**Title of Project:** Prevention of Bighorn sheep die-offs due to pneumonia

**Duration of Project:** July 1, 2014 – June 30, 2015

**Problem to be solved:** Pneumonia is the number one cause of death in bighorn sheep. Bighorn sheep are much more susceptible to pneumonia than are domestic sheep. Several die-offs of bighorn sheep have been attributed to pneumonia following contact with domestic sheep. Experimental commingling of bighorn sheep and domestic sheep also results in the death of bighorn sheep. *Mannheimia* (*Pasteurella*) *haemolytica* is an important bacterium that causes pneumonia in bighorn sheep. Studies in our laboratory with green fluorescent protein tagged *M. haemolytica* irrefutably proved transmission of this bacterium from domestic sheep to bighorn sheep. *M. haemolytica* produces a toxin known as leukotoxin that kills the white blood cells, causes lung damage, and death of the animals. Our earlier studies in bighorn sheep with *M. haemolytica* wild-type and leukotoxin-deletion mutant have revealed that the leukotoxin-deletion mutant causes only mild lesions, and does not cause the death of bighorn sheep. Therefore, leukotoxin is the most important virulence factor (weapon) that is produced by this bacterium. Most of the healthy domestic sheep carry leukotoxin-positive *M. haemolytica* as a commensal bacterium in their nasopharynx, and hence develop high titers of antibodies to leukotoxin, and therefore are resistant to pneumonia caused by leukotoxin-positive *M. haemolytica*. Most bighorn sheep do not carry *M. haemolytica* in their nasopharynx, and if they do, they carry mostly leukotoxin-negative *M. haemolytica* strains, and hence do not develop high titers of antibodies to leukotoxin. Therefore, transmission of the leukotoxin-positive strains of *M. haemolytica* from domestic sheep to bighorn sheep results in pneumonia and death of bighorn sheep. Another bacterium known as *Mycoplasma ovipneumoniae*, can predispose bighorn sheep to pneumonia caused by *M. haemolytica* and other bacteria. But the death of bighorn sheep is almost always caused by leukotoxin-positive *M. haemolytica* and other members of the Family *Pasteurellaceae*. Therefore, it is logical and prudent to direct our efforts towards protecting bighorn sheep from leukotoxin-positive *M. haemolytica* and other members of *Pasteurellaceae*.

We propose a two-pronged approach to prevent pneumonia in bighorn sheep:

1. Prevent transmission of leukotoxin-positive *M. haemolytica* from domestic sheep to bighorn sheep.

2. Vaccinate bighorn sheep to make them resistant to leukotoxin-positive *M. haemolytica* transmitted by domestic sheep. This can be easily done when bighorn sheep are captured for transplantation to a habitat to replace the animals that died in a pneumonia-related die-off.

**How will the problem be solved:**

**1. Prevent transmission of leukotoxin-positive *M. haemolytica* from domestic to bighorn sheep.**

*M. haemolytica* is an important pathogen that has been shown to consistently cause fatal pneumonia in bighorn sheep under experimental conditions. However, *Bibersteinia (Pasteurella) trehalosi* and *Pas-*

*teurella multocida* have been isolated from bighorn sheep pneumonic lungs much more frequently than *M. haemolytica*. These observations suggest that there may be an inhibitory effect of *B. trehalosi* and *P. multocida* on the growth of *M. haemolytica*. Recent studies conducted in our laboratory to investigate this phenomenon have, in deed, proven that *B. trehalosi* and *P. multocida* inhibits the growth of *M. haemolytica,* which enables us to develop a novel strategy to prevent transmission of *M. haemolytica* from domestic to bighorn sheep. We hypothesize that *intranasal administration of B. trehalosi will eliminate or reduce M. haemolytica from the nasopharynx of domestic sheep and hence will eliminate or reduce transmission of these organisms to* bighorn sheep. We have isolated several strains of leukotoxin-negative *B. trehalosi*. We propose to use these strains to eliminate leukotoxin-positive *M. haemolytica* from the nasopharynx of domestic sheep. It is important to note that we have already determined that the leukotoxin-negative *B. trehalosi* strains do not acquire the leukotoxin gene from the leukotoxin-positive *M. haemolytica* carried by the domestic sheep by a process known as "horizontal gene transfer." In the proposed study, we will determine whether replacement of leukotoxin-positive *M. haemolytica* that colonize the nasopharynx of domestic sheep with leukotoxin-negative *B. trehalosi* will eliminate or reduce the risk of transmission of *M. haemolytica* to bighorn sheep.

In the pilot studies performed earlier, *B. trehalosi* significantly inhibited *M. haemolytica* in the nasopharynx of domestic sheep. However, the degree of inhibition was not uniformly high in all the domestic sheep. Therefore, during the past year, we first inoculated the domestic sheep with an antibiotic to eliminate/reduce the *M. haemolytica* from the nasopharynx, and then inoculated *B. trehalosi* (See "Progress Report on page 8). This protocol enabled all four bighorn sheep to survive commingling with domestic sheep for 42 days. Unfortunately, two bighorn sheep died of unrelated causes (enteritis and trauma) on days 42 and 84, respectively. But the remaining two bighorn sheep survived commingling with domestic sheep for 100 days, which ended the first phase of the study. In the second phase of the study, the remaining two bighorn sheep were commingled with two domestic sheep that were not treated with antibiotic or *B. trehalosi*. Both these bighorn sheep survived commingling with the untreated domestic sheep for 100 days, which ended the second phase of the study. In the third phase of the study, these two bighorn sheep have been commingled with two domestic sheep that were positive for *Mycoplasma ovipneumoniae*, but not treated with antibiotic or *B. trehalosi*. This phase of the project is still ongoing, and will continue.

In addition, we will initiate experiments to identify the *B. trehalosi* protein(s) that inhibit(s) the growth of *M. haemolytica*. Identification of the inhibitory protein(s) of *B. trehalosi* will enable us to genetically engineer a strain of *B. trehalosi* that will produce larger amounts of the inhibitory protein(s). This genetically engineered *B. trehalosi* could be expected to inhibit *M. haemolytica* more effectively than the wild-type *B. trehalosi*.

**2. Vaccinate bighorn sheep to make them resistant to leukotoxin-positive *M. haemolytica* transmitted by domestic sheep.**

A previous experimental vaccination study conducted by us, as a "proof-of-concept" study, showed that if bighorn sheep can be induced to develop high titers of antibodies that neutralize the leukotoxin, and antibodies to other soluble antigens of *M. haemolytica*, they will resist the challenge with *M. haemolyti-*

*ca* and not develop pneumonia and die. For the first time ever, 100% of the vaccinated bighorn sheep were protected when challenged with a virulent strain of leukotoxin-positive *M. haemolytica*, whereas 100% of the un-vaccinated bighorn sheep died within 48 hours of challenge. In this study, however, the bighorn sheep received multiple injections of the vaccine, which is obviously not practical in the field. Therefore, we are focusing on the development of a vaccine that does not need to be injected multiple times, and more importantly, that could be delivered orally or nasally. We planned on two different approaches to come up with such a vaccine:
1. A live *M. haemolytica* strain that produces an inactive toxin (which induce the production of antibodies, but not cause disease).

In the studies performed during the previous year, a mutant of *M. haemolytica* that secretes an inactive leukotoxin (that does not kill the white blood cells of bighorn sheep) was tested as a vaccine candidate. This mutant did not induce satisfactory immune response against the toxin secreted by *M. haemolytica*. Therefore this approach has been abandoned. However, since two of the bighorn sheep died upon inoculation with the inactive toxin-producing mutant, in the coming year, we will investigate the molecular basis for the unexpected pathogenicity of this mutant.

2. A harmless virus that carries the gene for a segment of the leukotoxin and an outer membrane protein of *M. haemolytica* (when the virus replicates in the oral/nasal mucosa, the *M. haemolytica* antigens will also be produced).

During the past year, we have been successful in developing two bovine herpesvirus 1 (BHV-1) mutants which are attenuated (weak, and hence will not cause disease in bighorn sheep at all) (See "Progress Report on page 8). We have also demonstrated shedding, latency establishment and reactivation following BHV-1 inoculation in bighorn sheep. The virus shed for 9 days following inoculation, with peak shedding at the second day post-inoculation. This observation suggests that the vaccine virus from the vaccinated bighorn sheep is likely to be transmitted to the un-vaccinated bighorn sheep resulting in the vaccination of the un-vaccinated bighorn sheep in the wild. Also the animals remained healthy throughout the duration of the experiment. This suggests that BHV-1 is a good vector, in that it is able to replicate efficiently, while being safe. The latent virus in bighorn sheep underwent reactivation following dexamethasone treatment. This observation is especially important in our studies, since we are proposing that our vaccine will not require booster administration, but instead be capable of boosting by means of establishing latency and reactivation. In the coming year, we will develop the recombinant BHV-1 carrying the protective antigens of *M. haemolytica* (a segment of leukotoxin and an outer membrane protein). In the following year, this mutant will be tested for its ability to induce neutralizing antibodies against the leukotoxin, and its potential to serve as a vaccine candidate.

## Progress Report 2013-2014

1. Prevent transmission of leukotoxin-positive *M. haemolytica* from domestic sheep to bighorn sheep.

Animal studies in domestic sheep during 2012-13 gave encouraging results. Inoculation of *B. trehalosi* into the nasopharynx of domestic sheep reduced the number of *M. haemolytica* in them. However, reduction in the numbers of *M. haemolytica* was not uniformly high in all the domestic sheep. Therefore, during the past year (2013-14), we first inoculated the domestic sheep with an antibiotic to eliminate/reduce the *M. haemolytica* from the nasopharynx, and then inoculated *B. trehalosi*. This protocol enabled all four bighorn sheep to survive commingling with domestic sheep for 42 days. Unfortunately, two bighorn sheep died of unrelated causes (enteritis and trauma) on days 42 and 84, respectively. But the remaining two bighorn sheep survived commingling with domestic sheep for 100 days, which ended the first phase of the study. In the second phase of the study, the remaining two bighorn sheep were commingled with two domestic sheep that were not treated with antibiotic or *B. trehalosi*. Both these bighorn sheep survived commingling with the untreated domestic sheep for 100 days, which ended the second phase of the study. As the third phase of the study, we have commingled the two bighorn sheep with two domestic sheep that were positive for *Mycoplasma ovipneumoniae*, but not treated with antibiotic or *B. trehalosi*. This phase of the project is ongoing, and will continue.

2. Vaccinate bighorn sheep to make them resistant to leukotoxin-positive *M. haemolytica* transmitted by domestic sheep.

During the past year, we have been successful in developing two BHV-1 mutants which are attenuated (weak, and hence will not cause disease in bighorn sheep at all). We have also demonstrated shedding, latency establishment and reactivation following BHV-1 inoculation in bighorn sheep. The virus shed for 9 days following inoculation, with peak shedding at the second day post-inoculation. Also the animals remained healthy throughout the duration of the experiment. This suggests that BHV-1 is a good vector, in that it is able to replicate efficiently, while being safe. The latent virus in bighorn sheep underwent reactivation following dexamethasone treatment. This observation is especially important in our studies, since we are proposing that our vaccine will not require booster administration, but instead be capable of boosting by means of establishing latency and reactivating. In the coming year, we will develop the recombinant BHV-1 carrying the protective antigens of *M. haemolytica* (a segment of leukotoxin and an outer membrane protein). In the following year, this mutant will be tested for its ability to induce neutralizing antibodies against the leukotoxin, and its potential to serve as a vaccine candidate.

**Additional studies that were conducted during the past year, that were not listed in the proposal last year:** Understanding the reasons for poor lamb recruitment in the herds that suffered pneumonia die-off.

When a pneumonia die-off occurs, initially, bighorn sheep of all ages die. In subsequent years, the adult bighorn sheep rarely die, but the lambs continue to die year after year, and hence the herds do not grow in numbers. We are working on understanding the reasons underlying this phenomenon.

In 2012-13, we obtained four bighorn rams that survived the 2010 outbreak in Nevada, and commingled them with naïve (uninfected) bighorn ewes from our captive herd. These ewes got infected. The lambs born to these ewes also got infected, developed pneumonia and died. We are continuing our work on 'finger-printing' the organisms isolated from the Nevada survivors, and the commingled ewes from our herd, and the lambs born to them. This information will clearly tell us whether the survivors carry the harmful organisms and transmit to their lambs.

The survivor ewes may have immunity to the harmful organisms, but it may not be adequate to pass on to their lambs. We are also working on determining the levels of immunity carried by the survivor ewes and the lambs before they died. For the information to be meaningful, we should study survivor ewes from different geographical locations. Therefore, in 2013-14, we worked with another group of survivor ewes from Colorado, housed in Sybille, WY. Our objective was to treat the carrier ewes with an antibiotic to determine whether this treatment will protect the lambs born to these carrier ewes. But unfortunately, due to various reasons, this objective could not be pursued. This year, we have obtained six carrier bighorn ewes from Montana, and housed here at WSU. We will inoculate some of the lambs born to these carrier ewes with antibodies obtained from the serum of domestic sheep to determine the protective effects of the antibodies. This study should provide information useful in designing management strategies.