reported that the dairy industry accounted for nearly two-thirds of those losses, further confirming that it is more of an economic issue for dairy producers than beef; however, this doesn't negate the fact that it can have substantial impacts on the beef industry as well. While the work from Reichel et al. (2013) provides a more recent outlook and may, in fact, be a true representation, their experimental criteria ended up only yielding data from ten countries so a more expansive study may yield different results.

Host

Like other parasites, *Neospora caninum* requires a host to provide the proper environment to complete its life cycle. Literature generally refers to two types of host animals that house the parasite during different stages. The definitive host, also known as the primary host, is essential for the parasite to complete sexual reproduction, and the intermediate or secondary host is responsible for housing the parasite while it grows to the point of sexual maturity (Dubey et al., 2007). To date, far more intermediate hosts have been confirmed for *Neospora caninum* than definitive.

Definitive Host

Animals of the *Canis* genus have been identified as the definitive host for *Neospora caninum* (Donahoe et al., 2015). Dogs have garnered the most attention as being the primary host animals because of their popularity as both working dogs and pets (McAllister et al., 1998; Lindsay et al., 1999). Additionally, clinically and sub-clinically infected dogs are found world-wide, which technically could provide *Neospora caninum* with hosts on every continent (Dubey and Lindsay 1996). Antibodies have been isolated from dogs spreading from Argentina (Basso et al., 2001b), New Zealand (Reichel et al., 1998), Turkey (Coskun et al., 2000), Brazil (Gennari et al., 2002; Mineo et al., 2001), Italy (Cringoli, et al., 2002), Chile (Patitucci et al., 2001), Germany (Klein and Muller, 2001), Romania (Gavrea et al., 2012), and the United States (Cheadle et al., 1999). Rural farm dogs, in particular, have shown to be more regular hosts for the parasite. Multiple studies have confirmed that dogs housed on dairy farms had a much higher presence of antibodies than dogs from urban areas (Sawada et al., 1998; Wouda et al., 1999b; Basso et al., 2001a). Wouda et al., (1999b) concluded that the number of dogs on the farm also was highly correlated to antibody concentration in dairy cattle. A few years later,

Sanchez et al., (2003) supported this theory by showing that dairy cattle on farms with dogs expressed significantly higher antibody counts than dairy farms without dogs. Other members of the canine family have been identified as definitive hosts as well. Perhaps the most recognizable to North American cattle producers is coyotes (Gondim et al., 2004c). Coyotes are one of the primary predators to livestock in North America and are regularly in contact with domestic cattle (Gondim et al., 2004c). Because of their population density in some areas, along with their familiarity with cattle, they are certainly capable of contaminating feed sources with their feces. In addition to this, they are also carnivores that have a reputation for eating deceased livestock as well as afterbirth, which can propagate horizontal transmission. Gray wolves aren't as dense nationwide and seem to be more geographical in their population than coyotes, but nonetheless, they too serve as definitive hosts (Dubey et al., 2011). While they may not be a threat to the domestic cattle industry in the United States, it comes to no surprise that Australian dingoes have also been identified as definitive hosts (King et al., 2010). It is plausible to believe that there are more definitive hosts that are yet to be discovered. Due to the difficulties that come with studying non-domesticated animals, researching other possible hosts can pose certain limitations that make it extremely challenging to confirm new hosts.

Intermediate Host

A wide array of warm blooded animals has been identified as intermediate hosts. Because of their economic importance, cattle have been more extensively researched than many other species that may possibly serve as secondary hosts. Much of the original research conducted in cattle focused on dairy farms (Dijkstra et al., 2003; Hietala and Thurmond, 1999; Bartels et al., 2007; Mazuz et al., 2014; Gonzalez-Warleta et al., 2011).

Sheep and goats have been added to the list of ruminants that can harbor the parasite and get the infection (Barr et al., 1992; Gazzonis et al., 2016; Gonzalez-Warleta et al., 2014; Pena et al., 2007). Just prior to that, water buffalo were added to the list of animals that serve as a vector for the parasite (Reichel et al., 2015). This comes to no surprise because they share many commonalities with domesticated cattle, but it is interesting that the incidence of infection was as high as 48% in water buffalo (Reichel et al., 2015). Chickens are also among the list of domestic farm animals that have been identified as intermediate hosts (Costa et al., 2008). Unfortunately for farm chickens, they are often easy targets for predators and are occasionally eaten by animals such as dogs and coyotes, which could provide another explanation for the rapid transmission that takes place on some farms.

Wildlife is believed to be a key contributor to the spread of *Neospora caninum* because the infection has been identified in several wildlife species, and they are often preyed upon by many carnivores, which provides an obvious route of transmission. Red foxes may be one of the more interesting species identified as intermediate hosts for the parasite because it seems closely related to dogs, which are known as primary hosts for the parasite (Almeria et al., 2002), but do not share commonalities in their role as hosts for the parasite. In the same manuscript, the author states that *Neospora caninum* was detected in the brain of rural red foxes, but no oocysts were found in the feces. Schares et al. (2002) conducted another study to help clarify the role red foxes may have in the *Neospora caninum* life cycle. They did so by feeding tissue of clinically induced sheep and goats to both dogs and red foxes. Like the work of Almeria et al. (2002) concluded, Schares et al. (2002) provided clarification that red foxes are not final hosts for *Neospora caninum* but, rather, neutral intermediate hosts. Dubey et al., (1999) added to the list of wildlife by detecting antibodies in white tailed deer. Since then, tachyzoites have been

recovered from non-captive, white tailed deer (Vianna et al., 2005) along with conformation of transplacental transmission (Dubey et al., 2013). While this does not guarantee that all deer species can host the parasite it does reveal and add to the possibilities that may be uncovered later. Of the wildlife that have been researched, white tailed deer are likely to be major contributors in spreading the parasite in this region because deer carcasses are regularly eaten by dogs and coyotes, and it is well documented that these animals can become infected by eating tissue from infected animals (Dijkstra et al., 2001; McAllister et al., 1998). Further studies continue to find evidence of *Neospora caninum* in many other wildlife species. Donahoe et al. (2015) authored a review that compiled reports of either DNA or antibodies being found in over 20 species of artiodactyla (hooved animals with even number of toes), multiple species of perissodactyla (herbivores with either one or three hooves/toes on the hind foot), rodentia, lagamorpha (hares, rabbits, and pika), insectivora, proboscidea (elephants), cetacea (whales, dolphins, and porpoises), pinnipedia (carnivorous aquatic mammals), marsupialia, and birds. These reports are a far stretch from actually confirming Neosporosis in each of these species, but it helps illustrate the possibilities of just how broad the issues of *Neospora caninum* might reach. Once an animal is identified as a host, it is important to understand how it is transmitted from one host animal to another. Some of the listed species are often in direct contact with canines so it is easy to draw conclusions on how the transmission takes place, but others are more difficult to determine if they lend much to the transmission process of the parasite from definitive to intermediate hosts.

Transmission

Most literature uses the terms horizontal and vertical transmission to describe methods of *Neospora caninum* transmission (Bartels et al., 2007; Bartley et al., 2013; Davison et al., 1999;

Dubey et al., 2007; French et al., 1999). In regards to *Neospora caninum*, authors commonly describe all forms of post-natal transmission as horizontal, but this may be a bit deceiving because by definition, "horizontal transmission is the spread of an infectious disease from one individual to another susceptible contemporary" (Miller-Keane, 2003). Even though the majority of literature uses these two terms to describe transmission methods in cattle, perhaps the term "trophic infection" would be more appropriate for describing post-natal transmission because according to the Merck veterinary manual, cows are not actually capable of spreading it to one another post-natal (McAllister, retrieved 2019). With that said, a review of the literature suggests that researchers in this discipline continuously use vertical transmission to describe infections that occur in-utero via transplacental transmission and horizontal transmission when describing new infections that are initiated by the ingestion of new oocysts (Davison et al., 1999; Dubey et al., 2007; Bartels et al., 2007; Bartley et al., 2013). For post-natal transmission to take place, oocysts from a definitive host must be ingested by the uninfected animal (Bartley et al., 2013; De Merez et al., 1999; Dubey et al., 2007; O'Handley et al., 2002). It is important to note that only feces from a definitive host can cause an infection of a seronegative bovine animal. To date, there has been no diagnosed infections that were believed to be spread from one intermediate host to another. The most obvious routes of ingestion are feed stuffs, water sources, and pastures. While cattle can become infected after birth (De Merez et al., 1999; Trees et al., 2002), research has shown that vertical transmission is far more common in cattle (Barr et al., 1994; Hall et al., 2005). Studies have continually shown that naturally occurring vertical transmission rate in dairy cattle is regularly quite high and often times as high as 95% in infected herds (Davison et al., 1999; Shares et al., 1998). These results are consistent with many other species that are considered intermediate hosts. While vertical transmission is present in dogs,

according to Barber and Trees (1998), results are far more variable when trying to forecast the likelihood of vertical transmission in canines. Regardless of species, both vertical and horizontal transmission of *Neospora caninum* is considered to play a major role in spreading the parasite within herd.

Post-Natal Transmission in Cattle

The most common method of post-natal transmission in bovine animals comes after consumption of sporulated oocysts released by definitive host (Bartley et al., 2013). With no evidence of *Neospora caninum* infections being spread from one cow to another's unless it was through vertical transmission or colostrum, new infections are most likely to come from contaminated feed stuffs or pastures. For a cow to consume oocysts in a pasture setting, they would most likely need to consume oocysts that have been excreted onto the ground by a definitive host such as a dog or coyote. For this reason, there is some speculation that areas with denser stocking rates may provide a better opportunity for cows to graze over a contaminated area and, in turn, lead to a greater number of seropositive animals (Otranto et al., 2003). Sanderson et al. (2000) reported that herds that grazed cows during the summer had lower seroprevalence and implied that seasonal grazing patterns were linked to seroprevalence. A few years later, another article was published stating that farms with denser stocking rates and no summer grazing practices were also at a higher risk for seropositive animals (Otranto et al., 2003). Likewise, Dijkstra et al. (2002) provided evidence that cows being fed a mixed ration were more likely to be seropositive. This makes sense because often times hay, silage, and grain piles are attractive spots for wild animals to eat and sleep, making these areas more likely to acquire contaminated feces. Both of these results have helped lead to the belief that the coyote, dog, and dingo populations may be linked to a higher incidence of horizontal infections.

Although there are many other confounding factors, multiple researchers have linked dog populations to a higher incidence of seropositive cows (Dijkstra et al., 2002; Otranto et al., 2003).

In uncontrolled, commercial settings it is very difficult to prove exactly how newly infected animals develop the infection, but multiple clinical studies have induced new infections. Several approaches have been used to induce the infections. Since oral transmission most likely mimics the route of natural infection, some models have used an oral route to prove that oocysts can cause new infections in cattle (De Marez et al., 1999; Gondim et al., 2004a; Trees et al., 2002). Similarly, calves were clinically infected by being fed oocysts shed by dogs. That same study observed immune responses of calves' post infection (De Marez et al., 1999). After two to four weeks post infection, Neospora specific IgG1 and IgG2 antibodies were found in serum of infected cows, but were not present in the control group. Other oral infections have been induced in calves through Neospora contaminated colostrum (Davison et al., 2001). An additional study determined that calves born to seronegative dams and bottle fed contaminated colostrum developed serum antibodies, but after they were euthanized they showed no signs of pathological lesions and no evidence of the parasite (Uggla et al., 1998). To further support the idea that oral contamination through colostrum may take place, Moskwa et al. (2007b) confirmed that *Neospora caninum* DNA was present in the colostrum of infected cows; however, this is still not conclusive evidence that calves can be infected in this manner because DNA does not necessarily lead to infections (Dubey and Schares, 2011). Scientists have also inoculated cows via intravenous and subcutaneous methods and showed that both methods can lead to fetal death in pregnant cows, but they determined that the intravenous route caused more acute placental lesions and greater mortality (Macaldowie et al., 2004). The chances of this route of infection

taking place in an uncontrolled setting seem rather unlikely but do help prove horizontal transmission as a possibility. A dose-response effect has also been reported from heifers that received an intrauterine inoculant of tachyzoites (Serrano-Martinez et al., 2007). The experiment didn't necessarily produce a confirmed infection but did elicit specific anti-serum antibodies in some heifers.

Vertical Transmission in Cattle

Most literature uses the terms exogenous and endogenous transmission to describe transplacental transmission (Trees and Williams, 2005). Exogenous refers to new infections that take place after a pregnant dam ingests oocysts while endogenous transmission refers to persistently infected animals that experience resurgence of the infection during gestation (Dubey et al., 2007). As mentioned previously, the rate of endogenous transplacental infection is quite high in cattle. It appears that several of the key factors that determine the outcome of the disease are the maternal immune regulation in the placenta and the immunocompetence of the fetus at the onset of infection (Horcajo et al., 2016; Regidor-Cerillo et al., 2014). Vazquez et al. (2019) added to this list of factors by implying that the method of exposure and parasite dose also affects the outcome. They saw a dose-dependent effect on the parasite counts in the placenta and also in fetal brain tissues. There is no shortage of studies showing that seropositive cows are more likely to give birth to seropositive calves (Anderson et al., 1997; Schares et al., 1998; Davidson et al., 1999; Thurmond et al., 1997; Dijkstra et al., 2003). On the higher end, one study checked 154 seropositive cows and 124 seropositive heifers and determined that 95% of them gave birth to seropositive calves (Davison et al., 1999). A Canadian dairy study showed a more moderate vertical transmission rate of 40.7 % among seropositive cows and only 6.7% of seronegative cows gave birth to seropositive calves (Pan et al., 2004). It is worth noting that

these studies were from herds that already had a high prevalence herd. There have been reports of herds with a much lower rate of vertical transmission (Bergeron et al., 2000). Several publications are available outlining the incidence of infection at birth before nursing, indicating vertical transmission, and also some that insinuate post-colostral infections are possible. Not only is there multiyear studies that continuously linked serological infections from dam to offspring (Frossling et al., 2005; Bjorkman et al., 1996), but clinically induced infections have also yielded offspring that were born infected (Gondim et al., 2004a). This pattern has also been seen in other species of ruminants (McAllister et al., 1996); however, not all clinically induced infections resulted in transplacental infection. McCann et al. (2007) induced infections on 18 pregnant cows at three different stages of pregnancy (70, 120, and 210 days) and concluded that the only transplacental infections came from the group that was the furthest in gestation. They also only inoculated them with a modest dose of 40,000 oocysts. The previously mentioned project by Gondim et al. (2004a), was in agreement with McCann et al., (2007) that the rate of transplacental transmission elevated the later the cows were in gestation and as the inoculation dose increased. Albeit a smaller sample size, the previously mentioned project by Trees et al. (2002) induced persistent infections in cows by utilizing as little as 600 sporulated oocysts, but all the cows calved normally and no transplacental infections occurred. Together, these data imply that the outcome of infection may be greatly dependent on the timing of infection and the amount of oocysts consumed.

It is challenging to prove transplacental infections in aborted fetuses because often times the fetus is never found; however, there has been instances where PCR results detected that aborted calves were infected with *Neospora caninum,* which suggest they developed the infection in-utero (Yao et al., 2009). Histopathological studies that tested for lesion in CNS have

further validated this belief (Pescator et al., 2007; Kamali et al., 2014). The epidemiology is not completely understood, therefore there is still plenty to learn about how the placental transmission actually takes place, but several beliefs are offered as to how the parasite actually transmits to the offspring. In mid-late term abortions, the placental type 2 cytokines down-regulate the maternal type 1 T-cell cascade, consequently, the dam's immune system is compromised and the *Neospora caninum* population rises (Haddad et al., 2005). The sudden influx of *Neospora caninum* results in increased tachyzoite loads within the placenta and the calf (Haddad et al., 2005). At that point, the result of the infection is believed to be dependent on how secure and advanced the calf's immune system is. If the offspring's immune system is entirely compromised, then it will have severe tissue damage and lead to abortion. If its immune system is advanced enough, then they may be born alive but with neurological or encephalomyelitis challenges as well as low birth weights (Bryan et al., 1994; Innes et al., 2002; Haddad et al., 2005).

As discussed, there are difficulties in diagnosing mid-later term abortions caused by *Neospora caninum*, but at least there are occasionally recoverable tissues that can be tested. In the instance of early term abortions, it is even more challenging to prove *Neospora caninum* is responsible. Not only are there no fetal tissues to test, but those samples may not actually have detectable sign of Neospora yet. For example, abortions that take place in early embryonic stages may not actually be due to the parasite itself crossing the placental membranes and infecting the fetus, but rather the inflammatory response that comes from the mother's immune response. Haddad et al. (2005) outlined one possibility for this by describing a pro-inflammatory response where T helper type-1 cytokines at the maternal-fetal interface are harmful to the placental attachments. They support this theory by previous work that shows the mother's

immune system responds to the parasite antigen with an intense cell proliferation of IFN-gamma (Innes et al., 2002). In this instance, even though the offspring never actually developed an infection, the *Neospora caninum* parasite would still be responsible for the fetal loss.

To date, research has suggested that infected cows become persistent and remain infected for life; however, it is possible that some cows develop a latent infection over an extended time frame and their antibody levels drop below detectable thresholds (Dubey et al., 2007, Conrad et al., 1993). This may serve as one possible explanation as to why congenitally infected calves are occasionally born to seronegative cows.

Venereal Transmission in Cattle

At this point, venereal transmission seems highly unlikely (Osoro et al., 2009), but the notion that sires may serve as a source for transmission has not been completely ruled out. *Neospora caninum* DNA has been extracted from inherently contaminated bulls (Ferre et al., 2005; Ortega-Mora et al., 2003) and has also been traceable in frozen and extended bovine semen (Caetano-da-Silva et al., 2004). To coincide with these reports, there is an article available which outlined the possibilities of females developing infection from contaminated semen (Serrano et al., 2006). In this study, heifers were artificially inseminated using semen containing 10^7 *Neospora caninum* tachyzoites and responded by seroconversion and developing a specific IFN-γ response. They also reported traceable DNA was found in the blood, brain, lungs, liver, and uterine horn of some of the heifers. In contrast, heifers artificially inseminated with non-contaminated semen didn't experience any sort of response (Serrano et al., 2006). Six of the nine heifers made embryos that were all free of *Neospora caninum* DNA. Contrary to the work performed by Serrano et al. (2006), work performed by Canada et al. (2006) was unable to develop Neospirosis in cows through artificial insemination of semen contaminated with

tachyzoites. There are several possible explanations for these discrepancies in results. One is provided by another manuscript authored by Serrano-Martinez et al. (2007), which tested heifers and cows at several tachyzoite doses and concluded that cows were more difficult to induce infections in. Other possible explanations may have to do with the strain and dose used. Even though the aforementioned studies provide valuable insight, it is still far from confirming venereal transmission.

Canines Role in Transmission

To fully understand *Neosopora caninum* in cattle, researchers must continue to gather information on other hosts and their role in harboring and spreading the parasite. For now, one of the primary hosts studied is canine animals; therefore, they have been identified as key suspects associated with cattle infection rates. Much like the intermediate host already discussed, transmission in canines occurs both horizontally and vertically. Natural infection through vertical transmission has been reported by multiple sources (Bjerkas et al., 1984; Dubey et al., 1990) and has also been confirmed in experimentally induced infections (Cole et al., 1995; Dubey and Lindsay, 1989). Vertical transmission was actually identified as a point source for the infection in dogs before it was cattle. With that said, the likelihood of a transplacental infection seems to be more variable in dogs than in bovine animals (Barber and Trees, 1998) and seems to occur at a lower rate than in cattle (Dubey and Lindsay, 1996). Even more interesting is that not all offspring out of the same litter, born to seropositive bitches, are born seropositive (Dubey et al., 2005). This implies that transplacental infection affects some pups but not others, all in the same pregnancy. Much like in bovine animals, it may be that those particular fetuses were more immune suppressed than the non-infected pups, but it is still striking that they co-existed in the same pregnancy.

Horizontal transmission in canines occurs, but in a different manner than in cattle. Unlike intermediate hosts, scientists are still unsure if the consumption of oocysts can induce an infection in dogs (Dubey et al., 2007). It appears that canines develop post-natal infections by eating tissue contaminated with the tachyzoites (Lindsay et al., 1999). Oocysts have been recovered from dogs after being fed contaminated tissue from many sources including mice (Lindsay et al., 2001), guinea pigs (Schares et al., 2001), deer (Gondim et al., 2004b), calves (Gondim et al., 2002), sheep (Schares et al., 2001), and goats (Schares et al., 2001). They were not only animals of different species, but also different tissue types such as brains (Gondim et al., 2002), skeletal muscle (Schares et al., 2001), and placental tissues (Dijkstra et al., 2001). At least one study failed to recover oocysts from dogs that consumed Neosopora induced, aborted, bovine fetuses (Bergeron et al., 2001a), but the parasite has been discovered in placentas from naturally infected dams (Bergeron et al., 2001b; Shivaprasad et al, 1989). It may be possible that natural infections are more likely to occur after consuming placentas instead of the fetus itself because the placenta may have a heavier parasite load than the fetus.

As previously established, canine animals shed oocysts that are hazardous to other intermediate hosts that consume them, but it has been relatively difficult to confirm just how many oocysts are normally present in the feces of an infected dog. In what little data is available on naturally infected dogs, the results have varied from as little as a few oocysts (Basso et al., 2001a), to as many 114,000 per gram of feces in an older 13-year-old dog (Schares et al., 2005), and even upwards of one million in a yearling pup (Slapeta et al., 2002). Part of what makes it difficult is that oocyst shedding appears to be inconsistent (Dubey et al., 2007). In fact, McGarry et al. (2003) checked a follow up sample on a dog four months after the original sample revealed 84,000 oocysts per gram and discovered that the oocyst count had dropped drastically. Dubey et

al. (2007) also alluded to the thought that the species of tissue that the host dog consumed may have an association with the number of oocysts they shed. This thought is justified by previous work where dogs shed more oocysts after eating bovine tissue than they did after consuming mouse tissue (Gondim et al., 2002). Dubey et al. (2007) adds to their discussion by implying that the age of the infected host dog may also have an association with oocyst counts. Much like cattle, it appears that immuno-compentency of the infected dog may also play a role in the outcome of the infection because dogs immune-challenged with corticosteroids had altered oocyst production (Dubey et al., 2007; Lindsay et al., 1999; Lindsay et al., 2001).

Biology and Epidemiology

Neospora caninum is a tissue-dwelling coccidian and member of phylum Apicomplexa (Reid et al., 2012). There are three known infectious stages: tachyzoites, tissue cysts, and oocysts (Dubey, 2003). Tachyzoites and tissue cysts are found intracellularly in animals serving as the intermediate host (Al-Qassab et al., 2010; Khan et al., 2020). Tachyzoites are rod or banana shaped and measure roughly 6 x 2 µm in area. There have been varying reports pertaining to the size of tachyzoites and this may be due to the different stages and growth patterns (Dubey and Lindsay, 1996). They can be transmitted from an infected mother to the offspring via the placenta (Dubey, 2003). Al-Qassab et al. (2010) explains the parasite life cycle and shares that they originate from sporozoites that are formed from oocysts in the gastro intestinal tract. These sporozoites travel to host cells and begin to replicate. Once they replicate, they begin to migrate to neuro cells, macrophages, fibroblasts, vascular endothelial cells, liver cells, and renal tubular epithelial cells (Al-Qassab et al., 2010). Tissue cysts are most commonly present in the central nervous system but have also been found in other muscle tissue (Peters et al., 2001). They are generally round or oblong shaped with a solid wall surrounding them and

may measure up to 107 µm in length (Al-Qassab et al., 2010; Dubey, 2003). The tissue cysts are referred to as bradyzoites, and according to Al-Qassab et al. (2010), they develop following a cell mediated immune response that exposes tachyzoites to γ interferon (IFN-γ). These tissue cysts play a big role in the transmission of the disease because they are present in muscle tissue and placenta membranes that are often eaten by predators. After the definitive host ingests the tissue cysts, the bradyzoites are released and they attack the neural and skeletal muscle fiber cells where they then undergo asexual reproduction (Al-Qassab et al., 2010). This process propagates the life cycle of the parasite and then develops to create a newly infected definitive host. The definitive host then sheds unsporulated oocysts. The oocysts are very similar to *Toxoplasma gondii* and *Hammondia hammondi* that is common in felines. Unsporulated oocysts measure 10-11 µm in diameter (Perrucci et al., 2017), and this is very similar to the findings Lindsay et al. (1999) reported nearly twenty years earlier, as they stated that unsporulated oocyst were 11.7 x 11.3 µm. After the oocysts are expelled and shed in the feces of the host animal, they sporulate within a few days and become a hazard to animals that may consume them (Al-Qassab et al., 2010; Dubey, 2003). Although they typically shed cysts five to ten days after the infection and continue to shed around 10 days, there is some variation in the shedding patterns of canines. One of the determinates that may affect the number of cysts is the age of the host. One study showed that puppies expelled more oocysts than mature dogs (Gondim et al., 2002). That same author reported that dogs that consumed infected calf tissue had more oocysts than they did from infected mouse tissue which implies that maybe even the tissue type and source may affect the outcome of oocysts numbers. It is still unknown exactly how long the oocysts can survive under environmental conditions outside the host. It would be very beneficial to understand the details of survival outside the host and this may help explain why some areas have more infections than

others. It is plausible to believe that the oocysts are more survivable in some climates than others.

More recent research has focused on different strains and isolates of Neospora. In an article authored by Calarco et al. (2018), they reported that there is over 100 different identified strains of *Neospora caninum* isolates. Like many other research studies investigating disease, much of the literature began with treatments on mice in a lab setting. In mice, three strains referred to as NC-1, NC-2, and NC-3 have been applied to test how quickly they elicited an infection and the immune response. The NC-1 strand induced a faster and more severe immune response to the infection (Lindsay and Dubey, 1990), and NC-3 did not induce any clinical symptoms (Lindsay et al., 1995). McGuire et al. (1997) also reported measurable differences in mice as NC-2 caused a higher mortality rate than NC-Liverpool. Many of these isolates from other species, such as deer, are being applied to mice and may not perfectly represent an authentic situation. Nonetheless, it does provide insight on how much variation there may be and it does so in a much cheaper and controlled research setting. After all, financial limitations put restrictions on the amount of research that has been conducted in cattle. Also, there has been research that applied different isolates to cattle. Jimenez-Pelayo et al. (2019) investigated Nc-Spain7 and Nc-Spain1H isolates and found that certain strains, in this case Nc-Spain7, may be more potent and faster acting than others. Likewise, heifers inoculated with NC-1 experienced more fetal loss than NC-Spain1H (Rojo-Montejo et al., 2009). It is also noteworthy that some isolates may just be tougher and more survivable than others. Bradyzoites from NC-2 lived for 30 minutes in pepsin-HCL solution whereas NC-1 bradyzoites died in that time period (Lindsay and Dubey, 1990). The strain may also be correlated to the immune response of the infected animal as NC-Spain7 caused a faster and greater anti-*Neospora caninum* IgG response than NC1

(Caspe et al., 2012). To help understand these marked differences in strains, scientists have begun to study differences in strain potency at the genetic level. Garcia-Sanchez et al. (2019) used the NC-Liverpool genome, which is the only *Neospora caninum* genome currently available (Reid et al., 2012), to study gene expression differences between isolates in bovine macrophages. They illustrated that the Nc-Spain1H isolate enhanced the gene expression of surface antigens and bradyzoite-stage specific genes. Alternatively, the Nc-Spain7 increased the expression of genes linked to parasite growth and survival in macrophages. These same two strains showed differences in the expression of genes that are associated with host cell invasion and attachment, glideosomes, rhopties, metabolic process, and stress responses (Horcajo et al., 2017). The ability to distinguish between strains is valuable and has been useful in describing the differences often seen from case to case in animals infected with Neosporosis. Even though there have been clear differences in isolates and how they may affect the outcome of the infection and the immune response of the host, in bovine animals, there has not been much variation in antigens that are developed in response to the infection (Al-Qassab et al., 2010).

Valuable insight about the epidemiology of *Neospora caninum* has also been cultivated from prior knowledge about *Toxi gondii* and its affiliation with the Apicomplexa phylum. Apicomplexan parasites are different from many other parasites because of their intracellular characteristics (Calarco et al., 2018). They alter their hosts cell physiology by secreting effector proteins via secretory organelles (Calarco et al., 2018). These organelles are specific to Apicomplexan parasites and are referred to in literature as micronemes, rhoptries, and dense granules (English et al., 2015) and are believed to be released in a step by step cascade (Nam, 2009). It is hypothesized that adhesions that are synonymous with Neospora-infected animals are a result of micronemal proteins that are secreted following initial contact with the host cell

(Calarco et al., 2018; Cerede et al., 2005). Following the secretion of micronemal proteins, rhoptry proteins are released into the host cell cytosol (Calarco et al., 2018), which changes the function and allows the infectection to proceed. That step then leads to the creation of a parasitophorous vacuole (Talevich and Kannan, 2013). This vacuole has been heavily studied in toxoplasmosis and plays a critical role in the parasites' ability to develop while protecting itself from the attack of the host cell and eventually becomes the outer layer of the cyst membrane (Paredes-Santos et al., 2019). Following the development of the parasitophorous vacuole, the dense granules (*GRA*) then release *GRA* protiens, in which Calarco et al. (2018) imply it may be linked to nutrient acquisition. These granules are regular in tachyzoites and bradyzoites and comprise much of the circulating antigens. These *GRA* proteins offer resistance to the host IFN-γ response and allow for the parasite to develop a chronic infection (Fox et al., 2019). If *Neospora caninum* shares these same similarities, this research provides insight on how it creates a chronic infection within the host and how it reactivates during periods of immune deficiency. Referring back to the previous discussion about gene expression, profiling work performed by Garcia-Sanchez et al. (2019) may help provide clarity on why there are such drastic differences among strains. Their work showed that specific strains differed in their expression of genes which encoded for rhoptry proteins, which could definitely explain observed differences in parasite proliferation. More specifically, they illustrated that in Nc-Spain7 two genes related to the rhoptry kinase family were highly expressed (Garcia-Sanchez et al., 2019).

Host Immune Response

The exact mechanisms by which the *Neospora caninum* parasite migrates in the host tissue and how the host's immune system responds are still unclear, but several studies have produced helpful insight. It has been shown that inflammatory cytokines, specifically IFN-γ,

play a significant role in the defense mechanism that host animals use after the parasite enters the body (Almeria et al., 2017). The same publication describes an innate immune response that provides acute protection by expressing pattern-recognition receptors that induces the production of IFN-γ (Almeria et al., 2017). Similarly, *in vitro* studies suggest that pro-inflammatory cytokines, including IFN-γ (Innes et al., 1995), and tumor necrosis factor (TNF-α) (Yamane et al., 2000) limit proliferation of *Neospora caninum.* In addition, when pro-inflammatory cytokines such as IFN-γ were produced in elevated levels in the cow, the spleen and thymus harvested out of several recovered fetuses also yielded the same results (Bartley et al., 2012). Evidence is also available showing that infected cattle generate parasite-specific CD4+ T and natural killer (NK) cells which are capable of lysing cells that have been invaded by the parasite (Staska et al., 2003). In naïve cells, pro-inflammatory cytokine IL-17 is expressed in the presence of a *Neospora caninum* infection, and this may promote the role INF-γ. More recent work has focused on nucleotide-binding oligomerization domain receptors (NLR's) and their role in Neospora-infected macrophages. These cytoplasmic receptors have drawn attention because in the presence of pathogens, they induce an inflammasome complex's that help prevent the proliferation of the parasite (Wang et al., 2019). Wang et al. (2019) stated that NLR's help regulate the activation of caspase-1 in infected bovine macrophages and that caspase-1 was correlated to the number of parasites in the parasitophorous vacuole. Those same authors determined that ATP treatment of *Neospora caninum* infected bovine macrophages reduced the proliferation of newly infected cells, which may prove to be valuable insight for creating a vaccine. Other focal points of the immune systems response to *Neospora caninum* include extracellular traps (ET) that are derived from macrophages. These extracellular traps have been shown to play a key role in combating larval and other pathogens (Bonnee et al., 2014; Wong

and Jacobs, 2013); therefore, Wei et al. (2018) studied the role of macrophage-derived ET's and their role in controlling *Neospora caninum* in host animals. They provided some of the first data that suggest ET's may be part of the animal's defense against tachyzoites.

Scholars have attempted to understand exactly how recrudescence and reactivation occurs within cows. The recrudescense process occurs when bradyzoites convert to tachyzoites and is believed to take place around the middle and last stage of pregnancy (Horcajo et al., 2016). Much of what has been concluded about the recrudescence process has been mined from literature about *Toxi gondii* because of their similarities. In *Toxi gondii* the conversion takes place during the period when Th2 cytokines increase and Th1 cytokines become impaired (Horcajo et al., 2016; Luft et al., 1984). Horcajo et al. (2016) hypothesize that the normal Th2-biased immune response that takes place during pregnancy in cattle may result in a brief compromise that hinders the dam's ability to stave off a resurgence of *Neospora caninum*. This timeline would match and explain the reports that antibody levels increase during the final two stages of pregnancy (Nogareda et al., 2007) and also provide support for the theory that tachyzoites reach the placenta during this phase (Horcajo et al., 2016).

In regards to the dam's immune response and its role of maintaining the pregnancy, there have been several possible explanations for how the immune response to the parasite may lead to fetal death in pregnant hosts. During normal pregnancies, NK cells are dense up until the time of implantation and then become dominated by macrophages as gestation advances (Mor et al., 2006). As gestation continues, macrophages are recruited to the endometrium and are believed to aid in clearing apoptotic cells and controlling placental lactogen levels at the fetal-membrane interface (Fair, 2015). Previous research also reported that early in normal pregnancies individuals generate Th-1 and Th-2 cytokines (Sykes et al., 2012). In humans and mice, at some

point during the pregnancy, there appears to be a shift where the cytokine production becomes favored toward Th-2 production to the point where it predominates the final two trimesters of pregnancy (Raghupathy, 2009; Joachim, 2003). This normal up-regulation along with the increased INF-γ in response to the foreign parasite may lead to an imbalance that has adverse effects on the pregnancy (Almeria et al., 2017). Their study also suggested that other cytokines such as IL-12 and IL-10 are crucial for preventing an imbalance that leads to pregnancy loss. Another article proposes that the increase in Th-1 cytokines that is effective for protecting the dam may also result in an infiltration of CD4 cells and NK cells at the maternal-fetal interface that may be dangerous to the fetal tissue (Maley et al., 2006). Human research has shown that NK cells have the ability to convert to lymphokine-activated killer (LAK) cells that can lyse trophoblasts and lead to fetal loss (Loke and King, 2000). Raghupathy (2009) continues by describing the possibilities of NK cells leading to increased IFN-γ which then activates decidual macrophages that go on to produce nitric oxide and TNF-α. In this instance, Th1 cytokines would affect pregnancy by activating cellular effectors and damaging the placenta (Raghupathy, 2009). Additional research outlines the possibilities of stress altering the normal progesterone levels necessary to maintain a normal pregnancy. During pregnancy, progesterone is generally produced and binds to progesterone receptors, which causes progesterone-induced blocking factor (PIBF) to be released from lymphocytes (Joachim et al., 2003). This PIBF response is crucial for providing the Th2 biased immune response that was previously linked to pregnancy maintenance (Joachim et al., 2003). Their anti-abortive responsibilities begin with their effect on B cells and their ability to increase production of asymmetric, non-cytotoxic antibodies (Druckmann and Druckmann, 2005). Those same authors further validate the importance of PIBF listing three key roles in the immune response: alters lymphocyte secretion of cytokines

and leads to elevated production of non-inflammatory and non-cytotoxic interleukins, reduces cytotoxic cytokines, and blocks the cytotoxity of NK cells. They influence NK cells by stopping their degranulation and perforin release and also preventing their transformation into LAK cells (Druckmann and Druckmann, 2005), which were previously alluded to because of their role in human miscarriages. All of this information provides evidence that altered progesterone levels in response to stress could very easily compromise pregnancies in cattle. Joachim et al. (2003) tested this theory by adding a progesterone derivative called dydrogesterone to stressed mice. They concluded that stress caused lower levels of progesterone and PIBF in plasma and lowered progesterone receptors at the placental interface. By adding dydrogesterone they minimized the effect of stress on abortion rate and improved plasma PIBF levels. Interestingly, another study conducted a few years later found that progesterone treatments applied during mid-gestation in dairy cows with Neosporosis led to increased abortion rates (Bech-Sabat et al., 2007). The conflicting results are not completely clear, but a few obvious differences in methodology could possibly have influenced the outcome. The bovine study used cows *Neospora caninum*-infected cows with high titers and applied an intravaginal progesterone source. The other study applied dydrogesterone by injection and induced the stress by sound exposure. Regardless, stress induced alteration of steroid hormones is certainly capable of causing issues in animals infected with *Neospora caninum*.

The immune competence of the developing fetus may certainly have a role in the outcome of the pregnancy in infected animals. To some extent the offspring's immune system is correlated to the immune health of the dam, but research states that the fetus's immune system advances throughout gestation and is more capable of defending itself against a foreign pathogen as its own immune system progresses (Barrinton, 2001; Chase et al., 2008). Several projects

have verified that the effects of *Neospora caninum* infections are more severe in animals during the first stage of gestation, and it is at this point that absorption or mummification is more likely to occur (Williams et al., 2000; Gibney et al., 2008; Rosbottom et al., 2008; Regidor-Cerillo et al., 2014; Horcajo et al., 2016). If the dams are infected mid-gestation then the outcome is relatively unpredictable, but the majority of *Neospora caninum* induced abortions are reported during this period (Dubey et al., 2007; Almeria and Lopez-Gatius, 2013; Harcajo et al., 2016). This may be due to the fact that the innate immune system organized by phagocytic cells does not actually become fully mature until late gestation (Horcajo et al., 2016).

Several proposals have been offered to help describe the process by which *Neospora caninum* can lead to fetal damage, and it is easy to believe that all mentioned mechanisms may occur and possibly work together. There is certainly more work to be conducted, but the information that is currently available has served as the basis for vaccine production.

Vaccines

The production of a commercialized vaccine would be beneficial for negating the adverse effects that often result from *Neospora caninum* infections. There have been a few commercialized vaccines created, but the efficacy along with the benefits relative to the expense have been debated (Almeria et al., 2017). The debates on efficacy of the vaccine may depend on the expectations by which the vaccine is measured. For instance, preventing abortions is different from preventing infections. Several studies have shown vaccinations yield beneficial effects but there seem to be varying degrees of efficacy, and results are variable depending on the animal model tested, age, stage of pregnancy, and vaccine type.

Several vaccine types have been experimented on mice. Early studies showed *Neospora caninum* tachyzoites (10^7), administered intraparatoneally, did offer short term protection from

an acute infection, but they did not elaborate on the possible benefits for preventing abortions in pregnant dams (Ramamoorthy et al., 2006). Two popular surface antigens of *Neospora caninum*, NcSAG1 and NcSAG2, were tested in an intramuscular vaccine and did help prevent mice from developing symptoms (Cannas et al., 2003). This vaccine utilized a combination of DNA and recombinant antigen, but did not include abortion or pregnancy rates in their study (Cannas et al., 2003). With that said, there has been research that focused on preventing abortions and vertical transmission. Miller et al. (2005) administered live NC-Nowra tachyzoites prior to pregnancy and reported a reduction in transplacental infections from 76%-8%. They also used an injection crude lysate of NC-Nowra, which yielded a reduction in transplacental infections by 14%. The two forms elicited different responses. They reported the crude lysate induced only IgG1 antibodies, while the whole tachyzoites led to both an IgG1 and IgG2 response (Miller et al., 2005). Since that study, other strains of *Neospora caninum* have been used in vaccines with lessor results. Jimenez-Ruiz (2012) had difficulties proving efficacy when using NCGRA7, NCSAG4, NCBSR4, and NCSRS9.

Vaccine research on bovine models first focused the use of killed tachyzoites because it induced an IFN-γ response, much like the one observed in natural infections (Horcajo et al., 2016). The tachyzoites used did lead to an increase in IgG1 along with increased IFN-γ but was not effective for preventing infection in the offspring (Horcajo et al., 2016; Andrianarivo et al., 2000). The commercialized bovine vaccine utilized tachyzoite lysate to develop immunity, and there have been multiple studies that challenged the efficacy of this vaccine. Romero et al. (2004) conducted a study with 876 head of dairy cows and concluded that the vaccination with killed tachyzoites did reduce the abortion percentage from 20.8%-11.2%. In this study, they administered two 5 ml doses one month apart and began giving the first shots between 75-90

days of gestation (Romero et al., 2004). Since that study there has been more recent work that experienced different results. This New Zealand based study was performed on five dairy herds. In this study, the first vaccination was given earlier in gestation at days 30-60 but still administered in two doses one month apart (Weston et al., 2012). Their work reported the vaccine was beneficial for reducing abortions in one herd but not the remaining four. They went on to report that the vaccine resulted in more seropositive offspring, suggesting that it may increase the risk of vertical transmission. With that said, there was not another follow up study with those seropositive offspring to see if they were more likely to abort nor does this address the issue of preventing seronegative cows from seroconverting at a later date.

Live vaccines are certainly viable options for protecting against apicomplexan coccidian, but to date, they appear to present issues with vaccine expense and stability (Monney et al. 2011). Horcajo et al. (2016) outlined several live-attenuated vaccines that could be useful. Attenuated vaccines using both Nc-Nowra (Williams et al., 2007) and Nc-Spain1H (Rojo-Montejo et al., 2013) have been linked to fetal protection and decreasing vertical transmission. Interestingly, both these projects opted to vaccinate cattle prior to breeding rather than during gestation, which differs from several of the other articles previously reviewed. Those two studies also clinically induced infections in an experimental setting. Mazuz et al. (2015) conducted a case study using live tachyzoites in naturally infected cattle and discovered that NcIs491 reduced the abortion rate but did not reduce transplacental infections.

Scientists have begun exploring the possibilities of using subunit antigens for improving vaccine efficacy. Monney et al. (2011) referred to subunit antigens as a safer option with a longer shelf life. Apicomplexan profilins surfaced as antigen candidates because of their binding capabilities to toll-like receptors that propagate a series of signaling pathways believed to be

linked to IFN-γ and IL-12 production (Mansilla and Capozzo, 2017). This concept is supported by previous research by Wang et al. (2019) that focused on using nucleotide-binding domain and leucine-rich repeat proteins (NLRP), specifically NLRP3 inflammosomes, and their ability to eliminate parasite proliferation and growth. As mentioned earlier, *GRA* proteins that are released from the parasitophorous vacuole play a key role in preventing parasite growth; therefore, their usefulness for vaccine formulation has gained traction. In what limited work that has been conducted with *GRA* proteins, NcGRA7 vaccinations did reduce the parasite load in the brain of vaccinated cattle (Nishimura et al., 2013), but more work is needed to conclusively show that it will prevent abortions.

Other challenges may face vaccine production because coccidian vaccines often require adjuvants (Sander et al.; 2019). According to Sander et al. (2019), there are typically drawbacks with using prophylactic vaccines because they could possibly lead to unwanted residues in meat and milk. For this reason, plant based adjuvants may serve as safer options for compounding recombinant antigens and are likely to be studied more in depth moving forward. In regards to vaccines specifically for *Neospora caninum*, Mansilla et al. (2015) reported soya lecithin/β-glucan adjuvant had no negligible effects on the dam or offspring when used in pregnant cattle.

It is also important to note that the commercialized vaccines that have been used in the past can still show up as seropositive if tested with serological assays (Dubey et al., 2007). Because the serological test cannot distinguish between vaccinated animals and truly infected animals, all animals will have to be treated the same within a herd (Dubey et al., 2007). This could be difficult for trade purposes, but also for managers who are hoping to use serological tests to perform regular culling measures.

Diagnosis and Testing

There are several options available for testing for *Neospora caninum*, and each one comes with its own strengths and weaknesses. Most research either uses serological assays, polymerase chain reaction (PCR), or histopatholgy test to detect *Neospora caninum*. Evidence of antibodies, DNA, or tissue cysts is not necessarily synonymous with viable parasites (Donahoe et al., 2015), but it is generally the most realistic means of testing because, as a general rule, *Neospora caninum* is extremely difficult to isolate (Donahoe et al., 2015). Not only is it difficult to recover viable parasites in large enough numbers, but it can also take up to several weeks to distinguish parasite stages in cell culture (Conrad et al., 1993; Kim et al., 2000). Although it may be more applicable in wildlife specific strains, Donahoe et al. (2015) discuss the challenges of growing some isolates in cell culture. For these reasons, serological tests seem to be the most popular testing tool for *Neosopora caninum* (Donahoe et al., 2015) and multiple studies have reviewed the efficacy of a few of the more popular tests.

Serological Assays

Serological analysis in cattle is generally performed by the following assays: enzyme-linked immunosorbent assay (ELISA), indirect immunoflourescent antibody test (IFAT), *Neospora* agglutination test (NAT), and immunoblotting (IB). The ELISA assay can be used with milk or serum and some of the original studies showed ELISA as a viable test for diagnosing seropositive cattle. In fact, Almeria (2013) writes that it is one of the best tests for testing bovine animals at the herd and individual level. Pare et al. (1995) established that the ELISA test was more sensitive and specific than immunofluorescent antibody test (IFA). Another study comparing ELISA to IFAT determined that the ELISA test generated more accurate test results than IFAT at a cut off value of 0.200 (Frossling et al., 2003). At that cut-off

value, the ELISA test produced a sensitivity of 96% and specificity of 99%. The IFAT test had a specificity of 99%, but the sensitivity was only 78%. Their suggested optimal densities were ≤0.150 to ≥0.550. Wouda et al. (1998) also compared three different ELISA tests for their ability to detect antibodies in bovine sera. One was based on chemically-fixed, intact, tachyzoite antigens, one was based on sonicate lysate of whole tachyzoites, and they were compared against a detergent lysate of whole tachyzoites. They determined that all three tests were in agreement when they tested serum from cows that had recently aborted due to *Neospora caninum*; however, there seemed to be more variability when they tested serum from cows doing total herd screenings. These tests had different sensitivity levels, and it affected their consistency when testing chronically infected cows with low antibodies (Wouda et al., 1998). ELISA analysis using milk is still recognized as a useful tool but is less specific than serum. The milk test was also variable depending on how far in lactation the cow was (Schares et al., 2004). The usefulness of each test may also depend on what species are being tested. Donahoe et al. (2015) reported that ELISA and NAT are generally the test of choice for wildlife, because unlike other test, there is no need for species-specific subordinate antigens.

There are some drawbacks with the serological test. The antibody levels can fluctuate during pregnancy and also in cows with chronic infections (Dubey et al., 2007; Dubey and Schares, 2011). It is still unknown exactly when and how much they fluctuate, but it is plausible to believe that an infected cow could drop below the sensitivity level set by the test. There are also reports available that imply not all infected animals produce detectable amounts of antibodies (De Marez et al., 1999; Lindsay et al., 1999). In a study conducted on congenitally infected calves, four percent experienced a period from 9-18 months when their antibody level dropped below detectable levels and then elevated again after that period (Hietala and

Thurmond, 1999). Others have cited the extra time, labor, and equipment required to perform an ELISA test as another disadvantage (Liao et al., 2005).

Polymerase Chain Reaction

The PCR analysis is a highly sensitive test that is capable of testing tissues, as well as blood and other fluids (Donahoe et al., 2015). This analysis is useful for determining parasite stages and identifying the presence of *Neospora caninum* DNA (Almeria, 2013). According to Dubey and Schares (2006) the most commonly used markers for identifying *Neospora caninum* is the *Neospora caninum*-specific, repetitive Nc5 gene, along with the internal transcribed spacer one (ITS1) region of the rRNA gene. The brain seems to be the most common tissue tested because *Neospora caninum* is notorious for traveling to nervous tissues (Dubey et al., 2007). Even so, it does have the capability to set up in other organs and skeletal tissue (Almeria, 2013). In this instance, PCR on brain tissue only could risk missing the infections if it is located elsewhere. The expense of a PCR test is also a concern for producers who want to test for herd prevalence.

Histopathology

Histopathology is valuable for detecting lesions and also analyzing parasite distribution (Donahoe et al., 2015). Evidence of lesions gives visual support of the parasite. Confirmation of lesions provides support for *Neospora caninum* infections that may have already been detected through serology tests. Besides the fact that histopathalogy tests are more invasive than serological tests, it also requires recoverable tissue that may not always be available unless the animal is deceased and recoverable.

Management Strategies

Much like the majority of all other diseases, management practices can be performed to help mitigate the effects and incidence of Neosporosis in a herd. In regards to preventing the infection from occurring in the first place, testing and culling seems to be the fastest way and most cost-effective way to reduce *Neospora caninum* infections in a herd (Larson et al., 2004). This is assuming that the primary source of infection comes from vertical transmission that takes place during gestation. If the primary source of infection is stemming from trophic infections, then other practices should be considered to reduce the risk of contaminated feed and water sources. Rodent, varmint, and coyote control could help reduce the number of host animals that could possibly be shedding oocysts in feed sources such as silage piles, hay stacks, or commodity pits. Producers may also want to consider modifying grazing patterns for cows to graze during the summer, as there has been a strong correlation between Neosporosis and cows that have limited grazing during the summer months (Otranto et al., 2003). Less intensively grazed pastures and a lower stocking rate should also help reduce the risk of infection (Otranto et al., 2003).

There are also options for managing cows that are already infected. At least two studies have presented evidence that crossbred pregnancies are more likely to survive a *Neospora caninum* infection (Almeria et al., 2009; Lopez-Gatius et al., 2005b), suggesting that using semen of other breed types to impregnate infected cows may pay dividends for producers. Other reproductive techniques such as embryo transfer can be used as well. Due to the timing of vertical transmission in naturally infected animals, embryos can be removed and transferred into seronegative recipients and still result in a seronegative calf (Baillargeon et al., 2001; Landmann et al., 2002). Obviously, this would lead to additional expenses that may not be feasible for

commercial cattlemen, but it does provide an option for producers who place special value on an infected cow but want to avoid the risk that comes with an infected dam.

Heat Shock Proteins

Although good management accepts the responsibility of providing proper husbandry practices that protect livestock from stressful elements, the animals themselves also have an innate defense mechanism to help them cope with stress. Naturally, livestock often rely on the secretion of stress hormones and the cell-mediated production of heat shock proteins to help battle stress. While stress hormones are a beneficial coping mechanism for animals, they may also negatively impact normal functions, such as reproduction and immunity (Burdick et al., 2011). Heat shock proteins (HSP) are a class of conserved proteins that are hyper expressed in the presence of stress (Garrido et al., 2006). Although many of these proteins are always available in the cells, exposure to stress induces and intensifies the expression (Whitley et al., 1999). The exact mechanisms by which they operate are not completely known, but transcription and translations play an important role in the HSP response (Whitley et al., 1999). They were first reported in 1962 when they were discovered in *Drosophila* flies after noticing alterations in polytene chromosomes during exposure to heat and oxidative stress (Ritossa, 1962). Since then, they have been heavily researched and most widely known for their ability to mitigate stress-induced damage by binding to denatured proteins and counteracting proteotoxicity (Tower, 2011) and termed as molecular chaperones. These proteins exist in both prokerurotic and eukyrotic and are found in all cells of the body. Within the cell, they can be present in the cytosol, mitochondria, endoplasmic reticulum, and the nucleus (Kiang and Tsokos, 1998). Because they were first discovered in a study researching heat stress in flies, they earned the name heat shock proteins, but many other forms of stress have since then been linked to their

expression (Whitley et al., 1999). As research began to associate these proteins with other stressors, they became recognized for their role in aging, immune function, thermotolerance, and reproduction. It is now well known that there are several different types of heat shock proteins that appear to serve different roles in maintaining proper cellular balance. In humans, there have been associations between several health disorders and the abnormal supply of stress proteins (Whitley et al., 1999). The same has occurred in cattle studies, and because of it, HSPs may be used for biomarkers in health and reproduction (Tower, 2011). These biomarkers may be consistent with specific breeds and their physical characteristics. In fact, research indicates that some breed types synthesize HSPs more regularly, as Mullins et al. (2016) reported that basal levels of *Hsp27* and *Hsp70* are found in higher concentrations in the skeletal muscle of *Bos indicus* than *Bos taurus.* Understanding exactly how each family of HSPs impact normal bodily functions in all breeds of cattle could benefit the industry greatly. The classifications of HSPs are named for their molecular size (Whitley et al., 1999) and their unique characteristics are described below.

***Hsp*100-110**

Although this group of HSPs is the largest in terms of molecular weight, it is still the third or fourth most abundant of the heat shock families found in mammalians (Easton et al., 2000). *Hsp*100 is located in the cytoplasm, and *Hsp*110 is present in the cytosol and nucleus (Jee, 2016). *Hsp*100 is most well known for being a co-chaperone with *Hsp*40, *Hsp*70, and *Hsp*90 and is generally associated with refolding aggregates (Jee, 2016). Much like *Hsp*70, the *Hsp*110 family shares the same loop structure and is a key component of the immune response (Zou et al., 2016). *Hsp*110 also lend to cell survival by working with *Hsp*70 and GRA78

(Gething and Sambrook, 1992; Hartle, 1996). Zou et al. (2016) also concluded there may be endogenous immune-stimulation of large HSP directly following an injury.

*Hsp*90

The *Hsp*90 family ranges from 83kDA to 110kDA and have been studied extensively for their relationship in protecting the body from many diseases (Zuehlke et al., 2017). On the other hand, human medicine has discovered that tumor cells synthesize HSPs, which protect these cells from many types of treatments and therapies (Lang et al., 2019). These proteins are generally abundant at normal temperatures and get further induced as temperatures elevate above normal body conditions (Lindquist and Craig, 1988). Much like many of the HSPs, they are critical components in maintaining peptide integrity and aid in enzyme folding (Wiech et al., 1992). This group of proteins is present in the cytosol and nucleus (Whitley et al., 1999). Albeit in human HSPs, an extensive review by Zuehlke et al. (2017), explains the structural components of *Hsp*90. They feature a dimeric structure and each monomer has a N-terminal ATP-binding domain, a middle co-chaperone and client binding domain, along with a C-terminal dimerization domain (Zuehlke et al., 2017). Their chaperone capabilities come following a conformational change which is influenced by ATP (Zuehlke et al., 2017). When they are not bound to nucleotides the C-terminal domains are dimerized and remain in an open conformation (Zuehlke et al., 2017). Upon nucleotide interaction, the N-terminal domains undergo dimerization, which strengthens its ATPase competence and leads to a closed conformation (Zuehlke et al., 2017). After ATP-hydrolysis, *Hsp*90 reverts back to its open conformation. Those same authors explain the importance of co-chaperones in the function of Hsp90 during the ATPase cycle. They outline two important co-chaperones, $p50^{Cdc37}$ and Hop, that bind to the open conformation of *Hsp*90 and are crucial for avoiding kinase degredation and apoptosis. Perhaps a more intriguing

co-chaperone relating to reproduction is immunophilin because it recognized for impact on steroidogenesis (Zuehlke et al., 2017). With this, they also bind steroid receptors, protein kinases, intermediate filaments, microtubules, and actin microfilaments, while being a critical piece of glucocorticoid receptors (Jee, 2016). During the phase when the N-terminal undergoes dimerization and *Hsp*90 is in the closed conformation, it coordinates with immunophilin proteins at the C-terminal. Aside from their intracellular capabilities, many HSPs have extracellular functions which may be associated with their paralogs. In regards to *Hsp*90, it has two paralogs known as *Hsp*90-α and *Hsp*90-β, and it is the alpha paralog that is associated with the extracellular capabilities (Li et al., 2013). In humans, the *Hsp*90 that is secreted extracellularly appears to play an important role in medicinal research because of its significance in binding to surface receptors that mediate invasiveness (Lang et al., 2019). The full capacity of these extracellular components and their importance in livestock is yet to be determined, but there is a good possibility they play a bigger role than initially expected.

***Hsp*70**

The most conserved and most plentiful family of HSP's is *Hsp*70 (Lindquist and Craig, 1988). The *Hsp*70 gene is encoded by a single exon and is 1926 base pairs long, and the protein is comprised of 641 amino acids (Gade et al., 2010). Named for its molecular size (Whitley et al., 1999), this family of HSP's varies from 66 kDa to 78 kDA (Tavaria et al., 1996) and is well known for its role in thermoregulation (Lindquist and Craig, 1988). Along with their expression after heat exposure, they also offer protective properties during other forms of stress, including cold and oxidative stress (Lindquist and Craig, 1988). Interestingly, the expected half-life of *Hsp*70 induced by heat stress is expected to be approximately 48 hours (Mizzen and Welch, 1988). During stress exposures, protein denaturation occurs, but *Hsp*70 has the ability to

maintain the protein integrity by assisting in the folding process of new proteins and also refolding of damaged proteins (Mayer and Bukau, 2005). They act in the cell as in-house components of folding and signal transduction pathways and operate through the interaction of *Hsp*70 with hydrophobic peptide segments of proteins in an ATP-modulated system (Mayer and Bukau, 2005). Much like *Hsp*90, their primary characteristics include N-terminal ATPase domain, substrate binding domain, and C-terminal domain (Schlesinger, 1990; Kiang and Tsokos, 1998).

Hsp70 co-chaperones

Co-chaperones assist HSPs in binding to their target proteins (Mayar and Bukau, 2005). Research has identified several co-chaperones that work together with *Hsp*70, along with other HSPs, but not all are necessarily proven to be present in cattle. In mammalian species it is estimated that there are over 100 different co-chaperones (Caplan, 2003). It is believed that the majority of co-chaperones belong to two classes known as the J-domain proteins (JDP) and tetratricopeptide repeats (TPR) (Caplan, 2003). Over 70 JDPs have been identified in mammalian genomes and a couple hundred TPRs (Caplan, 2003). JDPs are responsible for mediating ATP hydrolysis-dependent locking in the binding region of *Hsp*70 (Mayer and Bukau, 2005). Human HSPs utilize several co-chaperones such as Bag proteins, Hip, HOP, and CHIP (Mayer and Bukau, 2005), but literature is sparser on exactly what co-chaperones cooperate with cattle HSPs.

Hsp70-2 & Hsc70t

Two products of *Hsp*70 are produced specifically in spermatogenic cells (Eddy, 1999). Expression of the *Hsp*70-2 gene occurs during the meiotic phase (Allen et al., 1988; Eddy, 1999; Rosario et al., 1992; Zakeri et al., 1988) and the *Hsc*70t occurs during the post-meiotic stages of

spermatogenesis (Zakeri and Wolgemuth, 1987; Maekawa et al., 1989; Matsumoto and Fujimoto, 1990). In mice, *Hsp*70-2 takes place directly following transcription in leptotene-zygotene spermatocytes (O'Brien 1987) and *Hsp*-2 mRNA is present in spermatids (Eddy, 1999). Because of its presence in male germ cells, it has been studied for its role in spermatogenesis. Eddy (1999) also describes the importance of maturation promoting factor (MPF) in the maturation of spermatocytes. The MPF is a dimer comprised of a catalytic subunit called Cdc2 and a regulatory subunit know as cyclin B (Dunphy et al., 1988). According to Eddy (1999), the Cdc2 gains protein kinase activity upon the binding of cyclin B, which propagates changes in Cdc2 phosphorylation. Apparently, this Cdc2-cyclin B complex is essential for spermatocyte development (Eddy, 1999). Due to its association with the HSP family of molecular chaperones, it is hypothesized that *Hsp*70-2 is a crucial chaperone for germ cell maturation. This hypothesis was supported when Zhu et al. (1997) added a recumbent *Hsp*70-2 to the gonads of mutant mice who regained their ability to form an active kinase resulting from the heterodimer of Cdc2 and cyclin B (Zhu et al., 1997; Eddy, 1999). It is not fully understood if *Hsp*70-2 has other functions and exactly what species require this protein, but the current research implies that it may serve as a key contributor to proper spermatogenesis.

***Hsp*60**

The *Hsp*60 family varies from 58-65 kDa (Whitley et al., 1999) and are large, oligomeric complexes that contain two stacked, heptomeric rings with a central cavity (Frydmann and Hatl, 1994). Similar to most other HSPs, they are hyper-expressed due to stress and are critical chaperones in folding, transport, and assembly of protein subunits (Neuer et al., 2000), but in the presence of stress they can lead to protein denaturation (Frydmann and Hatl, 1994). They primarily localize in the mitochondria (Jindal et al., 1989), but they also have cell surface

properties (Soltys and Gupta, 1996). Because of their location, they are believed to be one of the more important HSP directly related to reproduction (Neuer et al., 2000). Not only has there been a parallel between *Hsp*60 expression and spermatogenic function (Werner et al., 1997), but they also play a significant role in steroid metabolism in the placenta (Olvero-Sanchez et al., 2011). In humans, follicular fluid from patients undergoing in-vitro fertilization had detectable amounts of *Hsp*60 (Neuer et al., 1997). It is unclear the exact purpose for its presence in the follicular fluid, but it is easy to believe that it could be a natural expression during oogenesis.

sHSP

The molecular weight of sHSP range between 15-43 kDa and are commonly referred to as HSPβ (Jee, 2016). They have a high affinity for unfolded proteins and protect against larger, insoluble aggregates. They are not directly responsible for folding proteins, but they indirectly catalyze refolding by directing substrates to larger *Hsp*70 complexes (Cox et al., 2018; Lang et al., 2019). They have the ability to transition from shorter to larger oligomers and are believed to play more of a role in the vascular system (Whitley et al., 1999). Research has linked many of the sHSP to raspatory morphology and cardiac development (Jee, 2016). When released by the placenta during pregnancy, *Hsp*10 is known to be a suppressor of the mother's immune response (Noonan et al., 1979), and in humans, it is often used as a biomarker for endometrial cancer (Dube et al., 2007).

Regulation Through Heat Shock Factors

The regulation of HSPs is mediated by heat shock elements (HSE) that are positioned upstream of HSP genes and available in multiple copies (Akerfelt et al., 2010; Pelham, 1982). Inside the promoter of HSPs are binding sites for heat shock factors (HSF). These HSF provide a near-instant response to stress and alter from its monomer-state into trimer form (Lang et al.,

2019). The heat shock promoter is described as readily accessible and maintains an open chromotin nucleosome-free make-up (Wu, 1984; Mason and Lis, 1997). The primary two factors that bind to the promoter are GAGA factor and TATA binding protein of the TFIID complex (Lis and Wu, 1993). Earlier research showed that transcriptional regulation takes place after HSF binds to cis-acting elements made up of nGAAn pentomers known as HSE, which leads to the induction of HSP genes expression (Wu, 1984; Parker and Topol, 1984). Once the HSF is bound to the DNA, the binding domain recognizes the HSE in the major groove of the double helix (Wu, 1995). In mammals there are four classes of HSFs referred to as the following: HSF1, HSF2, HSF3, and HSF4 (Akerfelt et al., 2010). Akerfelt et al. (2010) referred to HSF1 as the master of HSP gene regulation in vertebrates because of its responsibilities in the transcriptional activation of HSP genes. HSF1 is abundant in most tissues and cell types but is not activated until it is exposed to stress (Akerfelt et al., 2010). As the main activator of molecular chaperones, HSF1 transcriptional activation of HSPs is believed to be rapid and robust (Vihervaara and Sistonen, 2014) and is activated by cyclic AMP-dependent protein during stress exposure (Choi et al., 1991). HSP1 is an inactive monomer that is held together by the binding of three leucine zipper domains that form a triple stranded coiled coil (Calderwood et al., 2010; Zou et al., 1998; Zou et al., 1994). Unfavorable conditions due to stress cause the unraveling of the dormant proteins. It accomplishes this by breaking the bonds between the leucine zippers and trimers interact with the first leucine zipper, which provides its DNA-binding affinity (Zou et al., 1994; Zou et al., 1995).

Further details have been unveiled about how HSFs operate within the promoter region of the HSP gene and provide insight on how stress can induce expression of the HSP gene. The promoters are generally made up of two classes of sequence elements referred to as the core

region and the TATA box (Greene and Kingston, 1990). The core region contains the transcription start site and extends to approximately 40 nucleotides on either side of start site (Weber et al., 1997). The TATA box is approximately 30 nucleotides upstream from the start site and has been linked to upstream activation sequences (Greene and Kingston, 1990). As previously discussed, the heat shock gene promoters contain HSE that possess three repeating nGAAn sequences (Calderwood et al, 2010; Wu et al., 1995). The binding sites for HSF1 trimers are generated from these HSE located in the promoter which generates the rapid response to stress by propagating the activation of transcription (Calderwood et al., 2010; Wu et al., 1995). As illustrated by Vihervaara and Sistonen (2014), it adds to its rapid capabilities by priming the promoter for activation by GAGA factor, transctiption factor IID, and transcriptionally engaged polymerase II. Following the stress encounter, HSF1 unites at the promoter and sparks the recruitment of mediator complexes and positive transcription elongation factor b (Lis et al., 2000; Park et al., 2001). In response to the stressful event, on the Hsp70 gene the carboxyl-terminal domain of the hypophosphorylated Pol II is phosphorylated, resulting in a hyperphosphorylated Pol II in the productive elongated state (O'Brien et al., 1994). Park et al. (2001) also imply that the key regulatory step in the transcription activation of heat shock genes is a result of the mediator complex that is recruited to the promotor. Other work has offered insight on HSP specific pathways. During oxidative stress, a subset of HSPs are expressed through JNK pathway and transcription factor Foxo (Wang et al., 2005) and the menin protein is crucial for prolonged exposure of HSPs (Papaconstantinou et al., 2005). As for the other HSFs, HSF2 is also important for the regulation of HSPs but is dependent upon HSF1 and operates in response to HSF1 by its recruitment to the HSP gene promoters (Ostling et al., 2007). They regulate HSF1-mediated gene expression by forming heterotrimers with HSF1 and progressing

to the target sites (Vihervaara and Sisonen, 2014). In addition to their responsibilities in HSE binding activities, HSF2 may also contribute to the regulation of the heat shock gene during embryogenesis in mice (Mezger et al., 1994). In terms of HSP production, the role of HSF3 and HSF4 are a little more unclear, but they are believed to play a role in other biological processes, such as organ development (Arkerfelt et al., 2010).

HSPs Role in Reproduction

Proper reproduction takes immense coordination of many physiological functions and can easily be altered by environmental stressors. As much as producers try to eliminate stress, most cattle are exposed to some sort of environmental stress at some point during the year. Most people focus on heat stress, but it can actually come in several forms including cold, nutrition, and health stress. An animal's ability to cope with these stressors can directly impact fertility and reproductive performance.

Germ Cell Production and Survival

Male fertility is an integral part of reproduction. As previously discussed, HSPs are present in spermatogenesis and have been associated with sperm cell survival post ejaculation (Dix et al., 1996; Huang et al., 2000). After ejaculation, some of earlier work performed by Huang et al. (2000) showed a correlation between low *Hsp*70 concentrations and reduced motility, morphology, and sperm concentration parameters. To support this work, Elliot et al. (2009) deduced that *Hsp*70 concentrations were linked to extended longevity and viability of spermatozoa. Additional research found that *Hsp*70 exist on the acrosome of ejaculated bull spermatozoa (Kamaruddin, 1998), which implies that it may serve a purpose in gamete interaction when fertilization is taking place (Matwee et al., 2001). Prior to copulation, other data has shown that HSPs are regularly expressed in the testicles during sperm development.

Not only has *Hsp*70-2 been evident in the maturation process of spermatocytes (Eddy, 1999), but both *Hsp*60 and *Hsp*70 have been shown to present on the cell surface (Neuer et al., 2000). According to Govin et al. (2006), *Hsp*70 also has a function in spermatid DNA-packaging proteins at the time of spermatogenesis. In mouse studies, the absence of *Hsp*70 effected the development of normal spermatocytes and increased cell death (Christians et al., 2003). On another note, in humans, endocervical cells exposed to semen resulted in transcription of *Hsp*70 (Neuer et al., 2000). It is certainly possible that the contents of the seminal fluid are capable of stress response. Neuer et al. (2000) offers a theory which explains the process by which the *Hsp*70 expression may inhibit the immune response of the female reproductive tract to the semen, and in turn, allow for the survival of spermatozoa.

Much like in male germ-cell production, oogenesis experiences HSP expression, and it is likely to depend on these events for survival during hyperthermic stress (Neuer et al., 2000). Heat stress can cause multiple abnormalities including multinuclear eggs and a larger sized first polar body (Baumgartner and Chrisman, 1981). Higher temperatures are capable of limiting the number of oocytes that advance to metaphase II and often results in lower fertilization rates (Lenz et al., 1983). It appears that the expression of HSP takes place during earlier steps of oogenesis, and then reduces as the oocyte fully matures and follicular differentiation has taken place (Curci et al., 1987; Curci et al., 1991). *Hsp*60 has been detected in follicular fluid of humans, but this was pre-ovulation (Neuer et al., 1997). In mouse oocytes, *Hsp*70 is present in preovulatory oocytes, as well, but resides following the breakdown of germinal vesicles (Curci et al., 1991). This could explain why mammalian oocytes are so vulnerable to elevated heat.

Fertilization and Embryo Development

The majority of progress that has been made on the understanding of HSP on embryo development has been conducted on species other than bovine animals. Due to limitations of *in-vivo* studies, most of the available literature appears to be *in-vitro*. It is still unknown to what extent other experimental models can be applied to bovine research; however, research in other species provides some groundwork for the possibilities that may take place in cattle. For example, *Hsp*60, *Hsp*70, and *Hsp*90 are all synthesized by murine embryos prior to implantation (Bensaude et al., 1983). Likewise, the induction of *Hsp*70 antibodies retarded the development of embryos to the hatched blastocyst stage (Neuer et al., 1998). A year following, work by Neuer et al. (1999) concluded that embryos cultured with HSP antibodies had a higher degree of DNA fragmentations.

In what literature is available on HSPs role in bovine embryo development, it appears to follow suit with what has been determined in other species. The first results presented focused on the effects of anti-*Hsp*70 on sperm-oocyte interaction (Matwee et al., 2001). They reported that anti-*Hsp*70 significantly reduced the number of spermatozoa tightly bound to the zona pellucida, suggesting that *Hsp*70 is beneficial to gamete interaction (Matwee et al., 2001). They also reported a significant loss in development when embryos were cultured in anti-*Hsp*70 at a concentration of 50 µg/ml; however, the response was dose-dependent because the effects were minimal at a concentration of 1µg/ml. They also discuss the possibilities of HSP present in the spermatozoa playing a role in early embryo development post fertilization. Since the activation of the embryonic genome does not begin at the onset of early cleavage, they may offer a formattable defense against stress during the early cleavage stage (Matwee et al., 2001). Although research has shown 2-cell bovine embryos do contain HSPs and synthesis does take

place that early in the presence of heat stress, Matwee et al. (2000) imply that it is probably not until the 4-cell stage that regulation at the transcription level takes place. It could also be that the role they play in luteal maintenance is related to embryo survival. Khanna et al. (1994) found that HSPs were key mediators in luteal regression, which could obviously impact development since its primary function is to produce the hormone responsible for maintaining pregnancy.

HSP Effect Steroidogenesis

Heat shock proteins play an important role in steroidogenesis which is directly related to many biological processes including reproduction. Like many other cells, steroidogenic cells respond to stress by increasing *Hsp*70 production (Murphy et al., 2001). Much like steroid cells, many inhibitors for steroid production such as PGF2α, ionomycin, and TNFα, also respond to heat stress by increasing *Hsp*70 (Khanna et al., 1995). That same author reported that *Hsp*70 negated the inhibitory effects of PGF2α on progesterone production by the corpus luteum (Khanna et al., 1995). Research has shown that heat shock effects alter steroid production by disrupting steroidogenic acute regulatory protein (StAR) expression at the transcriptional level (Murphy et al., 2001). They believe it does so by acting on the promoter that is responsible for basal and cAMP inducible expression of StAR (Murphy et al., 2001). To further explore the mechanisms by which the process of steroidogenesis copes with heat stress, Oka et al. (2017) reviewed the mechanisms by which HSF1 influences leydig cell steroidogenesis. They concluded that HSF1 maintains cholesterol transport and protects StAR protein. This, of course, has important implications in steroidogenesis in the male, as testosterone production starts with cholesterol transport into the mitochondria due to StAR protein in the leydig cells (Oka et al., 2017).

Polymorphisms in the Hsp70 Gene

Single nucleotide polymorphisms (SNPs) in the HSP gene have gained interest as potential biomarkers for forecasting stress tolerance in cattle. We know that HSPs are critical components to maintain normal cell functions, but polymorphisms within the genes have not been studied in its full capacity. Many researchers in the human field have detected an association between SNPs in the *Hsp*70 gene and human health concerns, primarily cancer (He et al., 2014; Partida-Rodriguez et al., 2010; Wang et al., 2010). Because of their relationship with protein stabilization, they have a beneficial relationship with several immune cells and different haplotypes seem to affect the person's response to potential stressors (He et al., 2014). Surprisingly, some SNPs in *Hsp*70 genes associated with an increased risk to cancer while other SNP's had no relationship (He et al., 2014). They reported that *Hsp*70-2 was associated with increased cancer risk but not SNPs in *Hsp*70-1. Their work agrees with that of Guo et al. (2011), who also failed to correlate SNPs in *Hsp*70-1 to cancer. Alternatively, other studies have reported an association with specific types of cancer (Partida-Rodriguez et al., 2010; Wang et al., 2010). In addition to cancers, other research has linked SNPs to health factors such as diabetic nephropathy (Buraczynska et al., 2009), and chronic obstructive pulmonary disease (Matokanovic et al., 2012). Research in this area is not as advanced in cattle as it is humans, but SNPs in the *Hsp*70 gene are likely to have similar associations in cattle.

In cattle, SNPs have been linked to multiple production traits that could directly correlate to the bottom-line profits of a cow herd. One of the more obvious associations was with thermotolerance (Bhat et al., 2016; Li et al., 2011). This association is shown in both beef (Bhat et al., 2016) and dairy cattle (Basirico et al., 2011; Li et al., 2011). Knowing of this association, alone could possibly explain the remainder of associations that have been discovered. For

example, SNPs in the promoter region were associated with the weaning weights of cross bred calves (Starkey et al., 2007). Their work highlighted a tendency between a cytosine deletion at base 895 and increased weaning weights. Other factors that could influence both growth and reproduction in cattle include milking parameters. SNPs in the *Hsp*70 gene have been shown to impact milking traits such as mastitis (Cheng et al., 2009). Both of those studies focused on traits that are of more importance once the live offspring is on the ground. Other work has shown an association between SNPs and overall reproductive traits that correlate to even getting a live calf. In the promoter region, SNPs were associated with pregnancy rates of crossbred cows (Banks et al., 2007). Similar research conducted by Rosenkrans et al. (2010), indicated that cows that were homozygous for the minor allele at transversion site A1125C or G1128T has lower calving percentages, than the cows that were homozygous for the major allele. In the same study, they concluded that cows with a deletion for cytosine at base 895 not only had decreased calving percentages but also had a later Julian calving date (Rosenkrans et al., 2010). This is the same deletion that Starkey et al. (2007) associated with heavier weaning weights in crossbred calves. These two studies could describe a potential conflict that takes place in selecting females in beef cattle herds. After all, it is likely that commercial cattlemen are tempted to keep females in their herd that raise the fastest growing calves with heavier pay weights, but Rosenkrans et al. (2010) imply that these cows may also be less productive in terms of reproduction. Although the majority of research has focused on cow production traits, SNP in the *Hsp*70 gene may also affect male fertility. In goats, SNPs in the *Hsp*70 were associated with sperm concentration and motility characteristics (Nikbin et al., 2014). They also correlated sperm cryopreservation characteristics to SNPs. SNPs in the *Hsp*70 gene also have an association with boar semen characteristics (Huang et al., 2002).

All of these traits could be a direct result of their ability to cope with thermal stress. In addition, SNPs in the HSP genes appear to have a breed type association, which would explain differences in heat tolerance between breeds (Lamb et al., 2007). There work determined that the majority of SNPs found were present in Brahman cattle (Lamb et al., 2007). As mentioned before, identification of SNPs in the *Hsp*70 gene could serve as a biomarker for selecting animals to suit specific production environments.

Summary

Both *Neospora caninum* infections and environmental stressors can be detrimental to reproductive performance in beef cattle. To make matters worse, it is possible for multiple stressors to work together. Due to the nature of *Neospora caninum* infections and the inflammation response that may come with it, along with the stress induced mechanisms of *Hsp*70, it is conceivable to believe that *Hsp*70 genotypes could be associated with the mother's immune response to the infection. Regardless, both of the two show capabilities of influencing pregnancy status and outcome, which justifies the need for additional research. Specifically, there is a need for research that will help producers in the United States know how to select cattle that will be suited for their environment.

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Incidence of *Neospora Caninum* Infection in Beef Cattle and its Relationship with Reproductive Performance

Abstract

Neospora caninum is an infectious protozoon that has been linked to reproductive failure. It is primarily recognized for its association with abortions, but also causes substantial economic losses by affecting milk yield, weight gains, and calf morbidity. Cost estimates are difficult to fully quantify, but global estimations suggest *Neospora caninum* is responsible for billions of dollars in losses annually. This study tested for the incidence of *Neospora caninum* in beef cattle located in the central region of the United States and evaluated the relationship between seropositive animals and production characteristics. The study consisted of two trials. Trial one was an epidemiology population-study testing for the incidence of the infection in open, beef replacement heifers (n = 1306), and it yielded an infection rate of 6.9%. Trial two utilized serum samples collected from breeding-age beef cattle (n = 500) and evaluated the relationship that location, age, breed, farm dogs, and feeding mixed rations had with the infection rate and pregnancy outcome. Based off a serology test, the infection rate was 9.6%. There was a relationship ($P < 0.05$) between state residency of cows, cow age, and seropositive infection rates, but not between the number of seropositive animals and breed, number of farm dogs, or feeding a mixed ration ($P < 0.1$). While serostatus had no effects ($P > 0.1$) on pregnancy rates across the population, in Oklahoma fewer ($P < 0.05$) seropositive cows were pregnant. Overall, this project provides evidence that *Neospora caninum* is prevalent in the central region of the Unites States and may explain decreased reproductive rates for cattle in some areas.

Keywords: Abortion, Cattle, Infection, Protozoa, Neospora caninum

Introduction

Neospora caninum is an infectious protozoon that is a member of the *Apicomplexan* family (Reid et al., 2012) and has been linked to reproductive failure in bovine animals (Hassig and Gottstein, 2002; McAllister et al., 1996). It is transmitted vertically by trans-placental contamination during gestation (Barr et al., 1994; Hall et al., 2005), and also after parturition, through the ingestion of contaminated food and water sources (De Merez et al., 1999; Trees et al., 2002). Economic ramifications from this disease are difficult to quantify, but global estimations have been as high as $2.38 billion annually (Reichel et al., 2013). The United States dairy industry accounted for $546.3 of this dollar figure, suggesting it can have substantial financial impacts on the United States agricultural economy (Reichel et al., 2013). It is widely recognized as one of the leading causes of abortion in bovine animals. Because of this, the majority of financial losses are attributed to abortions (Gondim et al., 2004), which have been reported as high as 90% within some herds (Dubey, 2003); however, *Neospora caninum* is also responsible for decreased milk yields (Hernandez et al., 2001; Romero et al. 2005), decreased gains (Barling et al., 2001), morbidity in offspring (Monney et al., 2011), delayed breed back (Waldner et al., 1998), and increased culling rates (Waldner et al., 1998).

Members of the *Canis* genus are recognized as the primary definitive host for the parasite (Donahoe et al., 2015), and they are responsible for shedding oocysts that lead to exogenous infections in cattle. Cattle, along with many other warm-blooded animals, serve as secondary hosts (Donahoe et al., 2015). Because of its similarities to *Toxoplasma gondii*, it has previously been misdiagnosed. It is also very difficult to diagnose due to the nature and timing of abortions because, often, fetal tissue is never recovered for testing. For this reason, it is likely that *Neospora caninum* is responsible for more reproductive issues than is ever recognized.

Much of the previous work has been performed on dairy cows and has taken place in other countries (Almeria et al., 2009; Bartels et al., 2007; Frossling et al., 2005; Romero et al., 2005; Lopez-Gatius et al., 2004, Dijkstra et al., 2003; Otranto et al., 2003; Hassig and Gottstein, 2002; Bergeron et al., 2000; Bjorkman et al., 2000; Bartels et al., 1999). Research is limited on the relationship *Neospora caninum* may have on the beef industry. While there is data available from studies that have taken place here in the United States, more work needs to be conducted domestically to determine the severity and outcome of the infection (Barling et al., 2001; Sanderson et al., 2000; Thurmond et al., 1995; Anderson et al., 1991). If producers can perform a serological test to identify and cull infected cows out of their herd and also as a selection tool for selecting replacement females, then it may pay dividends for improving reproductive success and financial gains. This study was conducted to test for the incidence of *Neospora caninum* infections in the central plains of the United States and to determine the relationship with reproductive performance.

Materials and Methods

Trial 1: Incidence of Neospora Caninum in Open Replacement Heifers

The first trial tested for the incidence of *Neospora caninum* in non-pregnant beef heifers (n=1306) being developed for replacements and also evaluated herd effects on the rate of seropositive animals. Blood samples were collected prior to breeding from four different herds located in Illinois, Kansas, and Missouri. All serum was tested using the ELISA test (IDEXX Laboratories, Inc., Westbrook, MA).

Trial 2: Incidence of Neospora Caninum in Beef Cows

Blood samples were collected from 25 beef cattle operations located in a five-state region of Arkansas, Kansas, Missouri, Ohio, and Oklahoma. The cows (n = 500) were randomly

selected from each herd, and all were from operations with at least 50 head. The samples were tested for pregnancy-status first using bioPryn Flex (Biotracking, Inc., Moscow, ID), and then 20 samples from each herd (10 pregnant and 10 open) were tested for *Neospora caninum* using the Idexx Neospora X2, ELISA test (IDEXX Laboratories, Inc., Westbrook, MA). Data for production characteristics also were collected for stage of pregnancy, cow age, number of farm dogs, forage type, and use of mixed-ration feeding.

Serum Preparation and Analysis

Whole blood was received in BD vacutainer blood collection tubes. Serum used for pregnancy status was prepared using the bioPryn Flex protocol (Biotracking, Inc., Moscow, ID). Serum for *Neospora caninum* testing was prepared using the IDEXX Neospora X2 protocol (IDEXX Laboratories, Inc., Westbrook, MA).

Statistical Analysis

Trial 1: This trial was an epidemiology study that utilized a randomized design to perform a general population scan. Differences were tested with general linear mixed models using the GLIMMIX model in SAS. When F-tests were different ($P < 0.05$), least squares means were separated using multiple t-tests with Tukey-Kramer adjustment.

Trial 2: The second trial was a randomized complete block design. Within each herd (blocking factor), animals were categorized by pregnancy status as "open" or "pregnant" and *Neospora caninum* "negative" or "positive". Differences were tested with general linear mixed models using the GLIMMIX model in SAS. When F-tests were different ($P < 0 .05$), least squares means were separated using multiple t-tests with Tukey-Kramer adjustment. For statistical purposes, breed type was categorized into two groups (straightbred or crossbred), and

cow age was categorized into four groups (1 = heifers less than 2 years, 2= 2 years old, 3 = 3 - 6 years old, and 4 = 7 - 12 years old).

Results

The incidence of seropositive animals in the first trial testing for *Neospora caninum* in open replacement heifers was 6.9% (Table 2.1). In trial two, the overall seropositive infection rate in the beef cow population was 9.6%. Only four of the twenty-five herds tested did not yield at least one positive animal. The state in which the herd was located had a significant ($P < 0.0001$) effect on the rate of infection (Figure 2.1). Infection rate by state for the states that had multiple herds tested were Arkansas (27.5%), Missouri (13.3%), Oklahoma (5.6%), and Kansas (4.3%). Arkansas differed ($P < 0.001$) from Oklahoma and Kansas (Figure 2.1). The overall infection rate for all states had no effect on pregnancy status nor the pregnancy outcome, except for in Oklahoma where seropositive animals had a lower ($P < 0.05$) percentage of pregnancies (Table 2.2). Seropositive infection rate was greatest ($P = 0.02$) for cows that were 7 – 12 years old (Figure 2.2). There were no differences ($P > 0.1$) observed for breed, number of dogs, or cattle fed a mixed ration.

Discussion

For years, *Neospora caninum* infection rates were thought to be primarily an issue for dairy cattle due to the nature of how they were confined and managed. Based on a commercial serological test, this study demonstrates that grazing beef cattle are susceptible to this parasite with an overall infection rate of *Neospora caninum* at 9.6%. It is possible that the incidence could be even higher than detected because antibody levels can fluctuate in seropositive animals (Dubey et al., 2007; Dubey and Schares, 2011; Hietela and Thurmond, 1999). Therefore, latently infected cows do have the ability to fall below detectable levels only to experience a

recrudescence during mid-late gestation (De Marez et al., 1999; Lindsay et al., 1999). Due to the nature and timing of our study, all cows were tested during the first 120 d of bull exposure, so if a cow had developed the ability to suppress the infection, they may not have experienced the resurgence of antibodies. Of the states we tested, Arkansas had the highest sero-prevalance with a startling figure of 27%. Other articles support the notion that infection rates can be highly geographical (Dubey et al., 2007). There are several explanations why infection rates may drastically vary in different regions. According to Otranto et al. (2003), it could be that stocking rates influence infection rates. While stocking rates can drastically vary within regions due to many variables, it is conceivable that Arkansas and Missouri pastures support more animal units per acre than Kansas and Oklahoma. If this theory is true, then it would match the results of our study in which Arkansas was the highest incidence of infection and Missouri was the second highest. Even though all of the herds tested were located in the central portion of the United States, there is still noticeable differences in climate and rainfall in this region. Yaniz et al. (2010), along with Wouda et al. (1999) showed that rainfall, humidity, and season could play a role in the infection rates. This is quite possibly because the environment becomes more favorable for the parasite survival outside the hosts. Of the states we tested, the states with the highest annual relative humidity and annual rainfall also had the highest infection rate (National Weather Service). Another explanation for the significant interaction observed between states is the potential for increased populations of definitive hosts, such as dogs, coyotes, and other wildlife in some states versus others. According to Dubey et al. (2007), the closer the farm is to areas of increased human populations, the greater the risk of infection. They suggest this may be due to the fact that coyotes and dogs are more likely to congregate in these areas.

Results from this study also revealed that age has a significant effect on serostatus. The two age groups with the most seropositive animals were the second-calf heifers and also the aged cows. If previous research proving that immunosuppressors can affect the dams serostatus is applicable to all beef herds, it is possible that heifers bred with their second calf and milking their first, along with aged cows, could experience the most nutritional stress, which could explain the age effects we observed.

Our study also revealed differences in serostatus between herds with one herd being as high as 50%. The herd in which open replacement heifers were developed was associated with *Neospora caninum* infection rates. One group of 61 potential replacement heifers yielded 19 (31.6%) seropositive heifers and another group was 33%. While the study on open replacement heifers had a total incidence of 6.2%, it is worth noting that the majority of heifers from that study came from a herd that had tested for *Neospora caninum* infection and culled routinely for the past three years. Heifers from other herds in that study had a seropositive rate of 10.2%. While this information is merely anecdotal, it is in agreement with research by Larson et al. (2004) that suggests that testing and culling is a beneficial management tool for reducing infection rates within a herd. In addition, previous research found that seropositive, first-calf heifers experienced a higher rate of abortion in their first pregnancy than seronegative heifers (Lagomarsino et al., 2019; Brickell et al., 2010; Thurmond and Hietala, 1997). Research from Brickell et al. (2010) not only concluded that they were at a higher risk of abortion in their first and second pregnancies, but also showed that they were more likely to have perinatal death loss. Interestinlgy, they didn't observe any differences in conception rates or age at first conception. Albeit in dairy heifers, another heifer trial found that 61% of seropositive heifers experienced abortions compared to only 3% of seronegative heifers (Weston et al., 2005). If this is applied to

the group of open heifers in our first trial, then that group would have likely bred up at similar rates, regardless of serostatus, and experienced pregnancy losses in approximately 55 of those seropositive females. Depending on the cattle market and how producers value their time, this figure would more than justify testing and culling by using serological tests prior to selecting replacements. This would not guarantee the females stay seronegative for life because they could be infected through trophic transmission at a later date, but it would serve as a valuable first step in managing the adverse effects of *Neospora caninim* infections.

Although previous research has concluded that seropositive cows are more likely to abort pregnancies than seronegative cows (Lopez-Gatius et al., 2004; Pare et al., 1997; Thurmond and Hietala, 1997), our results indicate that other factors such as geographical location and operation are related to the effects of seropositive status on pregnancy outcomes. There are studies that are in agreement with our results, showing that infected dams are not always at risk for pregnancy loss (Dijkstra et al., 2002; Romero et al., 2005). In herds with elevated endemic abortions, first-calf heifers are at greater risk for experiencing an abortion than cows that are past their first lactation (Thurmond and Hietala, 1997). Our study included a low percentage of first calf heifers that were seropositive, which may explain why our results differ from some of the previously published work. Perhaps the best explanation for this discrepancy is the possibility that cows used in this study were born seropositive. If this were the case, then statistics show they are less likely to lose their pregnancy than naïve cows that develop an infection after they are born (Williams et al., 2003). Endogenous *Neospora caninim* infections result in different outcomes than exogenous infections (Dubey et al., 2007). We do not know the previous reproductive status of the females tested in our study, but if they had aborted previously then their risk of abortion would have been lower during the timecourse of this study; abortion risks

decrease with subsequent pregnancies in cows that were born seropositive (Thurmond and Hietala, 1997). Management practices, for our collaborators that developed heifers in our first study, resulted in the culling of seropositive heifers; therefore, subsequent testing for longitudinal observations of serostatus and pregnancy outcomes was not possible.

The ELISA test has been referred to as the best test for detecting *Neospora* infections at the herd level (Almeria, 2013; Frossling et al., 2003; Pare et al., 1995); however, the commercially available ELISA for detecting pregnancy does not have the capability of staging pregnancies, so if *Neospora caninim*-infected cows lost a pregnancy early and bred back prior to pregnancy testing those individuals would not have been identified in our study. Even though there are reports available that support the theory of early pregnancy loss, the majority of *Neospora*-induced abortions were reported in mid-gestation (Williams et al., 2000; Dubey et al., 2007; Gibney et al., 2008; Almeria and Lopez-Gatius, 2013; Regidor-Cerillo et al., 2014). Recent research has focused on strain potency and their relationship with pregnancy outcome (Jimenez-Pelayo et al., 2019; Rojo-Montejo et al., 2009). Perhaps cattle in our study were exposed to different strains of *Neospora caninim* which could explain some of the variation in serostatus impacts on pregnancy outcomes in our study. Identifying *Neospora caninim* strains, their virulence and impact on bovine pregnancies in central USA is a topic for future research.

Lastly, the climate and weather patterns during the period when the project was conducted may have influenced the pregnancy outcome and lack of abortions in seropositive animals. Atleast one recent multi-year study concluded that "year" had a relationship with pregnancy outcome in seropositive animals (Lagomarsino et al., 2019). In our study, rainfall in the testing region was above average (National Ocean and Atmospheric Administrations); in turn, forage availability and quality may have been superior to most years. Fall-calving cows, in

particular, would have benefited from that increased rainfall and nutritional plane of cattle in this region. Stressors, including plane of nutrition, can compromise immune function and work in conjunction with *Neospora caninum* to increase the risk of abortion (Yaniz et al., 2010; Dubey et al., 2007; Bartels et al., 1999).

Conclusion

Neospora caninum infections in cattle can substantially influence reproductive success and profit margins. This study provides evidence that beef cattle located in the central region of the United States are at risk of developing *Neospora caninum* infections providing a possible explanation for unexplained fetal loss. The commercial kit used in our study to determine serostatus of *Neospora caninum* infection may serve as management tool for producers and veterinarians for culling decisions; however, additional research is needed to determine the most beneficial testing and culling protocols for management of the parasite.

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Appendix

Figures and Tables

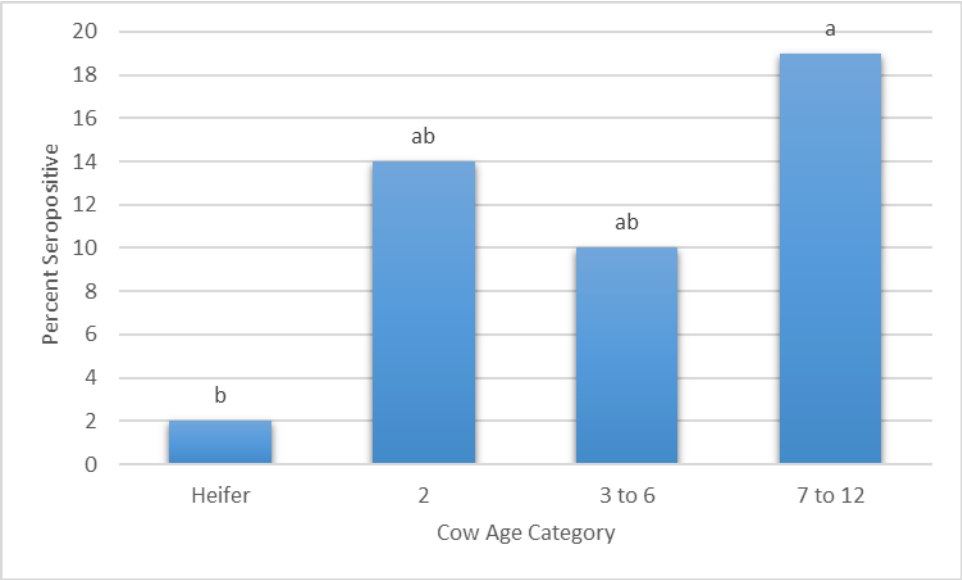

Figure 2.1 Percentage of cows, based on age classification, that were seropositive for *Neospora caninum*. Classifications were heifers less than 2 years of age (n = 107), females 2 years of age (n = 79), cows 3 to 6 years of age (n = 177), and cows 7 to 12 years of age (n = 77). Values in columns without a common superscript differ ($P < 0.05$).

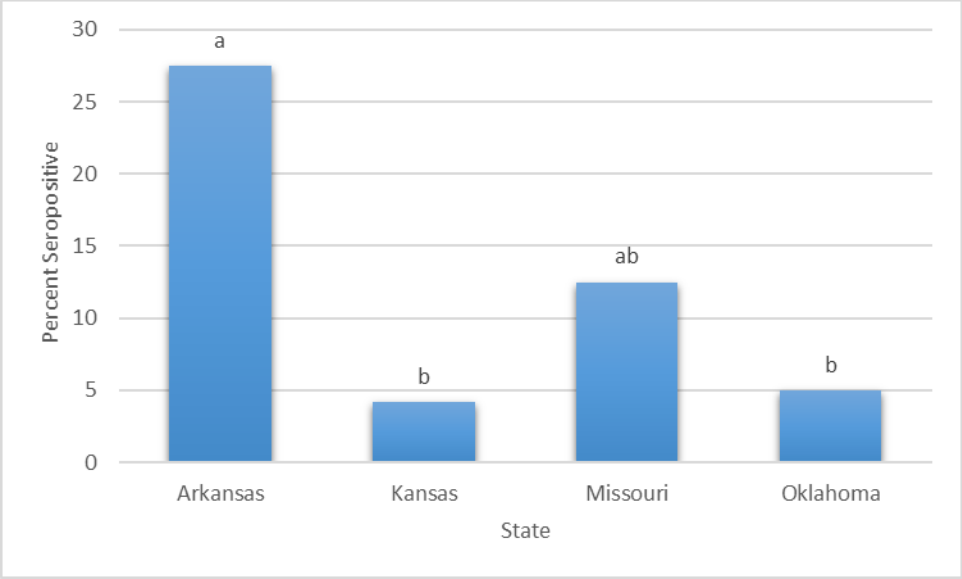

Figure 2.2 Percentage of cows that were seropositive for *Neospora caninum* by state of residency. Arkansas is represented by four herds (n = 80), Kansas is represented by seven herds (n = 140), Missouri is represented by four herds (n = 80), and Oklahoma is represented by nine herds (n = 180). Values in columns without a common superscript differ ($P < 0.05$).

Table 2.1 *Neospora caninum* seropositive incidence in virgin heifers (Trial 1).

Herd ID	State	# Tested	# Seropositive	% Seropositive
1	Illinois	61	19	31.1[a]
2	Kansas	313	35	11.1[b]
3	Kansas	85	3	3.5[cd]
4	Missouri	296	18	6.0[c]
5	Missouri	551	16	3.0[d]
Total	-	1306	91	6.9

[a,b,c,d]percentages without common superscript differ ($P < 0.05$).

Table 2.2 State effects on *Neospora caninum* infection rate and pregnancy status (Trial 2).

Pregnancy Status	Arkansas	Kansas	Missouri	Oklahoma	SEM	*P* value
Open	27.5[a]	4.3[b]	13.3[ab]	10[ab]	0.0575	0.0626
Pregnant	27.5[a]	4.3[b]	13.3[ab]	1.3[b]	0.0301	< 0.001
Total	27.5[a]	4.3[b]	13.3[ab]	5.6[b]	0.0389	0.002

[a,b]within rows, percentages without common superscripts differ ($P < 0.05$). For each herd within each state an equal number of pregnant and open females were tested for infection with *Neospora caninum*.

Relationship between Heat Shock Protein 70 and Beef Cattle Production

Abstract

Heat shock proteins are widely recognized for their role in protecting proteins and maintaining normal physiological functions in the body. Besides their protein chaperoning capabilities, they have been associated with profitability traits in cattle. This project tested the relationship between heat shock protein-70 (*Hsp*70) genotypes and beef heifer reproductive and blood characteristics. Whole blood was collected from virgin replacement-beef heifers (n = 165) and sent to a commercial laboratory for genotyping (Neogen Genomics; Lincoln, NE). Pelvic area (PA), reproductive tract score (RTS), and body weight (BWT) were determined pre-breeding. All heifers were synchronized, artificially inseminated (AI), and 14 days after AI clean-up bulls were placed with each group of heifers. Four single nucleotide polymorphisms associated with the *Hsp*70 gene [promoter (A1125C and C895D) and coding region (G1851A and G2033C)] were evaluated for their relationship with PA, RTS, WT, pregnancy status, and blood cell distribution. Minor allele frequencies were 40, 6.6, 3.6, and 14%; respectively for A1125C, C895D, G1851A, and G2033C. Mutation C895D was associated ($P = 0.04$) with the percentage of circulating lymphocytes (78.7 vs. 69.5; respectively CC and CD). Compared to homozygous (GG) heifers the heterozygous heifers at G2033C had larger ($P < 0.05$) PA but lower ($P < 0.05$) pregnancy rates (172 vs. 184 cm^2, and 91.8 vs. 78.1%; respectively). Neither A1125C nor G1851A affected ($P > 0.1$) heifer traits of interest. Mutations associated with *Hsp*70 impacted reproductive characteristics of beef heifers. Relationships among cellular mechanisms and phenotypic traits will be the subject of future projects.

Keywords: Heat Shock Proteins, Cattle, Reproduction, Genetics

Introduction

Profit potential of beef cattle operations relies heavily on the fertility and reproductive success of their cows and reproductive failure can be costly for cattle operations (Bellows et al., 2002). Non-pregnant cows along with cows that breed later than their contemporaries can be less profitable for commercial cattle producers (Prevatt et al., 2009). Additionally, the breeding rates of first-time heifers are traditionally lower than mature cows and age at first conception can affect lifetime productivity (Patterson et al., 1992). If livestock producers had access to a selection tool to help identify more fertile females prior to replacement selection it would serve as a valuable management tool.

Heat shock proteins (HSP) are molecular chaperones that are synthesized naturally in response to stressors and are important molecules for managing normal physiological functions (Tower, 2011). The HSP family of proteins have been related to oogenesis (Curci et al., 1987, Curci et al., 1991), gamete interaction during fertilization (Matwee et al., 2001), and steroidogenesis (Khanna et al., 1995; Murphy et al., 2001). At the organismal level, genotypes within upstream elements of the *Hsp*70 gene were associated with calving percentage and Julian calving date (Rosenkrans et al., 2010). Collectively, those reports suggest that *Hsp*70 genotypes may be predictive of reproductive success in beef cattle. The aim of this study was to determine the relationships between *Hsp*70 genotypes and reproductive success and blood cell distributions of virgin-beef heifers.

Materials and Methods

Yearling Angus and crossbred Angus-influenced heifers managed at two operations (Operation 1, n = 99; Operation 2, n = 66) had an average body condition score of six. Thirty days prior to artificial insemination (AI) body weight, pelvic area (PA), and reproductive tract

score (RTS; score 1-5, Pence et al., 1999). Heifers not meeting the minimum PA of 150 cm^2 and RTS of three or higher were culled from the project. All retained heifers were synchronized using the 14-day CIDR®+Prostaglandin protocol and timed AI at 66 h following the synchronization protocol (Johnson et al., 2013). Clean-up bulls were placed with the heifers 14 d following AI, and heifer pregnancy was determined by ultrasound at 90 d following AI. At the time of pregnancy detection, blood samples were collected and transported on ice to the lab where blood cell distribution was determined (Cell-Dyne 3500 hematology analyzer; Abbott Laboratories, Abbott Park, IL). Mutations associated with *Hsp*70 gene were genotyped (Table 3.1) from whole blood at Neogen Genomics (Lincoln, NE).

Statistical Analysis

Experimental design was a randomized complete block with operation serving as the block. Quantitative traits were analyzed with heifer as experimental unit, and main effects of operation and mutation site genotype. Each of the four single nucleotide polymorphisms (SNP; A1125C, C895D, G1851A, and G2033C) were analyzed independently by mixed model ANOVA (SAS Inst. Inc., Cary, NC). When F-tests were significant ($P < 0.05$) means were separated using multiple t-tests with Tukey-Kramer adjustment. Genotype associations with pregnancy status were determined using Chi-square tests.

Results

Identification of Polymorphisms

Table 3.1 presents base sequences for the four mutations associated with the *Hsp*70 gene that were evaluated in this study; two SNP sites were in the upstream elements (A1125C, and C895D) and two were in the coding sequence (G1851A, and G2033C). Minor allele frequencies were 40, 6.6, 3.6, and 14%; respectively for A1125C, C895D, G1851A, and G2033C (Table 3.2).

Base Position 1125

At 1125 bases upstream of the putative start site a transition (adenine to guanine) was identified and labeled as A1125C. Genotypes of A1125C (Table 3.2) were not associated ($P > 0.1$) with reproductive traits or blood cell differential counts (Table 3.3).

Base Position 895

An insertion/deletion was identified at 895 bases upstream of the putative start site and was labeled C895D. Genotypes at C895D were not associated ($P > 0.17$) with reproductive traits and most blood cell parameters. However, mutation C895D genotypes were associated ($P = 0.04$) with the percentage of circulating lymphocytes (78.7 vs. 69.5; respectively CC and CD; Table 3.4).

Base Position 1851

A transition of guanine to adenine was observed at base position 1851, located in the coding region (labeled G1851A). There were 140 heifers' homozygous guanine, 11 heterozygous heifers, and zero heifers that were homozygous for the minor allele (Table 3.2). Genotypes at SNP G1851A were not associated ($P > 0.16$) with reproductive traits or blood cell differentials (Table 3.5).

Base Position 2033

At base position 2033 in the coding region, a transversion of guanine to cytosine was detected and labeled G2033C. No homozygous minor allele heifers were observed at G2033C (Table 3.2). Compared to homozygous (GG) heifers the heterozygous heifers at G2033C had larger ($P < 0.05$) PA but lower ($P < 0.05$) pregnancy rates (172 vs. 184 cm^2, and 91.8 vs. 78.1%; respectively; Table 3.6). Heterozygous heifers also had a lower ($P = 0.04$) number of WBC.

Discussion

Previous research has demonstrated that *Hsp*70 polymorphisms are related to reproductive fitness (Rosenkrans et al., 2010; Ortega et al., 2016). Our study provides additional evidence that *Hsp*70 genotypes can be used to improve reproductive success in beef heifers that had previously met culling criteria for minimum PA and RTS. Heifers with the transversion modification of guanine to cytosine (G2033C) had lower pregnancy rates. Because fertility and pregnancy establishment are so crucial for economic success when raising beef cattle, uncovering genetic markers linked to pregnancy rates could be huge for the industry. Other scientists have recorded effects of G2033C genotype on multiple cattle production parameters. Specifically, Brown et al. (2010) reported differences in milk characteristics. Another project concluded that same SNP affected weaning weights and cow efficiency (Finney, 2018).

This is one of the first projects that related *Hsp*70 genotypes to PA. This is substantial because dystocia accounts for millions of dollars in economic losses annually (Patterson and Herring, 2017). Calf death loss is four to eight times higher in cows that experience difficulty in calving than in normal births (Patterson and Herring, 2017). Interestingly, the same group of heterozygous G2033C females that had a larger mean PA experienced lower pregnancy rates. While it would have been ideal for producers if the G2033C genotype benefited both PA and pregnancy rates simultaneously, reader is reminded that all heifers far exceeded the minimum PA for replacement beef heifers.

Lack of relationships between non-G2033C *Hsp*70 mutation sites and pregnancy rates in this study was not consistent with previous research. Cows with two cytosine bases at mutation C895D had higher calving percentages than C895D cows with one or zero cytosine (Rosenkrans et al., 2010). Since then, other studies have determined that deletions in the promoter region for

*Hsp*70 can affect embryo survival (Ortega et al., 2016) and can influence the number of cleaved embryos that advance to the blastocyst stage (Cochran et al., 2013). In the study by Ortega et al. (2016), the deletion appeared to benefit embryo development; however, more recently, it was discovered that cows with a deletion of cytosine at base 895 had a significantly later Julian calving date (Finney, 2018). Contrasting results in between these studies only validates the need for more research to be performed on this topic.

Even though there was variation amongst genotypes within the tested population, a strict culling process was performed on heifers prior to the study, which eliminated heifers that tested positive for *Neospora caninum*, had a PA less than 150 cm^2, and RTS lower than three. Because all of these traits have been linked to reproductive performance, it is reasonable to believe that the culling process reduced the amount of variation in genotypes we had in our test group and may have removed many of the poorer performing females. In future studies it would be valuable to include the females that failed to meet that criteria into the test population, but it is difficult to convince collaborators to retain heifers of questionable value.

Available research describing the association G2033C had on WBC data is limited. Even though there were differences observed in this study, according to Oliver et al. (2000), along with the Animal Health Diagnostic Center at Cornell University, the WBC data were within normal ranges for cattle. Therefore, the impact of this genetic relationship on herd health has yet to be determined, but it is good for the scientific community to know the relationship exists. The genetic relationships of *Hsp*70 and production of HSP in response to stressors and ultimately exhibited in phenotypic traits is a topic for future research. Results from this study support the need for more research describing the effects of *Hsp*70 genotypes on immune function and to

what extent this information can be used when cattle producers are making management decisions.

Conclusion

Reproductive performance and fertility is a critical component for economic success in a beef cow-calf operation (Bellows et al., 2002). The expense of purchasing or developing replacement heifers can greatly impact sustainability of a cattle enterprise (Perry et al., 2009). By identifying and culling low performing heifers from the herd prior to the expense of developing them as replacements, producers could increase their economic and operational efficiency. Results from this study support the previous work that *Hsp*70 genotypes are associated with cattle reproductive success and could be used in conjunction with other selection criteria for developing beef heifers.

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Appendix

Tables

Table 3.1 Mutations associated with Hsp70 gene that were evaluated in this study.

SNP[a]	Sequence	Reference
A1125C	GACCGCCCGAGGGGCACCAG[A/C]GCGTTCA GTTTTCGGGTTCC	Rosenkrans et al., 2010
C895D	GCCAGGAAACCAGAGACAGA[C/D]CCTACGC AGGAGTAGGTGGT	Rosenkrans et al., 2010
G1851A	GAAGAGCGCCGTGGAGGATG[G/A]CTTGGAA GTAAACAGAAACGGG	Brown et al., 2010
G2033C	CTGGCGGCTTTGGGGCTCAGG[G/C]CCCTAAA GGGGGCTCTGGGTGG	Brown et al., 2010

[a]Single nucleotide polymorphism occurred at the site indicated. The first letter represents the primary allele and the letter following the numbers indicates the minor allele.

Table 3.2 Genotypic distribution for polymorphisms associated with bovine *Hsp*70.

	Genotype Distribution[b]			
Polymorphism[a]	Homo	hetero	homo	MAF[c], %
A1125C	49	81	19	40
C895D	131	20	0	6.6
G1851A	140	11	0	3.6
G2033C	110	41	0	14

[a]Single nucleotide polymorphism occurred at the base indicated. The first letter represents the primary allele and the letter following the numbers indicates the minor allele.
[b]Homo = homozygous primary allele; hetero = heterozygous; homo = homozygous minor allele
[c]MAF = minor allele frequency

Table 3.3 Effects of *Hsp*70 SNP A1125C on beef heifer traits.

Item	AA	AC	CC	SEM	*P*-value
Number of heifers	49	81	19	-	-
Body weight, kg	353.9	359.3	371.0	6.3	0.27
Reproductive tract score	4.3	4.5	4.4	0.09	0.23
Pelvic area, cm^2	176.1	172.2	188.0	5.0	0.13
Pregnancy rate, %	89.8	85.1	100	-	0.17
Days pregnant	73.2	73.4	77.6	2.9	0.66
Blood cell differential					
WBC, $1x10^3/\mu L$	8.7	8.6	8.2	0.78	0.89
Neutrophil, % of WBC	6.7	9.5	7.3	1.9	0.34
Lymphocyte, % of WBC	78.7	76.9	75.1	3.2	0.74
RBC, $1x10^6/\mu L$	8.6	8.2	8.6	0.18	0.10
Hemoglobin, g/dL	13.3	13.1	13.4	0.8	0.63
Hematocrit, %	36.7	36.4	37.0	0.86	0.83
Platelets, $1x10^3/\mu L$	135.5	195.1	94.1	82.7	0.60

Table 3.4 Effects of *Hsp*70 SNP C895D on beef heifer traits.

Item	CC	CD	SEM	*P*-value
Number of heifers	131	20	-	-
Body weight, kg	358.7	362.2	6.1	0.71
Reproductive tract score	4.4	4.6	0.09	0.40
Pelvic area, cm^2	175.0	177.9	4.8	0.69
Pregnancy rate, %	87.0	95.0	-	0.35
Days pregnant	74.7	71.6	3.0	0.49
Blood cell differential				
WBC, $1x10^3/\mu L$	8.7	8.2	0.77	0.64
Neutrophil, % of WBC	7.9	11.2	2.0	0.24
Lymphocyte, % of WBC	78.7	69.5	3.1	0.04
RBC, $1x10^6/\mu L$	8.4	8.0	0.18	0.17
Hemoglobin, g/dL	13.2	13.0	0.26	0.70
Hematocrit, %	36.6	36.5	0.85	0.92
Platelets, $1x10^3/\mu L$	177.3	107.4	81.8	0.54

Table 3.5 Effects of *Hsp*70 SNP G1851A on beef heifer traits.

Item	GG	GA	SEM	*P*-value
Number of heifers	140	11	-	-
Body weight, kg	359.7	346.7	7.57	0.27
Reproductive tract score	4.4	4.7	0.11	0.19
Pelvic area, cm^2	175.5	173.8	6.1	0.89
Pregnancy rate, %	87.8	90.9	-	0.76
Days pregnant	74.3	73.1	3.6	0.84
Blood cell differential				
WBC, $1x10^3/\mu L$	8.5	10.4	0.93	0.17
Neutrophil, % of WBC	8.6	4.1	2.4	0.2
Lymphocyte, % of WBC	76.9	84.8	3.8	0.16
RBC, $1x10^6/\mu L$	8.3	8.7	0.23	0.28
Hemoglobin, g/dL	13.2	13.3	0.32	0.72
Hematocrit, %	36.5	37.3	1.03	0.61
Platelets, $1x10^3/\mu L$	167.2	136.1	99.8	0.83

Table 3.6 Effects of *Hsp*70 SNP G2033C on beef heifer traits.

Item	GG	GC	SEM	*P*-value
Number of heifers	110	41	-	-
Body weight, kg	355.9	367.7	4.9	0.1
Reproductive tract score	4.4	4.5	0.07	0.79
Pelvic area, cm^2	172.0	184.0	3.9	0.03
Pregnancy rate, %	91.8	78.1	-	0.02
Days pregnant	73.7	76.0	2.6	0.54
Blood cell differential				
WBC, $1x10^3/\mu L$	9.1	7.5	0.62	0.04
Neutrophil, % of WBC	7.9	9.4	1.6	0.49
Lymphocyte, % of WBC	77.6	76.4	2.6	0.73
RBC, $1x10^6/\mu L$	8.4	8.3	0.15	0.8
Hemoglobin, g/dL	13.2	13.0	0.22	0.37
Hematocrit, %	36.8	36.1	0.7	0.48
Platelets, $1x10^3/\mu L$	185.3	128.2	67.3	0.5

Conclusion

These projects were conducted in an effort to improve reproductive performance of beef cattle. We concluded that *Neospora caninum* is an infectious protozoon that is present in the central region of the United States and that serum tests can help identify seropositive animals in a herd. Geographical region, herd, and cow age contributed to variation in *Neospora caninum* infection rates. Future research could focus on different variables that may work in tandem with the infection to cause abortions and also what strains may be present in this region. Projects within this dissertation determined that *Hsp*70 genotypes were related to reproductive characteristics and lymphocyte populations of beef heifers. Together, these studies help identify potential issues that may affect reproductive performance of beef cattle in this region, and also provide insight on possibilities for identifying and selecting poorer-performing females. More research is needed to understand the full extent to which these techniques can be utilized, but both projects could be potential management tools to identify heifers and cows that are at a greater risk for reproductive failure.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Charles Rosenkrans

FROM: Craig N. Coon, Chairman

DATE: 9/14/15

SUBJECT: IACUC Approval

Expiration Date: Sep 10, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 16010: "Evaluating Stressors and Genotype on In Vitro Immune Response and Gene Expression in White Blood Cells of Cattle", you may begin work immediately.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Sep 10, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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