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Research article

Using transcriptomics to predict and visualize disease status in bighorn sheep (*Ovis canadensis*)

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Increasing risk of pathogen spillover coupled with overall declines in wildlife population abundance in the Anthropocene make infectious disease a relevant concern for species conservation worldwide. While emerging molecular tools could improve our diagnostic capabilities and give insight into mechanisms underlying wildlife disease risk, they have rarely been applied in practice. Here, employing a previously reported gene transcription panel of common immune markers to track physiological changes, we present a detailed analysis over the course of both acute and chronic infection in one wildlife species where disease plays a critical role in conservation, bighorn sheep (*Ovis canadensis*). Differential gene transcription patterns distinguished between infection statuses over the course of acute infection and differential correlation (DC) analyses identified clear changes in gene co-transcription patterns over the early stages of infection, with transcription of four genes—TGFb, AHR, IL1b and MX1—continuing to increase even as transcription of other immune-associated genes waned. In a separate analysis, we considered the capacity of the same gene transcription panel to aid in differentiating between chronically infected animals and animals in other disease states outside of acute disease events (an immediate priority for wildlife management in this system). We found that this transcription panel was capable of accurately identifying chronically infected animals in the test dataset, though additional data will be required to determine how far this ability extends. Taken together, our results showcase the successful proof of concept and breadth of potential utilities that gene transcription might provide to wildlife disease management, from direct insight into mechanisms associated with differential disease response to improved diagnostic capacity in the field.

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Introduction

The rate of newly emerging or re-emerging wildlife diseases has increased, impacting human, livestock and wildlife health and biodiversity (Daszak *et al.*, 2000; Dobson and Foufopoulos, 2001; Harvell *et al.*, 2002; Olden *et al.*, 2004; Cohen *et al.*, 2020). Understanding how host immune responses influence disease resistance and immunopathology is critical to elucidating disease dynamics and developing mitigation strategies (Bodhankar *et al.*, 2010; Plowright *et al.*, 2013; Dugovich *et al.*, 2017). Differences in host susceptibility may be due to differences in the host immune response (Faulkner

et al., 1995), which, in turn, can be influenced by factors such as nutrition, contaminants and other concomitant infections (Acevedo-Whitehouse and Duffus, 2009). Additionally, continued and prolonged activation of the immune system can be physiologically costly, potentially causing a reallocation of nutrients and energy away from other metabolic functions and resulting in reduction of fitness (Martin *et al.,* 2010; Graham *et al.,* 2011). A management regime effective at mitigating effects of an individual or herd response to infection may benefit from considering both immunological and ecological context.

Novel molecular approaches have yielded insights into human and wildlife disease at a variety of scales, revealing proximate and ultimate drivers of disease state and transforming understanding of pathogenesis, transmission and treatment (Pacis *et al.,* 2014; Eskew *et al.,* 2021). In particular, transcriptomics is one of the major avenues for comparing tissue/cell RNA expression profiles at various states of disease progression, helping to explain the complexities of the host immune response to infection (Zhang *et al.,* 2014; Blanchong *et al.,* 2016). Although similar analyses have only recently appeared in the context of wildlife disease and conservation science, and rarely as field-based studies, adoption of transcriptomics in wildlife disease will elucidate mechanisms that underlie host disease state and ultimately translate into more effective population monitoring, management and conservation (Blanchong *et al.,* 2016; Campbell *et al.,* 2018; DeCandia *et al.,* 2018; Bowen *et al.,* 2020). Although sometimes used interchangeably, gene transcription and expression technically refer to different 'events'; DNA is transcribed into mRNA and then translated into a functional protein (Alberts *et al.,* 2002). Transcriptomic approaches assess the quantity of mRNA produced, while proteomics assesses the quantity of protein synthesized (González-Fernández *et al.,* 2008). To avoid confusion, in this paper we use the terms transcriptomics/transcription/transcript to refer to the study of mRNA quantities.

Two broad pathways allow for investigation into gene transcription surrounding disease events: differential gene transcription and DC analyses (Ellison *et al.,* 2015). The more common of the two, differential gene transcription (or expression), describes changes in transcription levels of specific genes between particular host states (in our case, defined by disease status). This approach lets researchers identify genes that are up- or down-regulated in the focal contexts, but regards each gene as an independent entity. As a consequence, confounding among genes could lead to spurious signals about whether groups of genes are mechanistically linked during the change in host state. Additionally, genes that are not identified as differentially transcribed may actually be integral to disease state transitions. DC analyses, on the other hand, directly query gene–gene linkages by examining whether the correlation between gene pairs itself changes with changes in host state. Thus, DC analysis is better equipped to identify blocks of genes that move in tandem with one another as host state changes, adding resolution to inferences surrounding the underlying in-host processes.

The field of ecological or wild immunology has begun to address the complexity of immune responses in wildlife populations with the primary goal of understanding variation in immune function across individuals (Pedersen and Babayan, 2011). To identify causal relationships between immune function and disease status, experimental manipulations in wild populations will be essential (Pedersen and Babayan, 2011). A recent dataset assembled by Manlove *et al.* (in revision) lends itself to such an exploration, in the context of pneumonia infection associated with *Mycoplasma ovipneumoniae* in Rocky Mountain bighorn sheep (*Ovis canadensis*). *Mycoplasma ovipneumoniae* infections are thought to progress through a series of states. Infection is often followed by acute pneumonia primarily impacting the lower respiratory tract. As disease progresses, most animals completely clear infection, though a subset go on to become chronic carriers. Characterizing any immune functions that differentiate among acute, chronic and recovered animals is an important step towards understanding the aetiology of chronic carriers and developing efficient means to identify, and possibly treat, those animals.

Our primary goal was to use transcriptomic techniques to compare immune markers among animals in the context of both acute and chronic infection events, as part of a broader effort to gain information about the identification of and mechanisms behind disease state transitions in the bighorn sheep–*M. ovipneumoniae* system. First, we analyse patterns of differential gene transcription and differential gene correlation over the course of an acute disease event. We then extend our analyses to assess the ability of the same transcription panel to differentiate among chronically infected and recovered individuals following pathogen establishment. Our results shed new light on host physiological changes over the course of infection and suggest that gene transcription could add insight into the bighorn sheep pneumonia system, in both a diagnostic and a mechanistic understanding capacity.

Materials and methods

System overview: bighorn sheep pneumonia

Bighorn sheep pneumonia is a polymicrobial disease often initiated by introduction of the bacteria *M. ovipneumoniae* into otherwise naive bighorn herds (Besser *et al.,* 2008; Besser *et al.,* 2012). *Mycoplasma ovipneumoniae* introductions can precipitate population-wide disease outbreaks, producing acute all-age die-offs ranging from 0% to 90% of the population in size (Cassirer *et al.,* 2018). Although these die-offs are stark and impactful, the disease's longer-term effects are often more detrimental to population growth. While *M. ovipneumoniae* continues to circulate within the herd, each year's juvenile cohort is vulnerable to its own acute disease

event, leading to recruitment regularly below 20 lambs per 100 ewes (Cassirer and Sinclair, 2007; Cassirer *et al.*, 2013; Manlove *et al.*, 2016). Sustained transmission to lambs can completely halt population growth and limit the potential for post-die-off herd recovery. There are currently no vaccines for *M. ovipneumoniae*, and effective antibiotic treatment regimens are infeasible for most wild populations because those treatments often rely on daily injections of antibiotics over a week-long window.

Within a particular host, *M. ovipneumoniae* first invades the upper respiratory tract, where it can impede movement of the ciliary escalator and allow normally commensal microflora to invade the lower respiratory tract, producing an acute, polymicrobial pneumonia (Besser *et al.*, 2008). Following acute pneumonia, some bighorn hosts completely clear their *M. ovipneumoniae* infections, in the process acquiring a strain-specific immunity that protects them from future acute disease (Cassirer *et al.*, 2013; Plowright *et al.*, 2013) but not from periodic transient infection. Other bighorn hosts can become chronic *M. ovipneumoniae* carriers and act as local pathogen reservoirs that facilitate annual pneumonia events among lambs (Plowright *et al.*, 2017). The proportion of recovering adults that become chronic carriers appears to vary from herd to herd, but is often quite low (e.g. 10–15% of adult females). Transient infections among otherwise healthy adults are relatively common, however, especially in contexts where individuals aggregate into close-knit groups (Plowright *et al.*, 2017).

Both empirical and theoretical research indicate that removing chronically infected females can allow bighorn herds to rebound, even if individuals with transient infections are not removed (Garwood *et al.*, 2020; Almberg *et al.*, 2021). A number of state, provincial and federal wildlife management agencies are operationalizing these findings through 'test-and-remove' management efforts that aim to identify and remove chronic carriers. Carrier identification remains a logistical challenge, however. Currently, the most reliable method is to test each individual multiple times and classify animals as 'chronic' if they produce PCR-based evidence of *M. ovipneumoniae* infection at least twice over the course of 1 to 2 years (Cassirer *et al.*, 2018; Garwood *et al.*, 2020). The multiple-testing approach is logistically challenging in many field contexts. Each of the three common methods of bighorn capture, helicopter net-gunning (Krausman *et al.*, 1985), ground-darting with chemical immobilization darts and drop-netting at baited sites or natural aggregation points, has drawbacks including cost, time and human and animal safety.

Simply removing all animals that are PCR-positive after a single testing event, on the other hand, could result in removal of many more animals than is necessary to drive pathogen fade-out. This is problematic for herds already facing the usual challenges confronting any small population (Festa-Bianchet *et al.*, 2006) and may be completely untenable for subspecies and populations in federally targeted recovery programs (e.g. for Sierra Nevada or Peninsular bighorn sheep populations). Being able to differentiate between animals with transient and chronic infections after a single testing event would improve the feasibility and efficiency of nascent test-and-remove management efforts.

A variety of factors could help differentiate chronically and acutely infected adults. For example, some evidence suggests that chronic carriage is linked to age (Plowright *et al.*, 2017), genetics (Martin *et al.*, 2021) or expression of symptoms. None of these signatures has proven to be a reliable classifier in the field, however, nor have chronic carriers shown fundamentally different pathogen burdens (C_T scores) or serological signatures on conventional diagnostics than individuals with transient infections. Current *M. ovipneumoniae* management practices would benefit from additional metrics to help identify chronic carriers in a single animal handling event, and new management opportunities might emerge from a better understanding of the immune and physiological processes associated with chronic carriage (Cassirer *et al.*, 2018).

Here, we explored the ability of a targeted immune-centric gene transcription panel consisting of 16 genes to identify and shed light on the aetiology of both acute and chronic *M. ovipneumoniae* infections in bighorn sheep. Genes were chosen based upon two factors: (i) known genes relevant to the disease dynamics of *M. ovipneumoniae* in bighorn sheep and (ii) known genes relevant to similar disease dynamics in other species (Bowen *et al.*, 2020). Ultimately, the genes in our panel could be separated into groups based on primary function: reference (YWHAZ, S9), general immune function (CD69, IFNg, IL-10, IL1B, MyD88, TGFB, TNFa), immune system transcription factors (Gata3, T-bet), detoxification (AHR), muscle metabolism (AMPK), apoptosis (FADD), general stress (HSP70) and antiviral (MX1) (Table 1) (Bowen *et al.*, 2020). In similar studies identifying carrier states in wildlife, panels with as few as nine genes were capable of separating viral and bacterial diseases from latent infections (Miller *et al.*, 2017).

We analyse data arising from two disease contexts, one acute and one chronic. Our acute data come from a natural infection event surrounding a bighorn sheep translocation in late winter of 2020. A full detailing of the event can be found in Manlove *et al.* (in revision). Briefly, on 22 February 2020, 24 Rocky Mountain bighorn ewes were captured from the Rio Grande Gorge (RGG) herd in northern New Mexico and transported to Utah. Blood, nasal and oropharyngeal swabs were gathered from all 24 animals during capture, and samples were shipped to the Washington Animal Disease Diagnostic Laboratory (WADDL) to undergo diagnostic testing for *M. ovipneumoniae*. Laboratory results indicated that six of the transported animals showed evidence of *M. ovipneumoniae* exposure based on a PCR test (i.e. direct evidence of current infection), a cELISA test (i.e. antibody response indicating past exposure) or both (laboratory methods in Ziegler *et al.*, 2014). A cELISA percent inhibition of 40 served

Table 1: Genes selected for the transcription panel and their primary functions

Functional group	Gene	Gene function
Reference	YWHAZ	Reference gene—Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Mahakapuge *et al.*, 2016).
	S9	Reference gene—Ribosomal subunit S9 (Bowen *et al.*, 2007)
General immune function	CD69	Earliest inducible cell surface glycoprotein acquired during lymphoid activation. Involved in lymphocyte proliferation (Parham and Parham, 2014).
	IL-10	Interleukin-10 is an anti-inflammatory cytokine (Goldsby *et al.*, 2003). IL-10 has been linked with the ability of Mycobacterium to evade immune responses and mediate long-term infections in the lung (Redford *et al.*, 2011).
	Interleukin 1 beta (IL1B)	Inflammatory mediator (Parham and Parham, 2014).
	IFNg	Interferon gamma is a cytokine that is implicated in defence against viral pathogens (Schroder *et al.*, 2004) and has been found to be critical to protective immunity to Mycoplasma infections (Bodhankar *et al.*, 2010).
	MyD88	Research suggests that the MyD88-dependent TLR pathway may play a crucial role in sheep airway epithelial cells in response to *M. ovipneumoniae* infection (Xue *et al.*, 2015).
	TGF-beta (TGFB)	Transforming growth factor beta. Immunosuppressive cytokine (Parham and Parham, 2014).
	TNFa	Tumour necrosis factor alpha—a cytokine that plays a key role in the inflammatory response (Kalliolias and Ivashkiv, 2016).
Immune transcription factors	Gata3	A TH2-specific transcription factor controls transcription of cytokines Interleukin (IL) IL-4, -5 and -13 (Parham and Parham, 2014).
	T-bet	A TH1-specific T box transcription factor that controls the transcription of the hallmark TH1 cytokine, interferon gamma (Parham and Parham, 2014).
Detoxification	AHR	The AHR, once thought to primarily play a role in detoxification of anthropogenic contaminants, has more recently been shown to play a protective role against oxidative stress (Dietrich, 2016). One of the pathophysiological manifestations of the stress and physical exertion associated with wildlife capture is the breakdown of myoglobin in muscle, resulting in oxidative stress (Breed *et al.*, 2019). The molecular act of mitigation of oxidative stress is part of an organism's inherent biological stress defence (Breed *et al.*, 2019).
Muscle metabolism	AMPK	5′-AMP-activated protein kinase—a potent regulator of skeletal muscle metabolism (Jørgensen *et al.*, 2006). AMPK is activated during fasting and starvation and has a role in restoring energy homeostasis via promotion of glucose uptake and glycolysis, fatty acid uptake and fatty acid oxidation (Cohen *et al.*, 2017).
Apoptosis	FADD	Fas-Associated protein with Death Domain – a pivotal trigger in apoptosis (cell death) (Scott *et al.*, 2009).
General stress	HSP70	Heat shock protein 70 is produced in response to exposure to different kinds of environmental stress conditions, such as infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation) and thermal or other stress (Iwama *et al.*, 1999; Tsan and Gao, 2004). Heat shock proteins can activate the immune system by providing danger signals. In addition to being expressed in response to a wide array of stressors, heat shock proteins act as molecular chaperones (De Maio, 1999).
Antiviral	MX1	Although the Mx1 gene is generally thought to respond to viral infection (Tumpey *et al.*, 2007), studies have shown MX1 induction in the presence of Mycoplasma (Bierne *et al.*, 2012; Li *et al.*, 2015).

as the cut-off for classification as 'seropositive', consistent with the standards set by WADDL. Animals showing evidence of infection were euthanized. The remaining 18 animals that showed no serological or PCR evidence of infection were moved to a wildlife facility at Hardware Ranch, where they all were housed together in a solid pen, ~10 feet high and 50 feet in diameter. Three animals expired shortly after arrival at the pens, presumably due to stress from multiple sequential anaesthesia events; the analyses here reflect data from the remaining animals. Over the next 2 weeks, animals gradually began to display symptoms of pneumonia. Fourteen animals (all that remained except for one that evaded capture) were

Table 2: Categorization of Rocky Mountain bighorn sheep into disease groupings

	Pre-exposure	Early acute	Late acute	Early acute wild	Late acute wild	Recovered
	PCR-negative and cELISA % inhibition <40	PCR-positive and cELISA % inhibition <40	PCR positive and cELISA % inhibition >40	PCR-positive and cELISA % inhibition <40	PCR positive and cELISA % inhibition >40	PCR-negative and cELISA inhibition >40
RGG	$N = 6$ (1 M, 5 F)			$N = 4$	$N = 8$ (3 M, 5 F)	
Hardware Ranch 03/12/20		$N = 9$ (9 F)	$N = 3$ (3 F)			
Hardware Ranch 03/27/20		$N = 3$ (3 F)	$N = 8$ (8 F)			$N = 2$ (2 F)

While disease timing is well known for the Hardware Ranch animals, exposure time is not known at the RGG. Therefore, we categorized RGG animals as pre-exposure if they were PCR-negative and had cELISA % inhibition below 40. We categorized animals at RGG as early exposure wild if they were PCR-positive and had cELISA % inhibition values below 40. We categorized RGG animals as late acute wild if they were PCR positive and had % inhibition values above 40%. Animals with cELISA values above 40% that were PCR-negative were taken to be recovered.

subsequently chemically immobilized via a jab stick on 13 March. Nasal and oropharyngeal swabs and blood samples were gathered from each animal. The disease event continued, 2 animals died naturally and the remaining 12 animals were euthanized following a final sampling event on 26 March.

Following detection of *M. ovipneumoniae* among the translocated animals, parallel sampling of free-ranging bighorn sheep at the RGG source population was initiated via ground darting. Manlove *et al*. (in revision) found similarities in serological expansion patterns and timing of peak clinical signs between the source herd and the translocated bighorn sheep. While disease timing is well known for the captive animals, individual exposure times are not known at RGG. Therefore, we categorized RGG animals as pre-exposure ($N = 6$) if they were PCR-negative and had cELISA % inhibition below 40. We categorized animals at RGG as early acute exposure ($N = 4$) if they were PCR-positive and had cELISA % inhibition values below 40. We categorized RGG animals as late acute ($N = 8$) if they were PCR positive and had % inhibition values above 40. Animals with cELISA values above 40% that were PCR-negative were taken to be recovered. Exposure classifications for all Hardware Ranch and RGG animals are summarized in Table 2.

A completely separate set of animals was used for a pilot examination of the extensibility of our transcription panel to differentiate between chronic, recovered and unexposed bighorn sheep more generally. Twenty-seven California bighorn sheep (categorized in past taxonomies as a unique subspecies, *Ovis canadensis californiana*) were sampled throughout northern Nevada by the Nevada Department of Wildlife between 2017 and 2019. Five of these animals produced two or more positive PCR tests for *M. ovipneumoniae* over multiple years (in preparation for a test-and-remove effort similar to the one reported in Garwood *et al.*, 2020), and were thus categorized as known 'chronic carriers' (Plowright *et al.*, 2017; Cassirer *et al.*, 2018; Matt Jeffries, unpublished data). The remainder of the animals could be split into groups of 'unexposed' (i.e. both PCR-negative and serologically negative for *M. ovipneumoniae*; $N = 18$) or 'recovered' (PCR-negative and serologically positive; $N = 4$). Our objective was to understand how well gene transcription profiles might be able to differentiate among pre-exposed, acutely infected and recovered animals in the acute disease event, and chronically infected, recovered or unexposed animals in the Nevada-wide dataset.

Blood collection and RNA extraction

It is well documented that gene expression patterns differ by tissue (Li *et al.*, 2020). Although other tissue types may be more directly impacted by *M. ovipneumoniae* infection, the functional diversity in blood transcriptomes is comparable with transcriptomes from more traditionally sampled tissues (Banerjee *et al.*, 2021). Additionally, acquisition of blood samples is generally non-lethal and minimally invasive (Banerjee *et al.*, 2021). As such, we identified blood as the tissue sample of choice for our study. A 2.5-ml blood sample from each bighorn was drawn directly into a PAXgene™ blood RNA collection tube (PreAnalytiX, Switzerland) from the cephalic vein and then frozen at −20°C until extraction of RNA (Bowen *et al.*, 2012). The RNA from blood in PAXgene™ tubes was isolated according to manufacturer's standard protocols, which included an on-column DNase treatment to remove contaminating gDNA (silica-based microspin technology included in the PAXgene™ kit), and the extracted RNA was stored at −80°C until analysis. The RNA concentration was measured on a Qubit 3.0 Fluorometer using the RNA, DNA and RNA IQ Assay Kits (Life Technologies, Carlsbad, CA, USA). Samples were processed randomly (i.e. samples were not processed in batches according to location, age, sex or pathogen exposure status).

cDNA synthesis

A standard cDNA synthesis was performed on 2 μg of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript®, Qiagen, Valencia, CA, USA), 1 μM random hexamers, 0.5 mM each dNTP and

Table 3: Mean and standard error of $\Delta\Delta C_T$ values for bighorn sheep categorized into disease groups: pre-exposure (RGG, $N = 6$), early acute (Hardware Ranch, 3/12/20 $N = 9$, 3/27/20 $N = 3$), late acute (Hardware Ranch, 3/12/20 $N = 3$, 3/27/20 $N = 8$), early acute wild (RGG, $N = 4$), late acute wild (RGG, $N = 8$), recovered (Hardware Ranch, 3/27/20 $N = 2$)

	Pre-exposure		Early acute		Late acute		Early acute wild		Late acute wild		Recovered	
Gene	Mean	Std error	Mean	Std error	Mean	Std error	Mean	Std error	Mean	Std error	Mean	Std error
AHR	−2.33	0.49	−2.74	0.12	−2.76	0.10	−2.37	0.43	−3.12	0.19	−2.33	0.07
HSP70	−2.55	0.65	−3.59	0.18	−3.27	0.25	−2.57	0.69	−2.95	0.24	−3.90	0.54
MyD88	−3.20	0.77	−3.65	0.26	−3.28	0.19	−3.72	0.76	−3.55	0.18	−3.37	0.02
MX1	−2.69	0.64	−2.20	0.24	−2.25	0.16	−3.16	0.32	−2.53	0.19	−1.84	0.16
IFNg	−3.95	1.16	−6.21	0.15	−6.79	0.36	−4.17	1.41	−6.25	0.48	−7.32	1.20
IL1B	−2.53	0.54	−3.56	0.19	−3.19	0.13	−1.89	0.30	−2.68	0.19	−3.45	0.00
IL10	−4.86	1.53	−7.92	0.19	−7.78	0.40	−6.52	2.10	−8.53	0.58	−7.51	0.60
CD69	−5.11	1.68	−9.67	0.19	−9.32	0.57	−6.33	1.94	−9.24	0.90	−9.41	0.57
FADD	−3.50	0.96	−5.47	0.13	−5.03	0.13	−4.23	1.09	−5.03	0.16	−5.30	0.26
AMPK	−4.93	1.57	−6.20	0.33	−6.14	0.38	−6.63	1.91	−7.57	0.22	−6.83	0.11
TNFa	−2.37	0.56	−3.41	0.14	−3.15	0.15	−1.87	0.75	−3.02	0.19	−3.35	0.27
Gata3	−2.61	0.59	−4.58	0.14	−4.54	0.14	−2.58	1.08	−4.05	0.23	−4.52	0.62
Tbet	−2.96	0.70	−3.34	0.19	−3.37	0.19	−3.07	0.88	−3.33	0.29	−3.77	0.35
TGFB	−3.69	0.27	−4.24	0.13	−4.07	0.15	−3.30	1.19	−3.63	0.18	−3.90	0.04

10 units RNase inhibitor, in RT buffer (Qiagen, Valencia, CA, USA). Reactions were incubated for 60 min at 37°C, followed by an enzyme inactivation step of 5 min at 93°C and then stored at −20°C until further analysis.

Real-time PCR

Real-time PCR systems for the two individual, bighorn-specific reference genes and 14 genes of interest were run in separate wells; a summary description of every gene can be found in Table 1. Briefly, 1 μl of cDNA was added to a mix containing 12.5 μl of QuantiTect Fast SYBR Green® Master Mix [5 mM Mg^{2+}] (Qiagen, Valencia, CA, USA), 0.5 μl each of forward and reverse sequence specific primers and 10.5 μl of RNase-free water; total reaction mixture was 25 μl. The reaction mixture cDNA samples for each gene of interest and the reference genes were loaded into MicroAmp Fast Optical® 96-well reaction plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA, USA). Reaction mixtures containing water, but no cDNA, were used as negative controls; thus, approximately two individual bighorn samples were run per plate.

Amplifications were conducted on a QuantStudio 3 Real-time Thermal Cycler™ (Applied Biosystems, Foster City, CA, USA) using QuantStudio 3 Software. Reaction conditions were as follows: an initial hold stage of 95°C for 20 s, 40 cycles of 95°C for 1 s and 60°C for 20 s. The melt curve consists of 95°C for 1 s, 60°C for 20 s, 0.3°C per second temperature increase and then 95°C for 1 s.

The reference genes selected, YWHAZ and S9, were identified by Mahakapuge *et al.* (2016) and Bowen *et al.* (2007) as suitably stable reference genes. Briefly, stability of reference genes was evaluated and ranked using the web-based analysis tool RefFinder (https://www.heartcure.com.au/for-researchers/) (Chen *et al.*, 2015). Cycle threshold crossing values (C_T) for the genes of interest were normalized to the means of the reference genes.

Statistical methods

We transformed the qPCR data according to the 2^(-C_T') method (Livak and Schmittgen, 2001) as follows. First, we normalized values (housekeeping gene threshold crossing subtracted from the gene of interest threshold crossing). We then compared the normalized value of the target gene to the C_T of the calibrator sample (the lowest level of transcription for each gene). This gave us normalized transcription values for each gene relative to the maximum observed C_T across all samples. We then log-transformed the transcription values, and those log-transformed values served as the basis for all analyses going forward. We calculated means and standard errors of transformed values for all genes within each disease status group (Tables 3 and 4).

We used mixed effects statistical models to simultaneously estimate and account for the influences of age and sex on differences in gene transcription levels among bighorn disease groups (pre-exposure, early acute, late acute, early acute wild, late acute wild, recovered) in Rocky Mountain bighorn

Table 4: Mean and standard error of $\Delta\Delta C_T$ values for bighorn sheep sampled from the RGG, Hardware 3/12/20 and Hardware 3/27/20

	RGG ($N = 18$)		Hardware 3/12/20 ($N = 12$)		Hardware 3/27/20 ($N = 13$)	
Gene	Mean	Std Error	Mean	Std Error	Mean	Std Error
AHR	−2.67	0.21	−2.86	0.10	−2.58	0.11
HSP70	−2.72	0.26	−3.59	0.14	−3.41	0.26
MyD88	−3.43	0.30	−3.60	0.25	−3.37	0.19
MX1	−2.73	0.23	−2.25	0.23	−2.11	0.17
IFNg	−5.10	0.57	−6.48	0.14	−6.56	0.38
IL1B	−2.48	0.21	−3.59	0.19	−3.21	0.11
IL10	−6.93	0.79	−8.11	0.22	−7.41	0.30
CD69	−7.29	0.87	−9.73	0.20	−9.19	0.51
FADD	−4.34	0.41	−5.31	0.11	−5.22	0.15
AMPK	−6.48	0.69	−6.39	0.35	−5.95	0.32
TNFa	−2.53	0.26	−3.36	0.14	−3.27	0.14
Gata3	−3.24	0.34	−4.51	0.15	−4.65	0.13
Tbet	−3.21	0.31	−3.22	0.19	−3.45	0.15
TGFB	−3.54	0.26	−4.29	0.10	−4.07	0.13

Table 5: Scaling from coefficients of linear discrimination from the LDA fit to California bighorn sheep across Nevada

Gene	LD1	LD2
AHR	0.005	−0.031
HSP70	0.244	−0.053
MyD88	−0.985	−0.770
MX1	0.149	0.510
IFNg	0.177	−0.617
IL10	0.327	0.076
IL1b	0.190	0.238
CD69	−0.202	−0.026
FADD	−0.295	0.624
AMPK	0.291	0.519
TNFa	−0.122	0.418
Gata	0.102	0.465
Tbet	0.993	0.330
TGFb	−0.663	−1.311

sheep. Age was included as a categorical variable where the different categories represent a range of sheep ages. Each gene's transcription was treated as an independent outcome, and parameter estimates for all model effects were calculated using the lme4 package in R 2.8.1 (R Development Core Team, 2012).

We applied a covariance-adjusted linear discriminant analysis (Cochran and Bliss, 1948; Tu *et al.*, 1997) to assess whether the transcription profile could differentiate among four different disease groups (pre-exposure, early acute, late acute, recovered) after adjusting for two structural covariates that were not of interest in this analysis—source location (wild or captive) and sex. Because our objective was simply proof-of-concept, and because we were working with very limited sample sizes, we used data from all 43 samples over the course of the disease event to fit the model. We used the log-transformed relative expression measurements and made the covariance adjustment using the adjvec function in the TULIP package in R (Pan *et al.*, 2020). We then fit and visualized the linear discriminant analysis on the resulting covariance-adjusted variables using the LDA function in the flipMultivariate package (Displayr, 2022) (Table 5). Disease states were given prior weights proportional to their occurrence in the raw dataset, and a false discovery rate correction was applied across the entire ensemble for measured gene transcriptions and disease states in tandem.

We used differential gene correlation analysis to identify shifts in dynamic relationships among genes between different groups of bighorn sheep representing different disease states. We compared samples from the pre-exposure and early acute phase of infection, and between the early acute phase and late acute phase using the DGCA package in the R statistical computing environment (McKenzie *et al.*, 2016). We then calculated pairwise DCs using the pairwise DC function and extracted the pairs with the highest DC between the two sampling events using the cdTopPairs function. Pairwise *P*-values were adjusted using the Benjamini–Hochberg adjust-

ment to account for multiple testing. Next, we identified clusters of genes exhibiting similar transcriptional shifts by building networks in which each gene was a node, and each edge represented the inverse of the adjusted *P*-value in the change in pairwise correlation between the two genes the edge connected. We used an eight-step walktrap algorithm implemented through the cluster_walktrap function in igraph R package (Csárdi and Nepusz, 2006) to identify community structure, and then extracted the size and membership of all identified communities.

Finally, we took a first step towards considering expanded applicability of gene transcription for identifying chronically infected animals in the field. We quantified relations between *M. ovipneumoniae* chronic carriage and the genes in our panel using data from the California bighorn sheep described above. We followed methods identical to those described for the covariance-adjusted linear discriminant analysis applied to the acute disease event data, except that the set of states we considered here were 'unexposed', 'recovered' and 'chronically infected'.

Results

GLM ANOVA analyses identified sex as an influence on transcript levels of AHR and AMPK; females had higher levels of both AHR and AMPK transcription. Age influenced transcript levels of MyD88, MX1, FADD, TNFa (highest transcript levels were found in bighorn sheep ages 6–8) and HSP70 and Gata3 (highest transcript levels were found in bighorn sheep ages 2–4).

Discriminant function analysis of transcript patterns identified separation among the four disease groups defined for the acute disease event at RGG and Hardware Ranch (pre-exposure, early acute, late acute, recovered) (Fig. 1). The first function separated the acute disease states from the pre-exposure and recovered groups; the second function separated the early and late acute states from one another. In Fig. 1, each orange data point shows the correlation of the specified gene's relative transcription with the first and second covariance-adjusted linear discriminant functions. The blue points show the average score of individuals in each disease state on the first two discriminant functions. The first axis captured down-regulation of AHR, IFNg, IL10, CD69, FADD, AMPK and Gata3 in the acute disease states. The second axis captured a contrast between TNFa, TGFb and AHR vs. IL10, CD69 and HSP70: in recovered animals, TNFa, TGFb and AHR were high relative to IL10, CD69 and HSP70; in the pre-exposure animals, that pattern was reversed.

General temporal patterns in gene transcription

To better understand how transcription of the discriminating genes differed between the disease groups across the acute disease event, we visually examined patterns of differential gene transcription across all genes over the disease's progression (Fig. 2; note that here we separated free-ranging animals from animals in captivity to allow any non-disease associated differences in gene expression to emerge). Most of the genes that played key roles in the discriminant function also showed clear changes in transcription over disease states. Transcription of four genes (IL10, CD69, FADD and AMPK) dropped dramatically at the onset of disease and then stayed low throughout the disease event. IFNg, Gata3, TNFa and HSP70 all declined and continued to decline gradually throughout the disease event. One gene, MX1, increased over the course of infection. Transcription of IL1b peaked midway through the acute disease event. Transcription patterns in other genes were less clear.

Changes in gene–gene interactions from the pre-exposure to early acute phase

All genes exhibited moderate-to-strong positive correlations (>0.60; median = 0.91) within the six pre-exposure animals. Between the pre-exposure and early acute phases, six gene pairs exhibited significant changes at the 0.05 level in correlation and another 14 gene pairs exhibited changes significant at the 0.10 level in an analysis that adjusted for multiple testing. Of the pairs with significant changes in correlation, 18 of 20 transitioned from positive correlation to no correlation and 2 transitioned from a stronger to a weaker positive correlation. Genes involved in significant changes were Gata3 (involved in 6 of the significant pairwise changes), Tbet (6 changes), MyD88 (5 changes), IL1b (4), MX1 (3), TGFb (3), IL10 (2), HSP70 (2), TNFa (2), FADD (1), AMPK (1) and AHR (1). The walktrap algorithm identified two distinct communities in terms of DC from the pre-exposure to early acute phase. One community contained TGFb, AHR, IL1b and MX1, all of which showed either stable or increasing transcription from pre-exposure to early acute. All other genes clustered in a separate group, within which transcription declined. Most negative correlations during the early acute phase involved TGFb, Tbet or MX1 (and TGFb and MX1 were negatively correlated with one another).

Changes in gene–gene interactions from the early acute to the late acute phase

Only two gene pairs showed significant changes in DC between the early and late acute phases of infection (Fig. 3). IFNg-FADD and TNFa-Gata3 both switched from positive to no correlations. One community consisted of Gata3 and TNFa (which generally declined in transcription between the early and late acute phases); a second community contained IFNg and FADD, and all other genes grouped together in a third community. In the late acute phase, most negative correlations in gene transcription involved IL1b, IFNg or MX1.

A.

	Pre-exposure (n = 6)	Early acute (n = 16)	Late acute (n = 16)	Recovered (n = 2)	R-squared	P
AHR	-3.17	-3.30	-3.64	-3.03	0.12	1.000
HSP70	-5.87	-6.13	-5.94	-6.49	0.03	1.000
MyD88	-5.22	-5.27	-5.30	-5.16	0.00	1.000
MX1	-4.81	-4.68	-4.61	-4.28	0.02	1.000
IFNg	-3.27	-3.97	-5.22	-5.38	0.20	.642
IL10	-5.90	-8.65	-8.95	-8.53	0.20	.185
IL1b	-2.37	-2.17	-2.17	-2.23	0.01	1.000
CD69	-4.02	-6.85	-7.41	-7.06	0.18	.351
FADD	-4.29	-5.25	-5.39	-5.35	0.11	1.000
AMPK	-7.14	-8.74	-9.47	-9.79	0.15	1.000
TNFa	-2.74	-2.52	-2.90	-2.73	0.05	1.000
Gata	-1.81	-2.26	-2.84	-2.44	0.15	1.000
Tbet	-3.97	-4.04	-4.26	-4.60	0.03	1.000
TGFb	-3.50	-3.09	-3.24	-2.86	0.04	1.000

B.

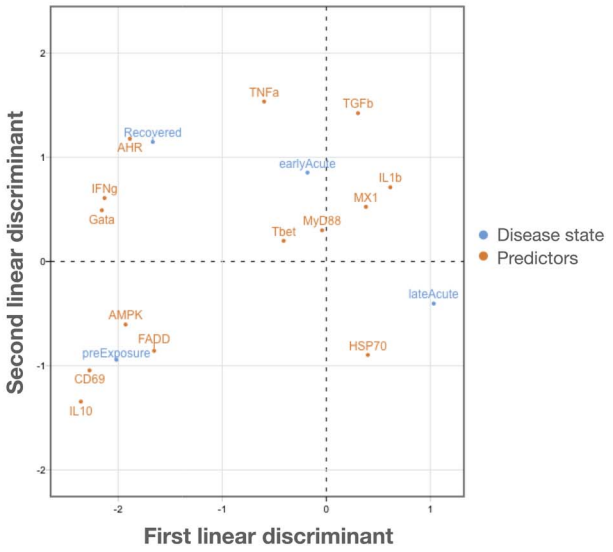

Figure 1: Covariance-adjusted discrimination between acute disease states in the longitudinally studied acute disease event at RGG and Hardware Ranch. The discriminant function was able to correctly classify 78.8% of individuals within the original dataset. Values in (**A**) represent the average transformed gene transcription across all animals within the disease state indicated by the column headers (disease states progress from pre-exposure to recovered from left to right). Colour coding in each row reflects whether the gene was up-regulated (increasingly blue) or down-regulated (increasingly red) in each group relative to the gene's average transcription across all disease states. Colour intensity indicates the magnitude of shifts in regulation, as determined by standard deviations above or below the mean transcription level. (**B**) Blue points show the average score of individuals within each disease state on the first two discriminant functions. Orange points show the correlations of each measured variable with the first and second discriminant functions (for example, IFNg is negatively correlated with the first discriminant function and positively correlated with the second function; IL10 is negatively correlated with both the first and the second discriminant function). Genes contributing most importantly to separation include AHR, IFNg, IL10, CD69, FADD, AMPK and Gata3.

Field testing the feasibility of chronic carrier detection in free-ranging bighorn sheep

Although we used identical data analysis methods in both the California bighorn-wide analysis and the Hardware Ranch/RGG acute disease event analysis, the linear discriminant functions were fit separately in each case (we did not simply apply the discriminant functions derived from the Hardware/RGG animals to the California bighorn gene transcription profiles). This is because the models considered disease states that emerge at different timescales: the timescale of acute disease progression in the Hardware Ranch/RGG case and the timescale of chronic infection establishment in the California bighorn analysis. In the California bighorn-wide analysis, the first discriminant function grouped all five carriers (100% of 'true positives') into a distinct group. The second discriminant function correctly separated three of the recovered animals into a distinct group, but one recovered individual was misclassified as unexposed. Loadings of each gene on the first linear discriminant functions are shown in Fig. 4A, and the location of each individual in that 2D space, along with their *M. ovipneumonia*e infection status, is shown in Fig. 4B.

Discussion

Worldwide, the rate of newly emerging or re-emerging wildlife diseases has increased, impacting human, livestock and wildlife health and biodiversity. While novel molecular approaches have yielded insights into human and wildlife disease at a variety of scales, revealing proximate and ultimate drivers of disease state and transforming understanding of pathogenesis, transmission and treatment, similar analyses have only recently appeared within the context of wildlife disease and conservation science, and rarely as field-based studies. Our primary goal was to assess the ability of a gene transcription panel to gain information about the identification of and mechanisms behind disease state transitions in a free-ranging wildlife disease system, *M. ovipneumoniae*-associated pneumonia in bighorn sheep.

We demonstrate the ability of an immune-targeted quantitative PCR to begin to elucidate molecular immunological mechanisms. Using two slightly different approaches (differential gene transcription and differential gene correlation), we were able to identify genes that were differentially transcribed between disease states as well as characterize the changing

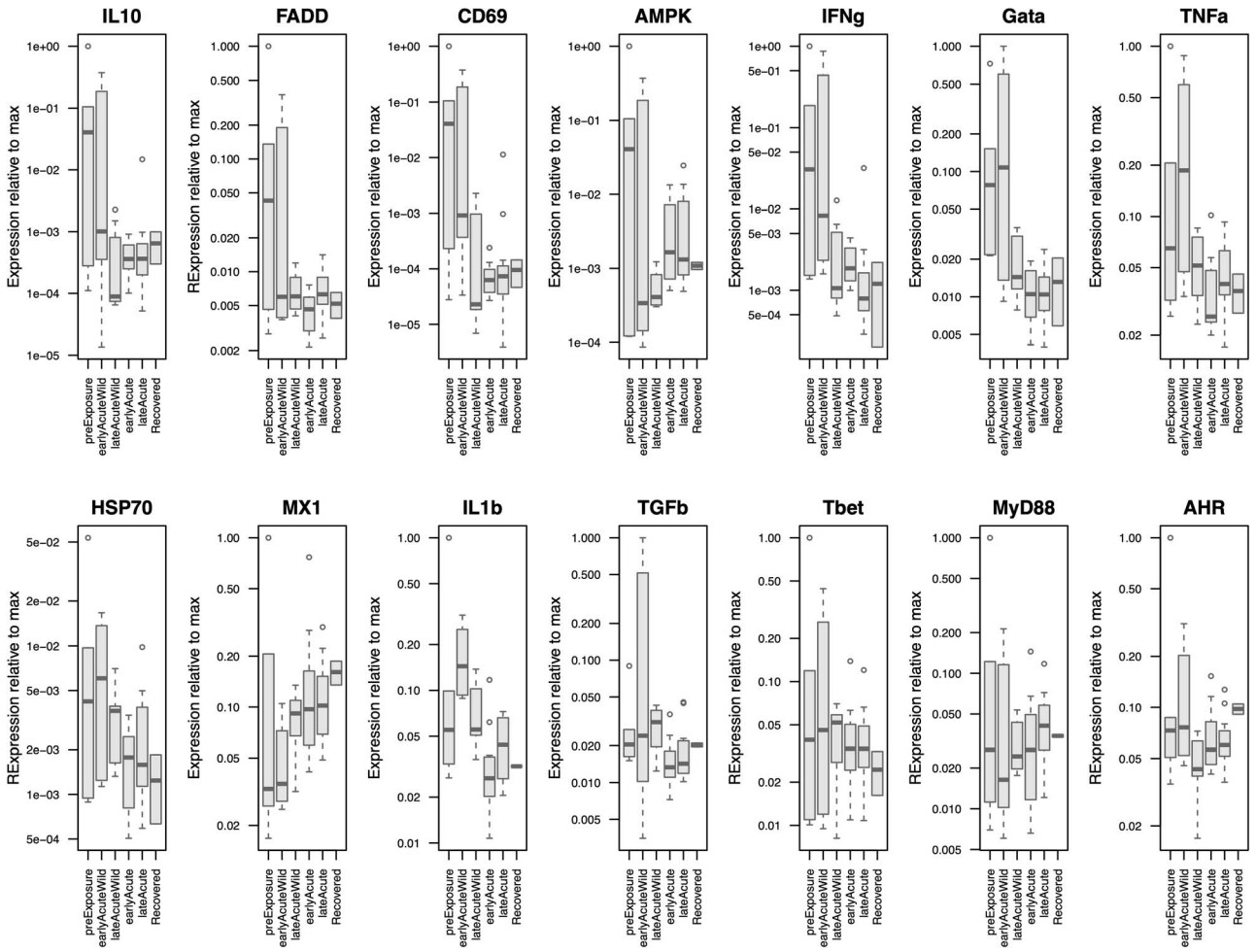

Figure 2: Differential transcription of the 14 genes over the course of the disease event. The first three boxes in each plot reflect transcription in the wild RGG herd. The last three boxes reflect transcription in the captive animals at Hardware Ranch. While disease timing is well known for the captive animals, exposure time is not known at RGG. Early exploration suggested that the 'late acute' phase at RGG may actually correspond best to the 'early acute' phase at Hardware Ranch (note the similarities in transcription for late acute wild animals and early acute Hardware animals at most genes).

relationships among genes as animals progress from one disease state to another. Although both approaches are used in human medicine, there are only a few studies employing this dual approach in wildlife (Ellison *et al.*, 2015). In particular, our analyses revealed patterns linked to a fundamental driver of this system's epidemiology: the existence of chronic carrier hosts. From a management perspective, refined versions of these molecular assays could serve as novel diagnostic tools for identifying chronic carriers, a critical need for effective management in this system. Moreover, our study demonstrates the potential for gene transcription profiles to shed new light on the aetiological mechanisms that generate chronic carrier individuals.

Differential gene transcription

One of the primary transcriptomic analyses in disease dynamics has been the identification of differentially expressed genes (DEGs) between different disease states (Zhang *et al.*, 2014). For example, molecular analyses described in human medicine show compelling associations between differential gene transcription and disease in many studies ranging from inflammatory disease to cancer (McLoughlin *et al.*, 2006). Increasingly, gene transcription-based diagnostics of wildlife are being used to explore immunosuppression and other immune-system impairments that can lead directly to disease or the increased risk of acquiring disease (Acevedo-

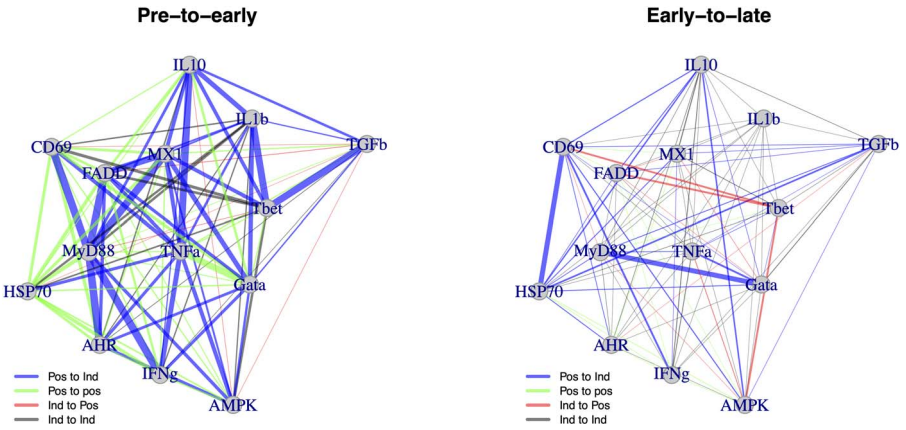

Figure 3: Differential gene–gene correlations between the pre-exposure and early acute phase samples (**A**) and between the early acute phase and late acute phase samples (**B**). Line width corresponds to the inverse of the Benjamini–Hochberg-adjusted *P*-value of the correlation's significance (relationships with stronger statistical support have wider lines). Colours represent how gene–gene correlations changed between disease states. Blue lines indicate pairs of genes whose transcription levels were positively correlated ('pos') in the earlier disease state, but independent or no relationship ('ind') in the later state. Green lines indicate pairs of genes whose transcription levels were positively correlated in both the first and the second state. Red lines indicate pairs of genes whose transcription was independent in the first state, but negatively correlated ('neg') in the second state. Grey lines indicate pairs of genes whose transcription levels were independent in both states shown.

Whitehouse and Duffus, 2009; Eskew *et al.*, 2021). As key indicators of pathophysiologic status, the earliest observable signs of health impairment are altered levels of gene transcripts (DEGs), evident prior to clinical manifestation (McLoughlin *et al.*, 2006), thus providing an early warning of potentially compromised health (Bowen *et al.*, 2020).

Although the induction of transcription in pro-inflammatory genes has been clearly associated with *Mycoplasma* infection (Borchsenius *et al.*, 2020), patterns of gene transcription in free-ranging RGG bighorn sheep in our study are consistent with a general decline in transcription in both pro- and anti-inflammatory genes. Notable exceptions are IL1b, a strong pro-inflammatory mediator, MX1, traditionally known to have a strong anti-viral association and TGFb, a strong anti-inflammatory gene—all of which increased in all bighorn sheep in this study as disease progressed (Table 1; Fig. 2). We identified lower transcription in captive/translocated bighorn sheep than in free-ranging RGG bighorn sheep in almost all genes except AMPK and MX1. It is possible that the relatively lower transcription levels of the genes in the Hardware Ranch bighorn sheep are a result of the stress associated with the attempted translocation and subsequent captivity. Stress can impede transcription of immune-related genes and has been shown to cause shifts in energy metabolism associated with capture stress in dolphins (Elenkov, 2004; Mancia *et al.*, 2008; Acevedo-Whitehouse and Duffus, 2009). Although potentially perplexing, as MX1 has traditionally been thought to play a strong role in anti-viral defence, increased transcription of MX1 in both groups of bighorn sheep is consistent with findings in controlled *Mycoplasma* studies in mice and pigs (Bierne *et al.*, 2012; Li *et al.*, 2015). Incidentally, MX1 has also been tightly linked to IL1b in a large IL1 network (Li *et al.*, 2015).

Differential gene correlation

Although traditional gene transcription methods mainly focus on DEGs, it has been widely recognized that genes do not work in isolation, but rather interact with each other within dynamic networks (interactomes) (Sahni *et al.*, 2013; Yu *et al.*, 2014). Differentially correlated gene pairs may well indicate the alteration of biological states even though the involved genes are not necessarily differentially transcribed (Lai *et al.*, 2004; Zeng and Zhang, 2013; Yu *et al.*, 2014; Zhang *et al.*, 2014). For example, an interacting gene pair can exhibit positive or negative correlation observed in the gene transcription profile. Changing of these gene correlations may correspond to the different states of a biological system (e.g. normal or disease state). Often the progression of disease is not smooth, but is more abrupt; changes in system state often occur at critical thresholds (i.e. 'tipping points') where the system abruptly shifts from one state to another (Chen *et al.*, 2012). The disease progression is sometimes broken into three stages or states: normal, pre-disease and disease (Chen *et al.*, 2012). One major advantage of DC analysis is its ability to help distinguish among disease states (Yu *et al.*, 2014).

A network-based analysis provides a systems-level understanding of the relationships within a network by focusing on gene modules rather than individual genes (Liu and Cai, 2017). The goal of DC analysis is different from the goal of differential transcription analysis. The DC approach identifies genes with varying co-transcription partners under different conditions, such as disease states; these identified genes are more likely to be regulators and can thus elucidate mechanistic links (Van Dam *et al.*, 2018). For example, different Th cell populations are responsible in determining the balance between protective and detrimental immune responses

A.

	Pre-exposure (*n* = 6)	Early acute (*n* = 16)	Late acute (*n* = 16)	Recovered (*n* = 2)	R-squared	*P*
AHR	-3.17	-3.30	-3.64	-3.03	0.12	1.000
HSP70	-5.87	-6.13	-5.94	-6.49	0.03	1.000
MyD88	-5.22	-5.27	-5.30	-5.16	0.00	1.000
MX1	-4.81	-4.68	-4.61	-4.28	0.02	1.000
IFNg	-3.27	-3.97	-5.22	-5.38	0.20	.642
IL10	-5.90	-8.65	-8.95	-8.53	0.20	.185
IL1b	-2.37	-2.17	-2.17	-2.23	0.01	1.000
CD69	-4.02	-6.85	-7.41	-7.06	0.18	.351
FADD	-4.29	-5.25	-5.39	-5.35	0.11	1.000
AMPK	-7.14	-8.74	-9.47	-9.79	0.15	1.000
TNFa	-2.74	-2.52	-2.90	-2.73	0.05	1.000
Gata	-1.81	-2.26	-2.84	-2.44	0.15	1.000
Tbet	-3.97	-4.04	-4.26	-4.60	0.03	1.000
TGFb	-3.50	-3.09	-3.24	-2.86	0.04	1.000

B.

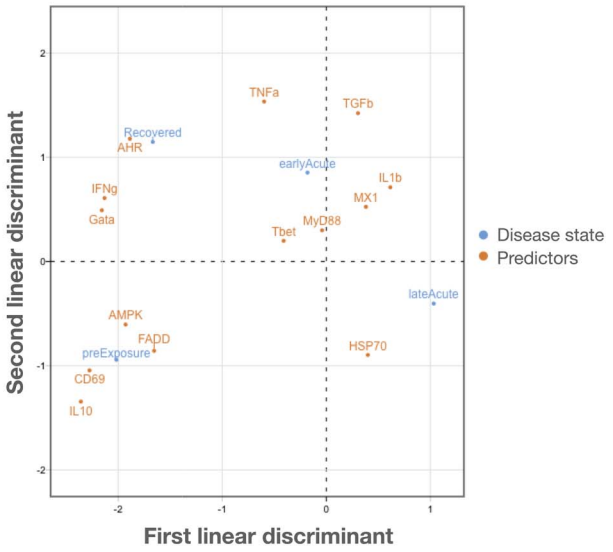

Figure 4: Covariance-adjusted discrimination among unexposed, chronic and recovered disease states in the cross-sectional California bighorn sheep data from Nevada Department of Wildlife. The discriminant function correctly classified 92.6% of individuals within the original dataset (including 100% of the chronically infected individuals). Values in (**A**) represent the average transformed gene transcription across all animals within the disease state indicated by the column headers (disease states progress from pre-exposure to recovered from left to right). Colour coding in each row reflects whether the gene was up-regulated (increasingly blue) or down-regulated (increasingly red) in each group relative to the gene's average transcription across all disease states. Colour intensity indicates the magnitude of shifts in regulation, as determined by standard deviations above or below the mean transcription level. (**B**) Blue points show the average score of individuals within each disease state on the first two discriminant functions. Orange points show the correlations of each measured variable with the first and second discriminant functions (for example, IFNg is positively correlated with the first discriminant function and relatively uncorrelated with the second function; MyD88 is negatively correlated with the first discriminant function and relatively independent of the second). Chronic carriers showed distinctly higher expression of MyD88 and FADD and distinctly lower expression of IFNg and Tbet than recovered and unexposed animals.

against *Mycoplasma*; Th1 and Th2 have opposing roles and exhibit strong effects on responses to *Mycoplasma* infection (Bodhankar *et al.*, 2010).

Although the time series in our study is limited and somewhat artificially manufactured, it allows for the study of transcriptional regulation of gene co-transcription networks during infection. In our artificial baseline (pre-exposure bighorn sheep), all genes exhibited strong positive correlations (>0.60; median = 0.91) within the six pre-exposure animals. Our networks analysing changes in gene relationships between pre-exposure and early acute as well as early acute to late acute represented a substantial decoupling of gene patterns. As we discussed earlier, and consistent with our differential gene transcription analyses, gene transcription patterns separated into two communities. One community contained TGFb, AHR, IL1b and MX1, all of which showed either stable or increasing transcription from pre-exposure to early acute, while all other genes clustered in a separate group, within which transcription declined. These relationships illustrate a phase shift with a concurrent reduction in initiation of a Type 1 immune response, anti-inflammatory activity, initiation of cell death and muscle metabolism.

A comparison of gene correlations between early acute and late acute phases revealed only two significant shifts in correlation (IFNg-FADD and TNFa-Gata3 both switched from positive to no correlations). These relationships illustrate a phase shift in which we identified decreases in initiation of a Type 2 immune response and general inflammatory responses.

The gene pairs identified in this analysis serve as examples of the utility of this method, indicating DCs between gene–gene pairs for genes that did not exhibit statistically significant differences in differential gene transcription analysis (i.e. identifying biologically meaningful associations). Although we can say with some certainty that the differentially correlated genes we identified contribute to transitions from one disease state to another, it is still not known how changes in correlation patterns can point to genes with critical capacity to guide a biological system into certain states/phenotypes. This reflects a broader underlying knowledge gap around whether we can infer causality or directionality between gene relationships and disease states (Thomas *et al.*, 2016; Van Dam *et al.*, 2018).

Study limitations

As with any study of a disease system operating in free-ranging wildlife, our analyses are confronted by limited replication. In particular, the small number of chronic carriers in

our longer-term dataset leaves open the potential for biassed inferences about differences in gene transcription between chronic carriers and individuals that cleared infection. This is why we emphasize the proof-of-concept nature of our work: while our results are intriguing and merit additional inquiry, they require replication (ideally, in chronic carriers arising across a variety of different environmental contexts) before being fully operational as a diagnostic tool. Given the critical role that chronic carrier identification currently plays in bighorn sheep management, however, the potential of the transcription profile to make that distinction goes beyond simple academic interest. Consequently, we believe that opportunities to expand the suite of data on chronic carriers are not outside the realm of possibility.

A second limitation is that our dataset does not represent all subspecies of bighorn sheep. While we assume that Rocky Mountain and California bighorns likely exhibit similar in-host responses to *M. ovipneumoniae* on the basis of consistent epidemiological descriptions of disease progression in both species, that assumption could be flawed. Importantly, our results would certainly require separate validation in the desert bighorn sheep subspecies, which exhibits more heterogeneity in response to disease due to mechanisms that are as-of-yet unknown (Cassirer *et al.,* 2018).

Field application

We took a first step towards considering field applicability of gene transcription for identifying chronically infected animals. Although more data are required to fully calibrate these patterns, the early results show promise. The California bighorn dataset consisted of 27 individuals, of whom 4 were identified as chronic carriers, 5 were classified as recovered and 18 were classified as unexposed. A major caveat to this analysis is that all chronic carriers arose from a single population, and no other animals from that population were available for inclusion. This means that the pattern reported here could be attributable to environmental as opposed to pathogen-associated factors. However, since our intent was simply to explore whether classification of disease status on the basis of the panel is feasible, we elected to proceed in hopes that our results might motivate additional exploration in this direction.

Implications for conservation and biodiversity

Wildlife managers and veterinarians have tried many techniques for controlling and mitigating respiratory disease in wild bighorn sheep populations; however, more effective strategies are needed to prevent pathogen introduction, induce disease fadeout in persistently infected populations and promote population resilience in bighorn sheep (Cassirer *et al.,* 2018; Almberg *et al*., in revision). To this end, better understanding in-host disease dynamics across populations with a focus on identifying factors associated with naturally occurring recovery would be useful for management (Cassirer *et al.,* 2018). With our transcriptomic panel of 16 genes, we achieved our goal of testing the use of transcriptomic techniques to compare immune markers within animals over the course of their infections, as well as beginning to identify mechanisms associated with disease state transitions in the bighorn sheep *M. ovipneumoniae* system. However, relatively recent advances in gene sequencing technology allow for the elucidation of an unprecedented breadth of genes. In the future, whole-transcriptome identification of genes and gene pathways associated with altered physiologic manifestations would enable us to design a more effective gene panel, providing greater insight into the causes of specific disease states (Eskew *et al.,* 2021).

From a management perspective, gene transcription may be a promising avenue for developing diagnostics that can differentiate between chronically and acutely infected animals. This is a major priority for current bighorn sheep disease management, which is increasingly built around identification and removal of chronically infected animals (Garwood *et al.,* 2020; Almberg *et al*., in revision). While our data are insufficient to definitively categorize animals as chronic vs. acute, our preliminary analyses suggest that the gene transcription panel employed here may have promise in this direction in the future.

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Author Contributions

L.B. and K.M. contributed equally to this paper and were responsible for concept, design, analysis, interpretation and writing. A.R., N.H. and P.W. were involved in conceptualization and provided valuable feedback and suggestions. S.W. processed all bighorn sheep samples and performed laboratory analyses.

Conflict of Interest

The authors have not identified any conflicts of interest.

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

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