LIFE HISTORY PERSPECTIVES ON PARASITE GENOTYPES, DISEASE OUTBREAKS, AND WILDLIFE HOSTS FOR *TOXOPLASMA GONDII* AND *SARCOCYSTIS NEURONA*

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CHAPTER I

INTRODUCTION

Molecular techniques for identifying and visualizing inter- and intra-specific genetic diversity in microbial pathogens have been paramount to efforts towards understanding the epidemiology, evolutionary biology, and life history parameters governing infectious diseases. Generally, pathogen genetic diversity is estimated from the direct or indirect analysis of nucleotide or amino acid sequences representing one or a few genomic loci or their encoded products (i.e. transcripts, proteins) among several pathogen isolates. The number of methods developed for pathogen genotyping is several. As a rule, higher resolution techniques, such as direct DNA sequencing, combined with a greater number of loci examined will increase the detectable level of inter and intra-specific resolution of pathogen isolates, often leading to more conclusive studies. An ability to distinguish pathogens at the species and sub-species level allows researchers to determine which pathogen species and strains cause disease and should therefore be the focus of prevention and treatment strategy development. This approach also permits tracing pathogens in the environment, which is important for understanding transmission dynamics. Furthermore, monitoring genetic diversity over time can give key insights to how pathogens evolve under pressure from natural (i.e. host immune responses) and artificial (i.e. drug or vaccine treatment) selection. The Apicomplexan parasites, which comprise a diverse group of important human and veterinary pathogens, have been no exception, as genotype and population genetic analyses have led to many important discoveries including identification of the genetic basis for drug resistance and virulence, strain associations with disease, and the mechanistic basis

for pathogen transmission and disease outbreaks (Beck et al., 2009). In this context, the focus of this study is two closely related Apicomplexans, *Sarcocystis neurona* and *Toxoplasma gondii*.

Sarcocystis neurona and *T. gondii* are both tissue encysting coccidia of the family Sarcocystidae and represent important disease threats to numerous mammalian and avian species. Both of these pathogens have complex lifecycles that involve a sexual stage in a definitive host and asexual stages in a range of intermediate hosts. The presence of life history traits that are permissive to both genetic recombination and clonal expansion has made efforts to define genetic diversity and structure in these organisms a non-trivial task and, in many respects, population genetic studies can still be considered in their infancy. Within the past decade, though, several genotypic data have accumulated for these parasites as higher resolution techniques have become more affordable and amenable (Elsheikha and Mansfield, 2007; Su et al., 2010). This has led to the realization that many of the initial and defining studies for *S. neurona* and *T. gondii* population genetics based on limited data are only rough approximations of the true complexity present in nature (Elsheikha and Mansfield, 2007; Su et al., 2010). In this study, past genotyping efforts are complied, analyzed, and expanded on in an effort to delineate the influences clonal propagation and sexual recombination have on shaping the genetic diversity and population structure of these parasites and the roles the life history traits that encompass these genetic processes play in disease. A special emphasis is given to study of parasite isolates from wildlife hosts, especially Southern sea otters, as an understudied but important parasite gene pool and host population with high potential to give overreaching insights to how and why disease outbreaks occur.

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preface. Chapter II is a review article that compiles and analyzes many recent *T. gondii* genotyping efforts, emphasizing those from wildlife hosts, to demonstrate the new insights these isolates have provided in the context of the history of discovery of *T. gondii* population genetics. Chapter III describes the development of a consensus mulit-locus genetic typing scheme for *S. neurona* and its preliminary application towards understanding emergence in the Southern sea otter population of the marine environment off the coast of California. A major theme in this chapter is how the better studied parasite, *T. gondii*, can be used as a model to guide studies of *S. neurona*. Since these parasites are closely related genetically, have similar lifecycles, and infect many of the same hosts, it is likely that the findings of a study involving one will apply to the other—an important point to keep in mind throughout this work. This principle is most evident in Chapter IV, in which genetic typing schemes are much more extensively developed for both *S. neurona* and *T. gondii* and applied to define a general mechanistic basis for the emergence of virulent, outbreak-causing clones for both pathogens. Finally, Chapter V presents the overall conclusions and future directions for research.

CHAPTER II

POPULATION GENETICS OF *TOXOPLASMA GONDII*: NEW PERSPECTIVES FROM PARASTIE GENOTYPES IN WILDLIFE

Preface

This chapter originally appeared as titled in the special issue, *Zoonoses in a Changing World*, of the journal, Veterinary Parasitology by Jered M. Wendte, Amanda K. Gibson, and Michael E. Grigg. JMW's contributions included conceiving and writing the manuscript and compiling and analyzing the data discussed herein, with the only exception being JMW's partial contribution to Table 1, the majority of which was compiled by AKG.

Toxoplasma gondii, a zoonotic protozoal parasite, is well-known for its global distribution and its ability to infect virtually all warm-blooded vertebrates. Nonetheless, attempts to describe the population structure of *T. gondii* have been primarily limited to samples isolated from humans and domesticated animals. More recent studies, however, have made efforts to characterize *T. gondii* isolates from a wider range of host species and geographic locales. These findings have dramatically changed our perception of the extent of genetic diversity in the *T. gondii* population structure and the relative roles of sexual recombination and clonal propagation in the parasite's lifecycle. In particular, identification of novel, disease-causing *T. gondii* strains in wildlife has raised concerns from both a conservation and public health perspective as to whether distinct domestic and sylvatic parasite gene pools exist. If so, overlap of these cycles

may represent regions of high probability of disease emergence. Here, we attempt to answer these key questions by reviewing recent studies of *T. gondii* infections in wildlife, highlighting those which have advanced our understanding of the genetic diversity and population biology of this important zoonotic pathogen.

1. Background

1.1 Lifecycle

Toxoplasma gondii is an apicomplexan parasite and the most extensively studied of the tissue encysting coccidia, a group that comprises many species of human and veterinary medical importance, including members of the genera *Sarcocystis*, *Neospora*, *Hammondia* and *Besnoitia*. The *T. gondii* lifecycle involves an asexual stage in intermediate hosts and a sexual stage in a definitive host, which may be any species of domestic or wild cat (Dubey and Frenkel, 1972; Hutchison, 1965). Cats are naturally infected through the oral route and, upon ingestion, *T. gondii* commences the sexual stage by differentiating into male and female gametes which fuse in the intestinal epithelium to form a fertilized oocyst (Dubey and Frenkel, 1972; Ferguson et al., 1974; Ferguson et al., 1975a; Ferguson, 2002; Ferguson et al., 1975b). Oocysts are then shed into the environment in the feces where they undergo meiosis during the process of sporulation, eventually producing eight haploid progeny that are enveloped in an environmentally stable infectious propagule (Dubey et al., 1970b; Ferguson, 2002; Frenkel et al., 1975). Over the course of one to three weeks, a cat may shed millions of oocysts, which are highly infectious orally to intermediate hosts and, to a lesser extent, other definitive hosts (Dubey, 1996, 1998, 2006; Dubey and Frenkel, 1972; Dubey et al., 1996; Dubey et al., 1970a).

The intermediate host range of *T. gondii* is vast, inclusive of essentially all warm-blooded vertebrates, both avian and mammalian (Dubey, 2008). When intermediate hosts ingest *T. gondii*, the parasite crosses the intestinal epithelium and differentiates into a rapidly dividing tachyzoite form which disseminates infection throughout the host (Dubey, 1998). If the host survives this acute phase of infection, the chronic phase is initiated when tachyzoites differentiate into slowly dividing, semi-dormant bradyzoites that form tissue cysts, typically in muscle or neural cells (Dubey, 1997, 1998; Dubey and Frenkel, 1976; Dubey et al., 1998). Tissue cysts can persist for the life of the host and are orally infectious to definitive hosts through carnivory (Dubey, 2001, 2006; Frenkel et al., 1970; Su et al., 2003).

Toxoplasma gondii is not, however, an obligately heteroxenous parasite and can propagate clonally, presumably indefinitely, by cycling among intermediate hosts. This can occur vertically through transplacental transmission from mother to offspring (Dubey et al., 1997b; Elbez-Rubinstein et al., 2009; Hide et al., 2007; Innes et al., 2009; Miller et al., 2008a), or, in rare cases during medical operations, horizontally through tissue transplant (Martina et al., 2010). Tissue cysts are also orally infectious to carnivorous intermediate hosts, permitting the parasite to bypass the sexual stage in the definitive host (Dubey, 2001, 2006; Khan et al., 2007; Su et al., 2003). This trait, along with its widely inclusive host range, has often been cited as unique to *T. gondii* among the tissue encysting coccidia (Grigg and Sundar, 2009; Khan et al., 2007; Sibley and Ajioka, 2008; Sibley et al., 2009; Su et al., 2003). However, many recent studies have shown that other tissue encysting coccidia, including *Neospora caninum* and *Sarcocystis neurona*, have much broader intermediate host ranges than originally thought, including both

mammalian and avian species and predators and their prey (Cheadle et al., 2001; Costa et al., 2008; Dubey et al., 2001a; Dubey et al., 2001b; Gondim et al., 2010; Mansfield et al., 2008; Miller et al., 2009; Rejmanek et al., 2010; Wendte et al., 2010b). Until more rigorous studies are completed, the possibility cannot be excluded that these related parasites also have an ability for carnivorous oral transmission among intermediate hosts.

Infection in the definitive host stage is also not always synonymous with sexual propagation. In the feline host, *T. gondii* readily differentiates into the asexual tachyzoite and bradyzoite forms, eventually developing into infectious tissue cysts just as in intermediate hosts (Dubey, 1997). Moreover, initiation of the sexual pathway does not preclude clonal propagation, as *T. gondii* has been found to lack predetermined mating types, permitting a single genotype to form both male and female gametes that fuse to yield progeny nearly identical to the parent (Cornelissen and Overdulve, 1985; Pfefferkorn et al., 1977; Pfefferkorn and Pfefferkorn, 1980). This process, termed selfing, has been shown to have important implications for the population biology and epidemiology of *T. gondii* and other tissue encysting coccidia (Vaudaux et al., 2010; Wendte et al., 2010a), and will be discussed further below.

1.2 Disease

Major manifestations of *T. gondii*-induced disease in humans include encephalitic, ocular, and pneumatic toxoplasmosis (Montoya and Liesenfeld, 2004). Based upon seroprevalence rates, it has been assumed that the majority of infections are asymptomatic, with disease mainly linked to congenital infection or an immune-compromised state such as AIDS (Montoya and Liesenfeld, 2004). However, it is

becoming increasingly appreciated that symptomatic disease can also occur in immune-competent individuals (Ajzenberg et al., 2004; Boothroyd and Grigg, 2002; De Salvador-Guillouet et al., 2006; Demar et al., 2007; Grigg et al., 2001b; Leal et al., 2007; Sacks et al., 1983). Many factors are likely to influence whether or not disease occurs including dose, parasite stage initiating infection, parasite genotype, host genotype, and various factors influencing host immune status, especially concomitant infection with other pathogens.

Descriptions of toxoplasmosis in wildlife are generally limited to post-mortem analyses except for well-monitored species such as endangered and captive or semi-captive animals. The clinical picture of toxoplasmosis in wildlife appears similar to that in humans, with lung, brain, and eye involvement prevalent in many cases, thus providing important parallels that have increased our understanding of human disease. Of particular concern are several reports of multi-organ involvement indicative of disseminated disease in rare and exotic species, perhaps signifying increased susceptibility in hosts that have only recently been exposed to this parasite through human activity. Several reports of toxoplasmosis in wildlife are summarized in Table 1 for the reader's reference.

2. *Toxoplasma* in wildlife

2.1 Molecular genotyping and population genetics

2.1.1 History

Several techniques for detecting and characterizing *T. gondii* genetic material either directly from infected host tissues or from parasites isolated via bioassay in mice, cats, or tissue culture have been developed. Here, we will discuss a subset of these

techniques and their relative advantages and disadvantages as pertinent to the subject matter at hand. We refer the reader to a recent extensive review of molecular genotyping of *T. gondii* for further details (Su et al., 2010). To adequately discuss the genetic characterization of *T. gondii* in wildlife, it will first be necessary to review the history of discovery and current viewpoints on the population genetic structure of this parasite.

The first picture of the *T. gondii* population structure came from isoenzyme and restriction fragment length polymorphism (RFLP) analyses (Darde et al., 1988, 1992; Sibley and Boothroyd, 1992b). Dardé and colleagues (1992) used isoelectric focusing of six enzymes to show that 35 *T. gondii* isolates could be grouped into just 5 distinct zymodemes, with the majority of isolates (56%) comprising just one zymodeme. At about the same time, Sibley and Boothroyd (1992b) used RFLP analysis of 27 samples at three loci to show that nine isolates that were virulent to laboratory mice comprised a distinct, identical genotype while a moderate amount of diversity was found in the remaining 21 samples. Clonality in the mouse virulent samples was confirmed with RFLP analysis of an additional seven loci (Sibley and Boothroyd, 1992b). These RFLP findings were later expanded to encompass a larger sampling of 106 isolates spanning 3 continents and several host species. Using six RFLP markers, ~84%, or 89 of 106 isolates were classified into just three clonal types (Howe and Sibley, 1995). This apparently clonal population structure was especially striking since, in a few cases, samples from diverse parts of the globe were identical at all six markers (Darde et al., 1992; Howe and Sibley, 1995; Sibley and Boothroyd, 1992b). These studies also prompted the conclusion that, despite presumably numerous opportunities for sexual recombination in widespread

feline definitive hosts, productive sexual crosses only occur very rarely in nature (Howe and Sibley, 1995).

Closer examination of the data presented in the studies described above, however, suggest other explanations aside from a strictly clonal population structure. It is important to note that the 1992 Dardé et al. and Sibley and Boothroyd studies shared nine isolates (9/62; approx. 30% of the total in each study). Moreover, of the 53 total isolates analyzed between the two studies, 47/53 (89%) were from either the US or France, 52/53 (98%) were from humans or domestic animals, and of these, 37/53 (70%) were from humans with congenital infections, AIDS patients, and/or humans with symptomatic disease (Darde et al., 1992; Sibley and Boothroyd, 1992b). Thirty-six of these 53 (68%) were again analyzed in the Howe and Sibley study (1995), which also comprised mainly human samples (68/106, 64%) from the US or France. A sample set that is highly biased towards a certain host species, towards cases of disease, or both increases the likelihood of over-sampling particular genotypes, skewing the population structure in favor of apparent clonality (Feil and Spratt, 2001). Moreover, even if parasite genotypes do not segregate according to host species or virulence, oversampling of a few genotypes does not preclude an underlying population of diverse parasite genotypes with high rates of genetic exchange, as has been described for organisms with so-called 'epidemic' population structures (Feil and Spratt, 2001; Maynard Smith et al., 1993). Epidemic here refers not exclusively to localized disease epidemics (though this is often the case), but instead to any circumstance where a particular genotype experiences enhanced clonal expansion relative to others in the population, at a local or global level, and is thus over-sampled (Maynard Smith et al., 1993).

Table 1. Toxoplasmosis in wildlife*

MAMMALS

Species	Captive?	Location	Diagnosis	Symptoms	Reference
Alouatta belzebul (red-handed howler monkey)	Y	Pernambuco State, Brazil	parasite isolation, PCR	prostration, diarrhea, hyperthermia	Pena (2010) Vet Parasitol *In Press*
Bettongi penicillata (woylie)		Manjimup reserve, Australia	PCR	neurologic symptoms	N. Parameswaran et al. (2010) Int J Parasitol 40: 635–640
Delphinapterus leucas (beluga whale)		Quebec, Canada	IHC	stranding, mortality	Mikaelian et al. (2000) J Comp Path 122: 73-76
Enhydra lutris (Northern sea otter)		WA, USA	IHC, bioassay, PCR-RFLP	convulsions, mortality, encephalitis	Lindsay et al. (2001) Vet Parasitol 97: 319-327
Enhydra lutris nereis (Southern sea otter)		CA, USA	IHC, serology, parasite isolation, PCR	stranding, encephalitis, mortality	Miller et al. (2004) Int J Parasitol 34: 275-284
Enhydra lutris nereis (Southern sea otter)		CA, USA	IHC, parasite isolation, PCR-RFLP	stranding, mortality	Cole et al. (2000) J Parasitol 86: 526-530
Felis margarita (sand cat)	Y	United Arab Emirates	IHC, serology, PCR	mortality, hepatitis, pneumonitis	Dubey et al. (2010) Vet Parasitol 172:195-203
Felis margarita (sand cat)	Y	Qatar	IHC, serology, PCR	anorexia, weakness, mortality	Dubey et al. (2010) Vet Parasitol 172:195-203
Lynx rufus (bobcat)		MT, USA	IHC, serology	mortality,myocarditis, hepatitis, encephalitis	Dubey et al. (1987) J Wildl Dis 23: 324-327
Macropus rufogriseus (wallaby)		Tasmania, Australia	parasite isolation	neurologic symptoms	N. Parameswaran et al. (2010) Int J Parasitol 40: 635–640

Marcropus eugeniii (Tammar wallaby)		New Zealand	serology, parasite isolation	poor health, mortality	Dubey and Crutchley (2008) J Parasitol 94:929-933
Marcropus rufogriseus (Bennett's wallaby)	Y	Spain	IHC	apathy, depression, emaciation, mortality, pneumonia, myocarditis, cholangiohepatitis, severe gastroenteritis, uveitis	Bermúdez et al. (2009) Vet Parasitol 160: 155-158
Marcropus rufogriseus (Bennett's wallaby)		New Zealand	IHC, serology, parasite isolation, PCR-RFLP	lethargy, emactiaion, incoordination,impaired vision,mortality, pneumonia, congestive heart failure	Dubey and Crutchley (2008) J Parasitol 94:929-933
Marcropus rufogriseus (Bennett's wallaby)	Y	PA, USA	IHC, serology, parasite isolation, PCR-RFLP	weakness, lethargy, loss of appetite, incoordination	Dubey and Crutchley (2008) J Parasitol 94:929-933
Marcropus rufogriseus (Bennett's wallaby)	Y	Argentina	IHC, serology, parasite isolation, PCR	mortality	Basso et al. (2007) Vet Parasitol 144: 157-161
Marmota monax (woodchuck)		NY, USA	IHC, RT-PCR	neurologic symptoms, weight loss, mortality, cerebral hemmorhage, encephalitis, myocarditis, hepatitis	Bangari et al. (2007) J Vet Diagn Invest 19: 705-709
Mustela vison (mink)		MI, USA	IHC, PCR	neurological symptoms (lameness, ataxia, head tremors, impaired vision)	Jones et al. (2006) J Wildl Dis 42: 865-869
Phoca vitulina richardsi (Pacific harbor seal)		CA, USA	parasite isolation	meningoencephalomyelitis	Miller et al. (2001) J Parasitol 87, 816-822
Rupicapra pyrenaica (southern chamois)		Pyrenees, Spain	IHC, serology	neurological symptoms, systemic toxoplasmosis with eye and lung pathology	Marco et al. (2009) J Vet Diagn Invest 21: 244–247

Sousa chinensis (Indo-Pacific humpbacked dolphin)		Queensland, Australia	IHC	mortality, pneumonia, emaciation, shark attack, peritonitis, pancreatitis, ocular pathology	Bowater et al. (2003) Aust Vet J 81: 627-632
Stenella coeruleoalba (striped dolphin)		Italy	ICH, serology	mortality, encephalitis, bronchopneumonia, pulmonoary atelectasis, consolidation, emphysema	Di Guardo et al. (2010) Vet Parasitol 47: 245-253
Suricata suricatta (meerkat)	Y	Perth, Australia	PCR	neurologic symptoms	N. Parameswaran et al. (2010) Int J Parasitol 40: 635–640
Tamiasciurus hudsonicus (American red squirrel)		IN, USA	IHC, RT-PCR	weight loss, mortality, pneumonia, encephalitis	Bangari et al. (2007) J Vet Diagn Invest 19: 705-709
Tursiops truncatus (bottlenose dolphin)		Russia	IHC, serology, parasite isolation, PCR, PCR-RFLP	disorientation, lethargy, anorexia, mortality, encephalitis	Dubey et al. (2009) J Parasitol 95: 82-85
*Vombatus ursinus (*wombat)		Tasmania, Australia	Parasite isolation, PCR	neurologic symptoms	N. Parameswaran et al. (2010) Int J Parasitol 40: 635–640
Vombatus ursinus (wombat)	Y	New South Wales, Australia	IHC, serology	anorexia, mortality, dyspnoea, tachycardia, neorological and respiratory lesions	Hartley (2006) Aust Vet J 84: 107-109
Vulpes cana (Blanford's fox)	Y	United Arab Emirates	IHC, serology	mortality	Dubey and Pas (2008) Vet Parasitol 153: 147-151
Vulpes lagopus (artic fox)		Svalbard	IHC, serology	jaundice, mortality, hepatitis, pneumonia, meningitis	Sorenson et al (2005) Res Vet Sci 78:161-167
Vulpes ruepelli (sand fox)	Y	United Arab Emirates	IHC	mortality	Pas and Dubey (2008) J Parasitol 94: 976-977

BIRDS **Species**	**Captive?**	**Location**	**Diagnosis**	**Symptoms**	**Reference**
Acridotheres (myna)	Y	Mexico	electron microscopy	inappetent, shivering, mortality	Dhillon et al. (1982) Avian Dis 26: 445-449
Anseranas semipalmata (magpie geese)	Y	TX, USA	IHC, serology	mortality, pneumonia, hepatitis	Dubey et al. (2001) J Parasitol 87: 219-223
Caloena nicobaria (Nicobar pigeons)	Y	South Africa	IHC	mortality	Las and Shivaprasad (2008) J S Afr Vet Assoc 79: 149-
Corus hawaiiensis ('Alala)		HI, USA	IHC, serology, parasite isolation	emaciation, depression, mortality	Work et al. (2000) J Wildl Dis 36: 205-212
Cyanoramphus (kakariki)	Y	Australia	IHC	loss of appetite, mortality	Hartley et al (2008) J Parasitol 96: 1424-1425
Eos bornea (red lory)	Y	LA, USA	IHC	pneumonia, depression, mortality	Howerth et al (1991) Avian Dis 35: 642-646
Eos cyanogenia (black-winged lory)	Y	SC, USA	serology, parasite isolation, PCR-RFLP	mortality, acute toxoplasmosis	Dubey et al. (2004) J Parasitol 90: 1171-1174
Eudyptula minor (penguin)	Y	Tasmania, Australia	IHC	anorectic, diarrhea,mortality	Mason et al (1991) J Parasitol 77: 328
Francolinus erckelii (Erckels francolin)		HI, USA	IHC	mortlality, necrosis	Work et al (2002) J Parasitol 88: 1040-1042
Haliaeetus leucocephalus (bald eagle)		unknown	IHC	respiratory distress, mortality, myocarditis	Szabo et al. (2004) J Parasitol 90: 907-908
Melanerpes carolinus (red-bellied woodpecker)		GA, USA	IHC, serology, PCR	lethargy, neurological symptoms (seizures), encephalitis	Gerhold and Yabsley (2007) Avian Dis 51: 992-994

Meleagris gallopavo(wild turkey)		WV, USA	IHC	emaciation, crusting dermatitis on head and neck, mortality, splenomegaly, multifocal necrotizing hepatitis and splenitis	Quist et al. (1995) J Wildl Dis 31:255-258
Meleagris gallopavo(wild turkey)		GA, USA	IHC, electron microscopy	mortality, pneumonia, splenomegaly	Howerth and Rodenroth(1985) J Wildl Dis 21: 446-449
Nesochen sandvicensis (nene goose)	Y	HI, USA	IHC	mortality, splenomegaly	Work et al (2002) J Parasitol 88: 1040-1042
Ramphastos sulfuratus (keel-billed toucan)	Y	Costa Rica	bioassay, parasite isolation, PCR-RFLP	mortality	Dubey et al. (2009) J Parasitol 95: 467-468
Serinus canaria (Roller canary)	Y	Victoria, Australia	IHC	blindness, nystagmus, ataxia, head rotation, mortality	Lindsay et al. (1995) Avian Dis 39: 204-207
Serinus canaria (canary)	Y	unknown	IHC, electron microscopy	chemosis, lethargy, torticollis	Williams et al (2001) Avian Dis 45: 262-267
Strix varia (barred owl)		Quebec, Canada	IHC	car collision, anorexia, inactivity, mortality, hepatitis	Mikaelian et al (1997) Avian Dis 41: 738-740
Sula Sula (red-footed booby)		HI, USA	IHC	weak, mortality, necrosis, inflammation	Work et al (2002) J Parasitol 88: 1040-1042

*Search terms used were: *Toxoplasma gondii*, mammal, bird, wildlife on ISI web of science and PubMed online databases

It is also possible that limitations in the discriminatory power of the markers used in these early studies missed much of the genetic diversity present. When Lehmann and colleagues applied higher resolution DNA sequencing analysis of loci, they identified increased levels of polymorphism among the genotypes of sixteen isolates, including twelve previously typed by the Howe and Sibley 1995 study (Lehmann et al., 2000). This study identified more allelic diversity among strains than that identified with RFLP analyses. Genetic diversity within the three clonal archetypes was also confirmed using high resolution microsatellite typing (Ajzenberg et al., 2002a; Ajzenberg et al., 2002b; Blackston et al., 2001). Ajzenberg and colleagues (2002a) found that among 83 archetypal isolates, mostly from human clinical cases, 72 distinct genotypes could be discerned using these highly polymorphic markers, highlighting their utility for distinguishing strains within clonal groups.

The re-analysis of previously typed strains also resulted in two important observations attesting to the importance of the sexual stage for *T. gondii*'s population structure. First, linkage disequilibrium, while significantly present even when correcting for effects of a geographically and temporally diverse sample set (Ajzenberg et al., 2002a), was not absolute across all loci, again supporting the notion that sexual recombination is a viable process in the *T. gondii* lifecycle (Ajzenberg et al., 2002a; Grigg et al., 2001a; Lehmann et al., 2000). Second, independent gene tree analyses of each typing locus revealed for the majority of strains only two groupings, instead of three, and that some strains could be assigned to either one group or the other depending on the locus examined (Grigg et al., 2001a; Lehmann et al., 2000). This suggested that members of the three clonal lineages defined by Howe and Sibley (1995) were actually

sibling progeny of one or a few genetic outcrosses, a finding that was later confirmed by more extensive analyses (Boyle et al., 2006). These findings revealed sexual recombination as a major force shaping the population structure of *T. gondii,* with important implications for the emergence of virulent strains (Grigg et al., 2001a) or strains associated with particular disease, for example ocular toxoplasmosis (Grigg et al., 2001b). It also became apparent that genetic exchange is a critical factor to account for when performing phylogenetic analyses, as it greatly increases the potential for phylogenetic non-congruency between loci, even in instances of moderate to high linkage disequilibrium, thus leading to conflicting (Blackston et al., 2001; Lehmann et al., 2000) or poorly resolved (Ajzenberg et al., 2002a) genetic clusters.

A more diverse population structure was later confirmed as typing techniques with increased resolution were applied to isolates more representative of *T. gondii*'s geographic distribution. Microsatellite typing of *T. gondii* isolates from humans and domestic animals in Brazil and French Guiana showed that many isolates comprise a genetically distinct population from that in the US and France (Ajzenberg et al., 2004; Lehmann et al., 2004). The increased levels of genetic diversity (Ajzenberg et al., 2004) and lower levels of linkage disequilibrium (Lehmann et al., 2004) detected in South American strains suggested that sexual recombination occurs more frequently in this population than in the US and France and, again, likely explains the poor phylogenetic resolution among these strains (Ajzenberg et al., 2004).

An apparently distinct and diverse gene pool in South America prompted additional studies from this area and others around the globe to gain a more comprehensive picture of genetic diversity in the *T. gondii* population. Su et al. (2006)

developed a standardized RFLP typing scheme based on nine mostly unlinked nuclear genomic loci and one apicoplast marker to reveal a wide diversity of strains isolated from 28 domestic cats in Brazil. Importantly, this study confirmed that clonality is not the rule and re-classified many strains that were typed previously only at a single locus, SAG2, via the method developed by Howe et al. (1997) under the assumption that a single locus was sufficient to type strains in a highly clonal population. Khan et al. (2006) also typed several human clinical and domestic animal samples from Brazil using a combination of RFLP typing and DNA sequencing at a single locus to reveal additional diverse genotypes. This study employed phylogenetic analyses of the typing data and identified poor bootstrap support for several branches and conflicting groupings of strains based on RFLP and DNA sequencing data. This study did not support a truly clonal population structure, and one plausible interpretation was that the clouding of genetic histories was attributable to recombination (Khan et al., 2006). RFLP analysis of additional human and domestic animal samples by Ferreira Ade et al. (2006) also revealed an admixture of archetypal alleles across loci, potentially indicating recombinant strains.

The increasing wealth of genetic data from diverse geographic locales facilitated several comprehensive re-assessments of the global *T. gondii* population structure. Lehmann and colleagues (2006) analyzed 275 strains isolated from chickens, representative of North America, Central America, South America, Europe, Africa and Asia, using a combination of six microsatellite markers and one gene coding marker. Their results showed a diverse array of genotypes that could be assigned to four distinct sub-populations: two exclusive to South America, one found on all continents sampled except South America and one found world-wide (Lehmann et al., 2006). Similar results

were revealed in a smaller-scale study that used eight intron loci to describe the population structure of 46 isolates from North America, Europe, and South America, many of which were previously typed in studies discussed above (Khan et al., 2007). This study also found evidence for structuring the *T. gondii* population into four sub-populations with the same geographic distribution as those found by Lehmann et al. (2006). Importantly, the methodology employed here permitted assignment of different parts of an individual isolate's genotype to different populations, thus accounting for the likely event of admixture between populations in a parasite with a viable sexual cycle (Khan et al., 2007). This revealed the intriguing result that nearly all genotypes showed some degree of admixture of two or more of the four ancestral populations (Khan et al., 2007).

This study also further sub-grouped isolates into 11 haplogroups by clustering representative subsets of isolates using neighbor-joining phylogenetic analysis of concatenated sequences and by comparing the relative admixture profiles of individual genotypes. However, evidence for recombination between these groupings (Grigg and Sundar, 2009; Khan et al., 2007) indicates that concatenation was inappropriately applied as the robustness of these haplogroups breaks down if loci are examined individually. There were also large discrepancies between the similarities in admixture profiles within an individual haplogroup. For example, genotypes in haplogroups 1, 2, and 3 had essentially identical admixture profiles to other members of their respective groups, but genotypes in haplogroups 4 and 5 had admixture profiles that varied widely in the extent to which each ancestral population contributed (Khan et al., 2007). It is unclear if these groupings represent static entities that can consistently be applied in future studies, or,

rather, points on a continuum that will blend together as more isolates are described. The diverse admixture distributions present among isolates, though, do reveal how the genomic position of markers chosen for genotyping studies can profoundly influence how a certain genotype is perceived to be related to ancestral parent types and extant strains.

Specific geographic locales outside the US and Europe have also been the focus of recent studies. Pena et al. (2008) described 125 isolates from chickens, cats, and dogs from Brazil using 10 PCR-RFLP markers to reveal 48 genotypes, many of which were unique to this region. Importantly, this study showed that the three dominant clones identified by Howe and Sibley (1995) were present at very low frequencies or even absent (e.g. 'Type II') from this region, and genetic analyses revealed a highly reticulated network indicative of uncertain genetic histories consistent with recombination (Pena et al., 2008). Additional unique Brazilian genotypes have been identified in similar studies of sheep and goats from this region (da Silva et al., 2011; Ragozo et al., 2010). Also, in Africa, Mercier et al. typed 69 strains isolated from domestic animals (mostly chickens) from Gabon with 13 microsatellite loci to reveal 27 genotypes that could be structured into two populations (Mercier et al., 2010). These results are consistent with those reported for African isolates by Lehmann et al. (2006), but no previously described isolates were included to identify whether the populations described here were the same or novel.

2.1.2 Toxoplasma *genotypes in wildlife*

Most of the studies discussed above only sampled or analyzed strains from a small fraction (i.e. humans and domestic animals) of the host range utilized by *T. gondii*.

It is telling, though, that a large majority of unique strains have been identified by studies of *T. gondii* in wildlife, advancing our understanding of the extent of genetic diversity in this species. Intriguingly, the few wildlife samples that were included in the Howe and Sibley 1995 study (2 white tailed deer, 2 bear, 1 rodent, 1 turkey, and 1 dove) were genetically distinct from the three major clones, with some of them potentially representing recombinants of the three clonotypes (Howe and Sibley, 1995). This suggested that a productive sexual stage for this parasite exists in nature and that wider sampling, more representative of *T. gondii*'s broad host range, would reveal much greater genetic diversity. In several genotyping reports of *T. gondii* in wildlife this has proved to be true.

In North America, multiple genetically diverse genotypes have been identified circulating in wild animals. From a cougar in Canada, Lehmann et al. (2000) identified a genetically highly divergent strain (from archetypal Types I, II, and III identified in Howe and Sibley (1995)) using DNA sequencing, and this result was later confirmed by RFLP typing with 10 markers (Dubey et al., 2008b). This same RFLP typing scheme, originally reported in Su et al. (2006), has been applied in other studies of terrestrial wildlife in the US and Canada to provide similar results. Eighty-one of the RFLP genotypes identified using this scheme have been classified according to a numbering system (i.e. RFLP genotype #1, #2, #3, etc.) on the public *T. gondii* genome database, Toxodb.org (Gajria et al., 2008). We will refer to RFLP genotypes using this classification, except for strains of the Type I, Type II, Type III, and Type X (see below) lineages (Toxodb.org designated RFLP genotypes 10, 1, 2, and 5, respectively) which we will refer to with their traditional 'Type' nomenclature. Strains that have an RFLP type

that has not been described on Toxodb.org will be referred to with their original isolate designation.

Of 31 total samples pooled from several studies, 16 were identified as non-archetypal, including 4/15 white-tailed deer, 2/2 bears, 1/2 skunks, 5/6 raccoons, and 4/6 coyotes (Dubey et al., 2008b; Dubey et al., 2010b; Dubey et al., 2007c; Dubey et al., 2008e). Some of these distinct strains likely represent recombinants of the three main clonal types based on their allelic combinations, but this cannot be confirmed without further sequencing analysis, since this typing scheme was specifically designed to detect polymorphisms inherent to the archetypal strains (Su et al., 2006). It is increasingly evident that apparent recombinants of the three archetypal lineages detected by RFLP analysis are often later re-classified by DNA sequencing as genetically distinct admixtures bearing many non-archetypal alleles (Boyle et al., 2006; Frazao-Teixeira et al., 2011; Grigg et al., 2001a; Khan et al., 2006; Su et al., 2010).

Toxoplasma gondii has also recently been identified as an important disease agent in marine mammals in North America (see Table 1 for details). Genetic studies of marine mammal isolates have contributed greatly to our understanding of how this terrestrial pathogen has infiltrated the marine environment. Miller et al. (2004) applied a combination of RFLP analyses and DNA sequencing to show that a genetically distinct strain termed 'Type X' was a major cause of disease in Southern sea otters stranding along the Morro Bay area of California. These results were corroborated by application of the RFLP typing to an additional 39 sea otter isolates to independently show that 'Type X' was the major strain causing disease in sea otters of Morro Bay (Sundar et al., 2008b). This result was contrasted with *T. gondii* strains isolated from sea otters near

Monterey Bay, CA that were mainly infected with 'Type II' strains or Toxodb.org designated RFLP genotype 3, which differs from 'Type II' at a single locus, Apico (Sundar et al., 2008b). This same 'Type X' genotype was later shown to be present in wild felid hosts in the adjacent terrestrial environment of California, as well as in a filter-feeding invertebrate collected near the shoreline (Miller et al., 2008b). This study provided evidence for a mechanism through which this terrestrial parasite could infiltrate the marine environment via land-to-sea run-off and bio-concentration of oocysts in prey species of sea otters and other marine mammals (Miller et al., 2008b). *Toxoplasma gondii* isolates from dolphins in the US (Dubey et al., 2008a) and Canada (Dubey et al., 2009a) have also been extensively PCR-RFLP genotyped, revealing two 'Type II' strains, one strain with RFLP genotype 3 (as designated on Toxodb.org), and one atypical genotype in four isolates analyzed. Overall, reports from wildlife in North America suggest diverse strains are circulating in this region despite apparent limited strain diversity in domestic animal and human samples (Howe and Sibley, 1995).

Wildlife *T. gondii* isolates from other parts of the world have been genetically characterized as well. The standardized RFLP scheme has been applied to isolates from synanthropic rodents, including a seronegative mouse and rat (Araujo et al., 2010), captive animals, including a red-handed howler monkey and a jaguarundi, and a free-ranging black eared opossum (Pena et al., 2010) from Brazil to reveal they were all infected with non-archtypal strains, two of which had not been identified previously. Also in Brazil, an extensive RFLP analysis was completed on isolates from 36 capybaras that identified 16 total genotypes, seven of which were unique to this sample set (Yai et al., 2009). Importantly, only five of the isolates could be classified as a classic archetypal

strain ('Type III'). However, without DNA sequencing, definitive classification as an archetypal strain is not possible in regions where genetic diversity is substantial. It is quite possible that these strains will harbor non-archetypal alleles and ultimately be resolved as genetically distinct admixtures. Three of the 36 samples were found to be infected with a mixture of *T. gondii* strains (Yai et al., 2009), which could promote the *T. gondii* sexual cycle in the definitive felid host. Genetic data has also been reported from the United Arab Emirates and Qatar showing two atypical and one classic 'Type II' genotype present in isolates from four sand cats (Dubey et al., 2010a). The unusually high prevalence of symptomatic toxoplasmosis in this felid species raises interesting questions as to the universality of felids as functional definitive hosts, though several factors could be contributing to disease in these cases (Dubey et al., 2010a).

This trend towards apparently increased genetic diversity from wildlife samples is not absolute, however, as recent studies of wildlife in France and Norway have reported high prevalence of archetypal strains, specifically the 'Type II' strain. From 45 French isolates, including 21 from wild boar (Richomme et al., 2009), 12 from roe deer, nine from fox, and one each from mouflon, red deer and mallard (Aubert et al., 2010), RFLP typed at three loci and microsatellite typed at six loci, only 'Type II' strains were identified. Similarly in the Svalbard archipelago of Norway, the majority of 55 total isolates from arctic foxes were either 'Type II,' 'Type III' or RFLP genotype 3 (Prestrud et al., 2008). It is possible that the low levels of diversity in this instance could be attributed to a founder effect, given the isolated nature of this location (Prestrud et al., 2008).

Genetic studies of *T. gondii* in Australia have been particularly limited, though interest in *T. gondii* in this area has risen due to recent reports of fatal disease in threatened marsupial fauna (Basso et al., 2007; Bermudez et al., 2009; Dubey and Crutchley, 2008; Hartley, 2006; Obendorf et al., 1996). Efforts have accordingly been made to identify parasite genotypes circulating in this area. Parameswaran et al. (2010) applied PCR and DNA sequencing to identify eight different non-archetypal and two recombinant strains from a wombat, a wallaby, two woylies, a mouse, a meerkat (from the Perth Zoo), and eight kangaroo samples. Two of the kangaroo samples and one woylie possessed only archetypal alleles at the few loci amplified indicating these animals were likely infected by 'Type I' or 'Type II' strains, and one kangaroo harbored a mixed-strain infection (Parameswaran et al., 2010). Non-archetypal or recombinant strains were also obtained from domestic animal samples (1 horse and 1 goat), along with classic 'Type I' strains (1 cat and 1 goat) (Parameswaran et al., 2010). That the recombinant strains were truly recombinants was supported by the high resolution of DNA sequencing in this case.

Overall, aside from studies from certain locales in Europe there appears to be a high level of diversity present in wildlife isolates, including those from areas previously reported to be genetically homogenous, such as North America. Whether this is suggestive of a distinct sylvatic cycle for *T. gondii* is an important topic that will be discussed further below.

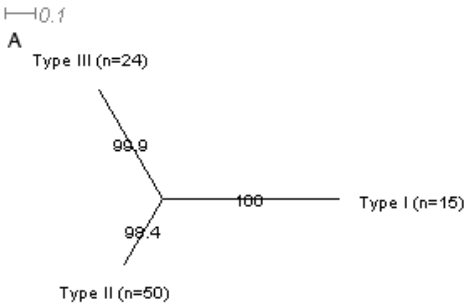
2.1.3 Population structure: reassessment

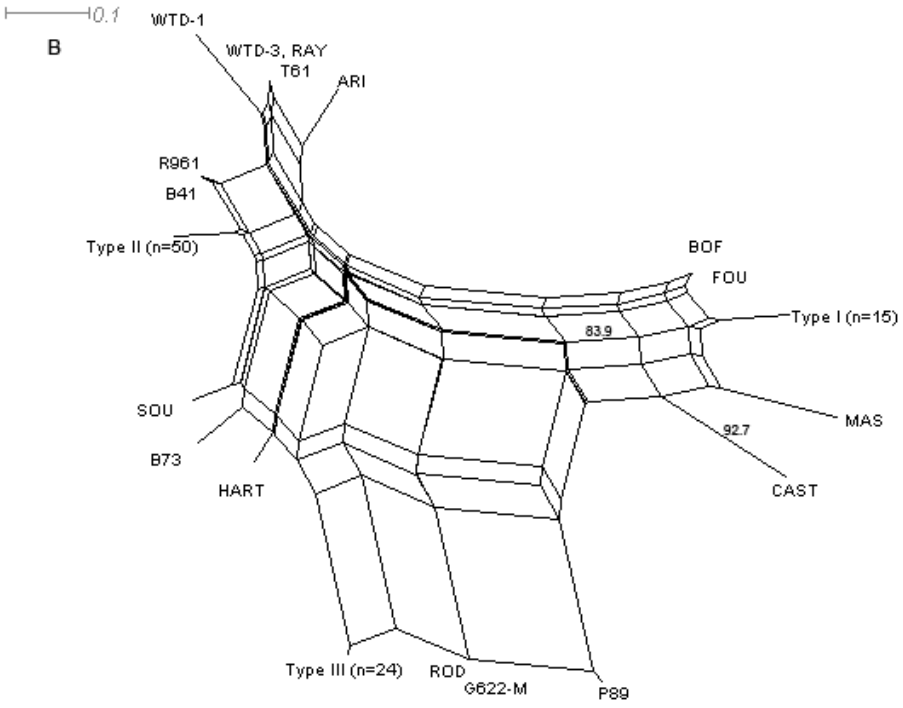
Considering the extent of genetic diversity and evidence for recombination present in wildlife samples, the picture that emerges for the population structure of *T. gondii* is more complex than a limited number of sexual events that have potentiated the emergence of just a few dominant clones (Khan et al., 2007; Sibley and Ajioka, 2008; Sibley et al., 2009; Su et al., 2003). Certainly the dominance of a limited number of genotypes in certain sample sets and from widely geographically dispersed locales is truly remarkable, but it does not necessarily lead to classification as a clonal population (Ajzenberg et al., 2002a; Howe and Sibley, 1995; Tibayrenc and Ayala, 1991). Based on the studies described above, the *T. gondii* population structure is in some cases highly clonal, in others it appears to be epidemic and overall, should be classified as intermediate, with clear influences from both clonal expansion of strains and sexual recombination (Lehmann et al., 2004; Lehmann et al., 2006). This would intuitively be the case for an organism with numerous mechanisms for clonal expansion in its lifecycle and a widespread host in which sexual recombination can occur. To what degree either clonal or sexual propagation can be detected in a sample set is dependent on several factors including: 1) the number and genomic location of genetic markers used, 2) the polymorphism present in the markers, 3) the resolution of the techniques used to detect polymorphism and 4) the host and geographic location of origin of the isolates.

To help illustrate these important points we re-analyzed the Howe and Sibley 1995 RFLP data using the Neighbor-Net method with SplitsTree4 software (Huson and Bryant, 2006). Though this method does not create a true phylogenetic history of genotypes, it has been applied in recent *T. gondii* genetic studies due to its advantages in

visually capturing the character conflicts in nucleotide data between genotypes that have undergone evolutionary events such as genetic exchange (recombination), gene conversion, or homoplasy and are therefore better represented by a network of nodes connected by multiple edges rather than as a bifurcating tree (Dubey et al., 2008c; Pena et al., 2008). First, we specifically input only those isolates that were members of the three clonal lineages, Types I, II, and III (essentially three data points) to illustrate the striking simplicity with which the vast majority (84%, 89/106) of the data can be described (Figure 1A). However, addition of the remaining samples (n=106 total), including those from wildlife, transforms the display into a complex network indicative of many potential pathways relating the additional strains to the clonal types and vice versa (Figure 1B).

As was reported previously (Howe and Sibley, 1995), implicating sexual recombination as the mechanism for convoluting relatedness among the genotypic data, opposed to events such as homoplasy, gene conversion, or other phenomena that can create conflicts in molecular data (Morrison, 2005), is supported by comparing allele combinations across loci. In total, eight of the 15 possible locus pair comparisons have at least one example of bi-allelic combinations compatible with a recombination (or at least reassortment) event in that all four expected combinations of alleles are present in the sample set (Figure 2). These data show evidence for recombination not only among the three main clonal types, but also between exotic strains (see SAG1 vs. 850 in Figure 2). As noted above, a potential caveat to interpreting strains as recombinants of the 3 main clonal lineages with RFLP typing is that certain of these isolates have been found to harbor unique alleles at the loci in question upon further sequencing analysis (Boyle et al., 2006; Frazao-Teixeira et al., 2011; Khan et al., 2006; Su et al., 2010). However, this


0.1
A
Type III (n=24)
99.9
100
Type I (n=15)
99.4
Type II (n=50)


0.1
B
WTD-1
WTD-3, RAY
T61
ARI
R961
B41
Type II (n=50)
BOF
FOU
Type I (n=15)
83.9
SOU
MAS
92.7
B73
HART
CAST
Type III (n=24)
ROD
G622-M
P89

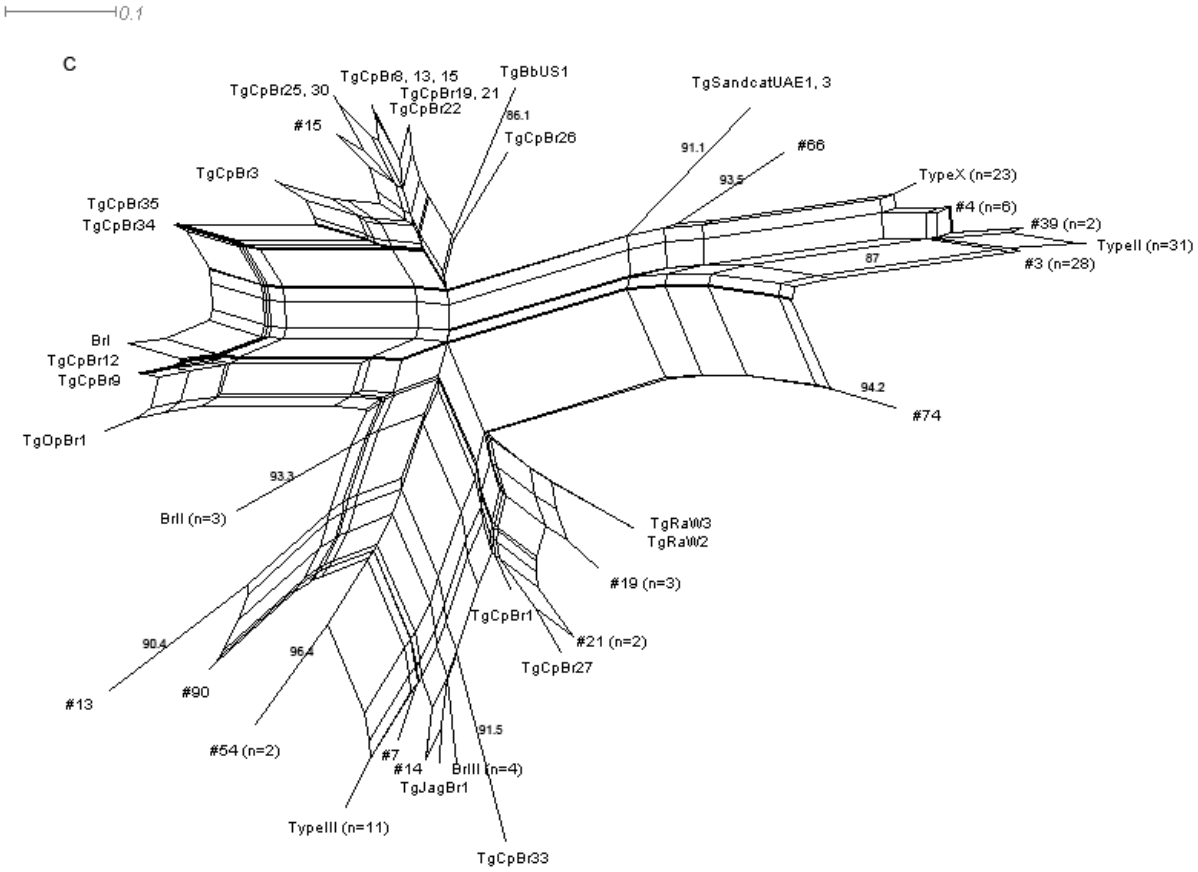

0.1
C
TgCpBr8, 13, 15
TgBbUS1
TgCpBr25, 30
TgCpBr19, 21
TgCpBr22
TgSandcatUAE1, 3
#15
86.1
TgCpBr26
91.1
#66
TgCpBr3
93.5
TypeX (n=23)
TgCpBr35
TgCpBr34
#4 (n=6)
#39 (n=2)
TypeII (n=31)
87
#3 (n=28)
BrI
TgCpBr12
TgCpBr9
94.2
#74
TgOpBr1
93.3
BrII (n=3)
TgRaW3
TgRaW2
#19 (n=3)
TgCpBr1
#21 (n=2)
90.4
96.4
TgCpBr27
#13
#90
91.5
#54 (n=2)
#7
#14
BrIII (n=4)
TgJagBr1
TypeIII (n=11)
TgCpBr33
0.1
D
Type X
#4 (n=17)
#3 (n=36)
TypeII (n=66)
87.2
TgPgUs_182
99.9
TgPgUs11
97.9
98.9
#7 (n=3)
82.9
#8 (n=8)
TypeIII (n=49)

Figure 1. SplitsTree4 analysis of *Toxoplasma gondii* RFLP data using the neighbor-net joining method. A. Input of only those strains which were classified by Howe and Sibley (1995) as one of the three main archetypal clones (Types I, II, and III) by 6 RFLP markers demonstrates the remarkable simplicity with which 84% (89/106 *T. gondii* isolates) of the isolates analyzed in this study can be described. **B.** Input of the complete data set from Howe and Sibley (1995), including the limited number *T. gondii* isolates from wildlife, reveals an underlying complex population structure, despite the simplicity indicated in (A). The multiple edges connecting different nodes indicate the many possible pathways that can describe relationships between genotypes, likely indicative of recombination. **C.** Analysis of 147 wildlife isolates typed 11 RFLP markers by various studies* reveals a slightly different and more complex population structure than that presented in B, highlighting the importance of including a sample set more inclusive of *T. gondii*'s expansive host range and utilizing typing schemes with higher resolution. Compared to B, which includes mostly samples from humans and domestic animals, Type II and III strains are still revealed as dominant clones, but Type I strains are totally absent. Additional dominant clones are revealed in wildlife samples as well, including Type X. High genotypic diversity related through a highly reticulated network again supports a viable and frequent sexual cycle for *T. gondii* in nature. **D.** Analysis of 182 isolates from pigs in the US RFLP typed with 11 markers by Velmurugan et al. (2009) reveals a much more complex picture of the *T. gondii* population structure for domestic animals in the US than that originally reported in Howe and Sibley (1995), reiterating the importance of more extensive genotyping. Similar to the wildlife samples, Types II and III are dominant clones and Type I strains are absent. Additional clonal types dominate as well, including RFLP genotypes 3, 4, and 8**. *Wildlife RFLP data was obtained from Arajuo et al. (2010), Dubey et al. (2010a, b, 2009a, 2008a, b, d, 2007c), Pena et al. (In Press), Prestrud et al. (2008), Sundar et al. (2008), Yai et al. (2009). **Numbers refer to RFLP genotype designations reported on Toxodb.org. Bootstrap values (1000 replicates) are only shown for those edges with greater than or equal to 80% support. Note: Samples with mixed strain infections or incomplete typing data were removed from the wildlife data set.

is not consistently the case (Parameswaran et al., 2010), and it is likely that at least a certain percentage of isolates are truly recombinants of either the archetypal clonal lines or archetypal and atypical strains. In fact, sequencing analysis of strain MAS, included in the 1995 study, confirmed that it harbored alleles that were likely the result of meiotic recombination (Grigg et al., 2001a). In contrast, strain P89, originally classified as a I/III recombinant (Howe and Sibley, 1995), was later found to likely represent one of the parent lineages that gave rise to the archetypal I, II, and III lineages (Boyle et al., 2006).

Intriguingly, when we analyzed more recent RFLP data pooled from the various studies of wildlife isolates discussed above, a somewhat different picture emerges (Figure 1C). Among the 147 isolates analyzed, 'Type II' and 'Type III' are still dominant clones, accounting for 42/147 (29%) of the samples. However, Type I strains are absent and

other clones are found to dominate, including 'Type X' (23/147, 16%) and RFLP genotype 3 (28/147, 19%), which together account for more samples than Types II and III. The overall genotypic diversity is also greater, with 36 distinct genotypes present in the 147 wildlife isolates (0.24 genotypes per isolate) compared to 14 in the Howe and Sibley 1995 data (0.13 genotypes per isolate), highlighting the effects a more encompassing sample set and more sensitive genotyping markers can have in changing perception of the genetic structure.

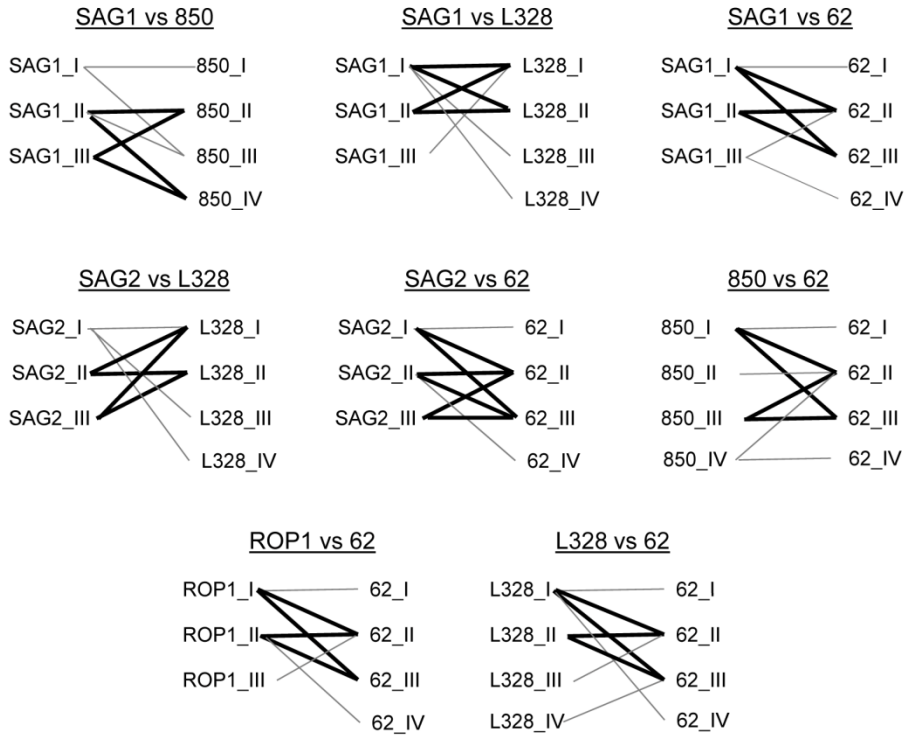


Figure 2. Bi-allelic analysis for recombination among *Toxoplasma gondii* isolates. Lines connect allelic combinations present in the RFLP genotypes reported by Howe and Sibley (1995) to describe 106 *T. gondii* isolates. Bold lines indicate the hour glass shape expected when all four combinations of alleles that would be expected after a recombination (or reassortment) event are present in the isolates. Bi-allelic combinations that did not show evidence for recombination include: SAG1 vs SAG2, SAG1 vs ROP1, SAG2 vs 850, SAG2 vs ROP1, 850 vs ROP1, 850 vs L328, and ROP1 vs L328 (not shown).

Until now, the consensus view of the global population structure of *T. gondii* based on recent studies in Europe, the Americas, Africa, and, to a limited extent, Asia (Khan et al., 2007; Lehmann et al., 2006; Mercier et al., 2010) was of a diverse array of genotypes derived from infrequent admixture among four ancestral populations and punctuated over time by recombination and clonal expansions (Sibley and Ajioka, 2008; Sibley et al., 2009). The degree to which recombination or clonal propagation has been interpreted as shaping *T. gondii*'s population structure has varied depending on the study and the samples analyzed, likely indicating that no study has truly analyzed a sample set fully representative of the *T. gondii* population. Indeed, many recent and unique strains described from both domestic animal and wildlife sources, such as those described in Brazil (da Silva et al., 2011; Frazao-Teixeira et al., 2011; Pena et al., 2008; Pena et al., 2010; Ragozo et al., 2010; Yai et al., 2009), the US (Dubey et al., 2010b), and Australia (Parameswaran et al., 2010) were not included in these previous global analyses (Khan et al., 2007; Lehmann et al., 2006). In fact, in Brazil alone at least 88 unique genotypes have been reported based on RFLP typing (da Silva et al., 2011) and only a small fraction of these have been incorporated in studies mapping the *T. gondii* population genetic structure. A combination of increased sample sizes that are more representative of *T. gondii*'s host range and of more extensive genetic data derived from each isolate will lead to further and potentially extensive revisions of the current consensus.

3. Sylvatic and domestic cycles: do they exist?

The high diversity in *T. gondii* genotypes isolated from wildlife samples as compared to those from domestic animals raises the question as to whether distinct gene pools exist for domestic and sylvatic hosts. The relevance of this question derives from

concern that when distinct parasite gene pools overlap, most likely due to human environmental encroachment, disease outbreaks may occur in either human/domestic animal or wildlife populations due to exposure of hosts to novel parasite genotypes (Bengis et al., 2004; Cleaveland et al., 2001; Daszak et al., 2000; Thompson et al., 2009). Unfortunately, no sampling efforts or genetic analyses of *T. gondii* have been systematically applied in a way that would allow for rigorous comparison of domestic and sylvatic gene pools. It is possible that wildlife samples are perceived to be more genetically diverse than domestic samples because: 1. more extensive and sensitive typing has been applied to the more recently acquired wildlife isolates and/or 2. a highly clinically biased sample set has been used repeatedly in multiple studies to represent strains in domestic animals and humans.

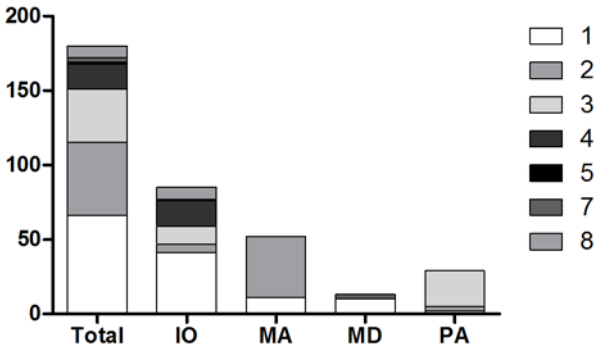
We have compiled some recently reported data to help illustrate factors that likely will need to be accounted for when addressing this complex but important question. First, it is evident that genotype prevalence can vary both between host species and over geographic distributions. It has been noted previously that parasite genotype distributions differ between humans and domestic animals (Howe and Sibley, 1995), which is an important factor in determining which host species should be used to represent the domestic cycle. Additional evidence for this emerges when we re-analyze recently reported RFLP data for 182 isolates from pigs in the US (Velmurugan et al., 2009) using the SplitsTree4 software as described above. Comparing the network generated for the pig isolates (Figure 1D) to that for the data reported in the Howe and Sibley 1995 study (Figure 1B), in which the domestic cycle was largely represented by human isolates, reveals some important differences. "Type II' and 'Type III' are also dominant clones in

the pig population, but, in contrast to the human population, 'Type I' strains are absent, confirming what was reported originally (Howe and Sibley, 1995). It is also notable that other clones absent from the human sample set, including RFLP genotypes 3, 4 and 8, are quite prevalent in these pigs, but this difference may be attributable to the higher resolution typing applied to the pig sample set. Overall, the distribution of dominant clones in the pig data is more similar to that in the wildlife sample set (Figure 1C), but differences exist here as well, including the greater number of unique genotypes and the increased prevalence of 'Type X' strains (largely due to sea otter samples) in the wildlife data.

Geographic effects can also play a role in shaping our perception of which parasite genotypes are associated with which host species. This is certainly the case on a global level (e.g. North vs. South America), but has been noted on more local levels as well. For example, in Gabon, Mercier et al. (2010) provided evidence for distinct *T. gondii* populations between chickens acquired in different towns, which ranged from 105 to 510 kilometers distant. As mentioned above, Sundar et al. (2008b) also found distinct strain compositions between the sea otters of Monterey Bay and Morro Bay, California, which are separated by approximately 200 kilometers. Similarly, when we re-examined the pig isolate data reported by Velmurugan et al. (2009) with pig genotypes classified by state of origin, different proportions of genotypes were found in different geographic sub-groupings (Figure 3).

In contrast to these data, however, Lehmann et al. (2004) found no difference measured by the F_{st} statistic between chicken isolates from two locations over 600 kilometers apart in Brazil, perhaps indicating more expansive transmission in this region.

Figure 3. RFLP genotype distribution among pig samples from the US. Colored blocks indicate the proportion of *T. gondii* isolates from pigs from various states in the US that are accounted for by RFLP genotype numbers 1-5, 7, and 8 (Numbers refer to those reported on Toxodb.org). Markedly different genotype distributions from an identical host species among the 4 states illustrates the importance of geographic effects on strain prevalence. Pig RFLP data is from Velmurugan et al. (2009). Note: isolates not typed at all loci were removed from the analysis. IO=Iowa, MA=Massachusetts, MD=Maryland, PA=Pennsylvania



Brazil is also one of the few locations where recent sampling efforts have identified a significant number of wildlife and domestic animal isolates with a comparable typing scheme in a localized area. In the state of Sao Paulo, a standardized RFLP typing scheme has been applied to isolates from 36 capybaras (Yai et al., 2009), 19 dogs (Dubey et al., 2007a), 44 cats (Pena et al., 2008), 16 sheep and 10 goats (Ragozo et al., 2010). Using the capybara samples as representative of wildlife, we compared the proportions of shared genotypes between wildlife and domestic carnivores (cats and dogs) (Figure 4A) and between wildlife and domestic herbivores (sheep and goats) (Figure 4B). In both cases there were several genotypes that were unique at the host species level, but comparing capybaras to domestic carnivores revealed that the genotypes for 22 out of the 33 capybara samples (mixed infections excluded) were also found in the domestic animal samples in contrast to only five isolates sharing genotypes with domestic herbivore samples (Figure 4A and B). Though it is unclear whether these results could be attributed to geographic differences within the state of Sao Paulo, they do give weight to the notion

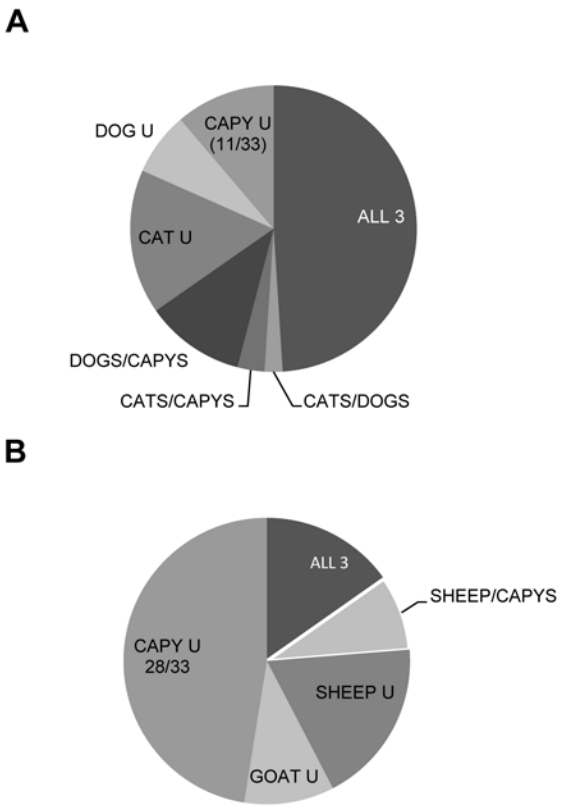


Figure 4. Comparison of *Toxoplasma gondii* genotypes from wildlife and domestic carnivores and herbivores in Sao Paulo state, Brazil. A. RFLP genotypes from *T. gondii* isolates from capybaras (representative of wildlife), cats and dogs (representative of domestic carnivores) were analyzed for their distribution among seven categories: 1. Genotypes found in all three species (ALL3); 2. Genotypes found in cats and dogs only (CATS/DOGS); 3. Genotypes found in cats and capybaras only (CATS/CAPYS); 4. Genotypes found in dogs and capybaras only (DOGS/CAPYS); and genotypes that were unique to 5. Cats (CAT U); 6. Dogs (DOG U); and 7. Capybaras (CAPY U). Results show that many of the isolates possessed genotypes that were unique to their respective species of origin within this sample set, though nearly half of the isolates possessed a genotype found in all three species. Only 11 of the 33 total isolates from capybaras possessed a genotype that was not also found in a domestic carnivore. **B.** The same analysis was conducted to compare the capybara sample set to domestic herbivores, represented by sheep and goats. In contrast to the domestic carnivore comparison, nearly all the capybara isolates (28/33) possessed a genotype that was not found in domestic herbivores. All isolates were obtained from animals from Sao Paulo state, Brazil. Capybara data was from Yai et al. (2009), cat data from Pena et al. (2008), and dog data from Dubey et al. (2007a).

that defining the *T. gondii* population circulating in domestic animals could be highly dependent upon which host species are chosen to represent the domestic cycle.

Overall, it is unclear to what extent distinct *T. gondii* gene pools exist between domestic and sylvatic cycles. It is likely that the answer to this question could vary with geographic location and host species chosen to define the respective cycles. Another potential caveat is variation in parasite genotype frequency over time. A systematic longitudinal sampling and genotyping effort across

several host species over an urban to rural gradient in diverse geographic locations would do much to help address these concerns and provide more definitive conclusions.

4. Conclusions and Future Directions

The most important conclusion that can be drawn from recent genotyping efforts of *T. gondii* isolates from wildlife is that the so-called 'exotic' or 'atypical' strains are not insignificant anomalies in the population structure of this parasite, but rather important members of the gene pool that provide a much better representation of the vast host range utilized by this parasite. There are clearly genotypes that dominate in the *T. gondii* population in addition to the originally identified Types I, II, and III, even in areas previously thought to contain only these original genotypes, such as North America. Future efforts to define the genetic structure of this parasite should incorporate these newly found complexities while realizing that this will likely lead to refinement of many pre-conceived notions of simplicity in *Toxoplasma* population genetics.

It will also be important to gather empirical and experimental evidence for the inferences drawn from these new genetic studies of *T. gondii*. This is especially true for the debates over the extent to which clonal expansion and sexual recombination occur in nature and over the mechanistic basis for how certain clones come to dominate in the population structure. As discussed above, there are many potential caveats in extrapolating from a clonal sample set to conclude that a population is clonal in nature (Feil and Spratt, 2001; Maynard Smith et al., 1993). Likewise, the extent of recombination can be over-estimated depending upon the sampling techniques used (Awadalla, 2003; Prugnolle and De Meeus, 2010). Given the vast and widespread

population of definitive felid hosts (approx. 90 million in the US alone (Dabritz and Conrad, 2010)), it is very likely that a frequent and productive sexual cycle exists for this parasite, despite a predominance of certain clones. Support for or against this possibility could be gained by attempting to determine the extent of co-infections in future sampling efforts, especially those which represent prey species of felids, because a simultaneous infection of two or more *T. gondii* strains is necessary for a productive cross to occur. In this regard, it is worth noting that the number of studies that have reported at least some mixed strain infections in a wide variety of hosts, including cats, has increased greatly since more sensitive genotyping techniques have been applied (Al-Kappany et al., 2010; Aspinall et al., 2003; Boughattas et al., 2010; Dubey et al., 2007b; Dubey et al., 2009b; Dubey et al., 2006a; Dubey et al., 2006b; Dubey et al., 2006c; Dubey et al., 2005; Elbez-Rubinstein et al., 2009; Lindstrom et al., 2008; Mercier et al., 2010; Parameswaran et al., 2010; Ragozo et al., 2010; Sundar et al., 2008b; Yai et al., 2009). This is in spite of the fact that the majority of studies still obtain isolates through bioassay, a technique that has been shown to preferentially isolate certain strains over others (Lindstrom et al., 2008), and only test tissues from a single organ, even though evidence exists that different strains localize to different organs (Dubey, 1997; Saeij et al., 2005). Techniques now exist that provide the sensitivity needed to allow extensive parasite genotyping directly from multiple host tissues to adequately address this question (Opsteegh et al., 2010).

Another possibility would be to directly test oocyst samples for multiple genotypes using these more sensitive typing techniques. It would also be of great benefit to experimentally verify whether a mixed strain infection in a single intermediate host is necessary for outcrossing in the felid host, or if consumption of multiple, singly-infected

prey over the course of the one to three week patent period of oocyst shedding would suffice. For studies of *T. gondii* infections in wildlife, it is also highly relevant to determine sources of infection and examine whether differences exist between domestic and wild felid species in their fecundity and ability to promote sexual outcrossing and/or self-mating for *T. gondii*. Clearly there is still much information to be gathered that could shape our perception of the likelihood and frequency of productive sexual crosses in nature.

Regardless of the ambiguities surrounding the extent of sexual recombination in the *T. gondii* population, it is undeniable that certain clones have expanded and persist in both domestic and wildlife populations over time. How and why this has occurred has been the focus of several studies over the past decade due to concerns that the mechanistic basis of clonal expansion may represent a threat for the emergence of virulent genotypes. Of the many mechanisms that exist in the *T. gondii* lifecycle for clonal propagation, it was originally proposed that the three archetypal strains, Types I, II, and III (Howe and Sibley, 1995), were unique among *T. gondii* strains in that they had recently acquired the ability for enhanced oral transmission of tissue cysts among intermediate hosts, thus allowing for clonal transmission that bypassed the sexual stage in the definitive host (Su et al., 2003). This hypothesis was based on limited laboratory studies and showed that tissue cysts from a small sample (3 isolates) of non-archetypal strains had greatly reduced oral transmission among laboratory mice compared to Types I, II, and III (Su et al., 2003). However, with additional sampling, it has since been shown that virtually all isolates of *T. gondii*, even rare genotypes, are capable of oral

transmission among intermediate hosts (Khan et al., 2007), suggesting this trait may not be solely responsible for the global dominance of certain genotypes.

A case has also been made suggesting that vertical transmission, either transplacentally or through ingestion of milk, may play an important role in maintaining clonal dominance of certain strains in nature (Johnson, 1997). This hypothesis was advanced based upon knowledge of the presumably important role transplacental transmission plays for infection with the related parasite, *Neospora caninum*, in domestic cattle and dogs (Johnson, 1997). Numerous studies have demonstrated that *T. gondii* is capable of vertical transmission in a variety of hosts, including humans, but few have examined the possibility that strict vertical transmission is maintained over several generations in natural host populations. High prevalence of *Toxoplasma* in certain hosts that are geographically isolated from definitive felid hosts, such as the arctic fox populations mentioned above (Prestrud et al., 2008), are intriguing scenarios to speculate whether vertical transmission is important, but evidence has yet to be gathered to support this hypothesis. Much remains to be done to delineate the relative roles of vertical versus oral, carnivorous transmission in maintaining clonality in nature.

Given the much greater infective potential inherent to the definitive host stage (a single infected cat can shed hundreds of millions of oocysts into the environment (Dubey, 2001)), we recently tested whether self-mating during this stage was a viable mechanism for the expansion of a single genotype in nature. We extensively genotyped oocyst samples recovered from a reservoir linked to a waterborne *T. gondii* outbreak in humans in Brazil and demonstrated that they were an identical genetic clone (Wendte et al., 2010a). Combining these results with serologic typing evidence from infected people

(Vaudaux et al., 2010) confirmed that the outbreak, which was attributed to ingestion of oocyst contaminated water, was indeed clonal and apparently the result of a selfing event in a felid definitive host (Wendte et al., 2010a). Since well over a hundred people were affected by this outbreak, this result demonstrated the major role selfing in the definitive host can play as a potential mechanism for clonal expansion of a disease-producing genotype in nature as compared to vertical transmission or oral transmission via carnivory (Wendte et al., 2010a). Importantly, self-mating was also the cause of a devastating clonal outbreak of the related parasite *Sarcocystis neurona* that caused a point-source mass mortality event in a threatened Southern sea otters due to oocyst/sporocyst contamination of waterways; a result that expands the explanatory scope of selfing as a mechanism of clonality to other tissue cyst coccidia (Wendte et al., 2010a).

The genetic basis for the expansion of certain clones has also been proposed in population genetic studies. The archetypal clones have been associated with certain alleles for the rhoptry kinase protein, ROP18, associated with virulence in the mouse model (Khan et al., 2009) and a monomorphic chromosome Ia (Khan et al., 2007), leading to the conclusion that these loci may contribute to the success of these clonotypes in nature. Fortunately, the identification of a genetically distinct, dominant clone circulating in wildlife, 'Type X' (see Figure 1C), should allow future comparative genomic analyses to further refine potential candidate genes accounting for the success of certain genotypes to carry forward in experimental studies.

Notably, while this manuscript was under review, a study was published further characterizing many 'Type X' and apparently 'Type X-like' strains at five intron and 3 antigen loci with a variety of phylogenetic and population analyses techniques (Khan et

al., 2011). This study grouped 'Type X' and many closely related strains into a new 'haplogroup 12' according to the previously published methodology of Khan et al. (2007). Similar to our discussion above of the 2007 study, close examination of the data reveals that consistent delineations between certain haplogroups break down to varying degrees depending on the analysis technique used (Khan et al., 2011). In fact, inconsistent delineations between 'haplogroup 12' ('Type X') and 'haplogroup 2' ('Type II') due to a bi-allelic inheritance pattern at many of the loci analyzed led to the conclusion that 'haplogroup 12' ('Type X') strains are the result of a cross between 'Type II' and a distinct ancestral type (Khan et al., 2011). This intriguing conclusion again speaks to the importance of the sexual cycle in the *T. gondii* population structure as a mechanism for the emergence of new strains that can go on to dominate clonally. It is also telling that what were once considered minor (and sometimes insignificant) genetic differences by RFLP and other typing schemes among 'Type X' genotypes compared against 'Type II' genotypes were indicative of major genomic level diversity. As the field moves forward with much more extensive, whole-genome level analyses, it is likely that several strains once thought to be identical clones will be found to be comprised of multiple, diverse genetic backgrounds.

In many ways, genotyping studies of *T. gondii* in wildlife have caused researchers to re-consider established viewpoints of the population genetic structure and relative roles of the various lifecycle stages in shaping the population biology of this important zoonotic pathogen. Yet much work remains to be done to uncover the extent and implications of the parasite genetic diversity circulating in wild animal populations and the degree to which sylvatic and domestic cycles are synonymous or distinct. Future

studies addressing these issues will be highly relevant to efforts aimed at minimizing disease in both wild and domestic populations.

5. Acknowledgements

Thanks to all members of the Grigg lab for helpful discussions.

CHAPTER III

LIMITED GENETIC DIVERSITY AMONG *SARCOCYSTIS NEURONA* STRAINS INFECTING SOUTHERN SEA OTTERS PRECLUDES DISTINCTION BETWEEN MARINE AND TERRESTRIAL ISOLATES

Preface

This chapter was originally published as *Limited genetic diversity among* Sarcocystis neurona *strains infecting Southern sea otters precludes distinction between marine and terrestrial isolates* in the journal Veterinary Parasitology 169 (2010) p. 37-44 by Jered M. Wendte, Melissa A. Miller, Amandeep K. Nandra, Scott M. Peat, Paul R. Crosbie, Patricia A. Conrad, and Michael E. Grigg. It is reprinted here with permission from Elsevier, license # 2413111083345. JMW was the majority contributing author for designing and conducting experiments, compiling and analyzing data, and writing the manuscript.

Sarcocystis neurona is an apicomplexan parasite identified as a cause of fatal neurological disease in the threatened southern sea otter (*Enhydra lutris nereis*). In an effort to characterize virulent *S. neurona* strains circulating in the marine ecosystem, this study developed a range of markers relevant for molecular genotyping. Highly conserved sequences within the 18S ribosomal gene array, the plastid-encoded RNA polymerase (RPOb) and the cytochrome c oxidase subunit 1 mitochondrial gene (CO1) were assessed for their ability to distinguish isolates at the genus and species level. For within-species

comparisons, five surface antigens (SnSAG1-SnSAG5) and one high resolution microsatellite marker (Sn9) were developed as genotyping markers to evaluate intra-strain diversity. Molecular analysis at multiple loci revealed insufficient genetic diversity to distinguish terrestrial isolates from strains infecting marine mammals. Furthermore, SnSAG specific primers applied against DNA from the closely related species, *Sarcocystis falcatula,* lead to the discovery of highly similar orthologs to SnSAG2, 3, and 4, calling into question the specificity of diagnostic tests based on these antigens. The results of this study suggest a population genetic structure for *S. neurona* similar to that reported for the related parasite, *Toxoplasma gondii*, dominated by a limited number of successful genotypes.

1. Introduction

Protozoal encephalitis is a major cause of mortality in southern sea otters, a federally listed threatened species (Miller et al., 2010; Thomas et al., 2007). *Sarcocystis neurona,* the agent responsible for deadly neurologic disease in horses (Dubey et al., 2001a), has been shown to cause fatal disease in sea otters and other marine mammals such as Pacific harbor seals (Dubey et al., 2003c; Kreuder et al., 2003; Lapointe et al., 1998; Lindsay et al., 2000; Lindsay et al., 2001; Miller et al., 2010; Miller et al., 2001a; Miller et al., 2004; Miller et al., 2001b; Mylniczenko et al., 2008; Peat, 2005; Thomas et al., 2007). Despite the importance of *S. neurona,* current research efforts addressing neurologic disease in sea otters have focused primarily on the zoonotic pathogen, *Toxoplasma gondii*. *Toxoplasma gondii* is a closely related tissue cyst-forming coccidian that shares many life history traits in common with *S. neurona.* It is likely that mechanisms resulting in the establishment of these terrestrial pathogens in the marine environment are very similar and may have selected for unique, marine-adapted strains. Studies on *T. gondii* for instance, discovered a novel genotype X associated with fatal disease in sea otters

(Miller et al., 2004). Evidence for the presence of Type X strains was also found in intermediate and definitive hosts inhabiting the adjacent terrestrial environment as well as a marine dwelling, filter-feeding invertebrate, which is a major staple in the sea otter diet (Miller et al., 2008b). These findings, combined with laboratory studies confirming the ability of filter-feeding invertebrates to concentrate and harbor infectious *T. gondii* oocysts (Arkush et al., 2003; Lindsay et al., 2004), suggest a mechanism of land-to-sea flow involving oocysts shed by definitive felid hosts being washed to sea via freshwater runoff, with subsequent bio-concentration in tissues of filter-feeding invertebrates consumed by sea otters. Whether the same is occurring for *S. neurona* has not been examined because high resolution, DNA sequence-level molecular tools to discriminate among *S. neurona* isolates are lacking.

In an effort to better characterize strains of *S. neurona* circulating in the marine environment, and to establish meaningful genotyping methods, this study developed genetic markers with potential for identifying and distinguishing parasites at the genus, species and sub-species level. We then applied these markers to determine and quantify the genetic variability of *S. neurona* isolated from southern sea otters in comparison with strains isolated from other marine and terrestrial mammal hosts.

2. Materials and methods

2.1 Parasite DNA

Sarcocystis neurona DNA samples were obtained from infected tissues collected at necropsy or from isolates obtained from infected tissues using cell culture, resulting in a total of 25 marine and 5 terrestrial mammal samples. The marine mammals included 22 sea otters (*Enhydra lutris nereis*) (Miller et al., 2010; Peat, 2005) and three harbor seals (*Phocina vitulina*) (Miller et al., 2001b), all of which died with systemic protozoal infections and were found stranded along the Pacific coast of California, USA (see Table 2 for details). Terrestrial isolates

Table 2

Sarcocystis neurona isolate genotypes

			Genetic Marker					
			SnSAG1-5-6	SnSAG3		SnSAG4	SN9	
				239	1056	592	(GT)n	
Sample	Origin	ATOS		C	C	C		Geno-type
SO3483	CA	820	5	·	·	·	17	**I**
SO3485	CA	827	5	·	·	·	17	
SO3501	CA	915	5	·	·	·	17	
SO3892	CA	917	5	·	·	·	17	
SO4135	CA	824	5	·	·	·	17	
SO4151	CA	825	5	·	·	·	17	
SO4160	CA	814	5	·	·	·	17	
SO4166	CA	827	5	·	·	·	17	
SO4167	CA	827	5	·	·	·	17	
SO4168	CA	832	5	·	·	·	17	
SO4169	CA	819	5	·	·	·	17	
SO4171	CA	819	5	·	·	·	17	
SO4285	CA	806	5	·	·	·	17	
HS2224	CA	na	1	G	T	·	-	**II**
SO3106	CA	318	1	G	T	·	18	
HS1531	CA	na	1	G	T	·	18	
SO3528	CA	292	1	G	T	·	18	
SO3629	CA	276	1	G	T	·	18	
SO3866	CA	309	1	G	T	·	18	
SO4181	CA	303	1	G	T	·	18	
H1 (SN1)	CA	na	1	G	T	·	18	
H2 (SN3)	CA	na	1	G	T	·	18	
H3 (EPM3)	CA	na	1	G	T	·	18	
HS1423	CA	na	5	·	·	G	14	**III**
R1	WI	na	5	·	·	G	14	
R2	WI	na	5	·	·	G	14	
SO3523	CA	141	6	·	·	G	14	**IV**
SO3639	CA	299	6	·	·	G	14	
SO4178	CA	1135	5	·	·	·	18	**V**
SO4194	CA	21	1	·	·	·	14	**VI**

Table 2. Samples were assigned multi-locus genotypes based on the presence or absence of SnSAG1, 5 or 6 and the differential segregation of alleles for SnSAG3, SnSAG4, and Sn9. Alleles for Sn9 were assigned based on the number of GT repeats resolved by DNA sequencing. Six total genotypes were resolved. Sea otter stranding locations and the resulting isolates are identified by an ATOS ('as the otter swims') number which describes the carcass recovery site to the nearest 0.5 km location along the California coast. The numbering system begins north of San Francisco and increases numerically from north to south. Genotype II was found exclusively in or near Monterey Bay and Genotype I dominated to the South in Estero Bay, California. Genotype IV was recovered over a more dispersed area in the Northern half of the sea otter range in central California. SO: sea otter; HS: harbor seal; H: horse; R: raccoon; na: not available.

included three from California horses (*Equus caballus*) (Marsh et al., 1996; Marsh et al., 1999; Peat, 2005) diagnosed with equine protozoal myeloencephalitis (EPM) and two strains from presumably asymptomatic raccoons (*Procyon lotor*) isolated in Wisconsin, USA (Sundar et al., 2008a). To test for specificity, primer sets were also tested on DNA from the related parasites *Sarcocystis falcatula*, *Sarcocystis cruzi*, *Sarcocystis campestris*, *Toxoplasma gondii*, and *Neospora caninum*.

2.2 PCR amplification and sequencing

Intra-specific variability in SnSAGs has been reported previously (Crowdus et al., 2008; Howe et al., 2008), so we decided to exploit this property and apply these markers against the *S. neurona* infected samples to resolve strain genotype differences. To maximize the likelihood of detecting strain-specific polymorphisms at these loci, primer sets were designed to amplify the majority of the open reading frame from all known SnSAG loci (SnSAG1-5). SnSAG1 and SnSAG5 are encoded by two different, paralogous genes. Strains of *S. neurona* possess one or the other gene (Crowdus et al., 2008). Primers (designated SnSAG1-5-6) were developed within conserved sequences of the two genes in order to amplify both of these mutually exclusive surface antigens. To differentiate isolates that encoded SnSAG1 from those that encoded SnSAG5, SnSAG1-specific primers were developed.

Primers were designed using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000) based on the following GenBank sequences: SnSAG1 (AY032845), SnSAG2 (AY191006), SnSAG3

(AY191007), SnSAG4 (AY191008), SnSAG1-5-6 (AY170620). Primer sequences are provided in Table 3. Previously published primer sets used include those for the cytochrome c oxidase subunit 1 (CO1) mitochondrial gene (Inagaki et al., 1997), internal transcribed spacer region 1 (ITS1) of the nuclear ribosomal gene array (Miller, RH, Grigg ME et al., unpublished), the apicoplast-encoded RNA polymerase (RPOb) (Dubey et al., 2003a), and microsatellite marker Sn9 (Asmundsson and Rosenthal, 2006). Highly conserved, multi-copy loci, such as CO1, ITS-1, and RPOb were utilized to identify and distinguish *S. neurona* strains infecting

Table. 3 Primers

Marker	Forward Primers	
	External	Internal
SnSAG1	GGAGGTAAGTGTTGGCGGTA	-
SnSAG2	AGCGGCGTTTTCAGATTGTA	-
SnSAG3	TCAAGGACGTTTTTCCCTGT	CCCTGCCTTTCTGGTCTCTT
SnSAG4	AATACCATACCTCGGCGTCA	-
SnSAG1-5-6	TGCTGCATCATTAGGGTCAG	-
RPOb	TAGTACATTAGAAATCCCTAAAC	†GCGGTCCAAAAAGGGTCAG TGGATATGATWTWTGAAGATGC
CO1	#TYTTGTTYTTYGGICAYCCIGARGTITA	-
Sn9	CTGCTGCTAGCGGACTCTCT	*CGCCAAAAGACTCACAAACA
	Reverse Primers	
	External	Internal
SnSAG1	TCCCGTTTTGGAACAGTAGG	-
SnSAG2	AAAACGAAGGCAAGTGTGCT	-
SnSAG3	CTCTGCATGCTGCAATGAAT	TTCTCCCCAAAGACCATCTG
SnSAG4	TCAAATGGCTGTCTCCACAA	-
SnSAG1-5-6	GCTGTGGGAGTAAGCAGGAT	-
RPOb	TCWGTATAAGGTCCTGTAGTTC	†GCGGTCCCAAAAGGGTC AGTCCTTTATKTCCATRTCT
CO1	#AARTGIGCIACIACRTARTAIGTRTCRTG	-
Sn9	*ACGCGCCTAAACGTGAATAG	-

Primer sets developed in this study using Primer 3 (Rozen and Skaletsky, 2000) are listed from 5' to 3'. See Materials and Methods for GenBank Acc. numbers of sequences used for primer design. (-): Primer set not developed. † (Dubey et al., 2003a) #(Inagaki et al., 1997) *(Asmundsson et al., 2006).

marine mammals from infections by related Apicomplexans at the genus and species level (Dubey et al., 2003a; Miller et al., 2010; Peat, 2005). Mitochondrial CO1 and apicoplast RPOb are extranuclear loci relevant phylogenetically as a marker to trace maternal inheritance and document sexual events in *S. neurona* life history. RPOb also has been used previously to distinguish *Sarcocystis* at the genus and species level (Dubey et al., 2003a). Sn9 is a polymorphic microsatellite marker that has been used successfully for intraspecific analysis of *S. neurona* (Asmundsson et al., 2006; Sundar et al., 2008a). To increase the sensitivity of previously published RPOb and Sn9 primer sets, a set of forward and reverse primers external to those published by Dubey et al. (2003a) for RPOb were developed, and Sn9 primers were made into a hemi-nested set by the addition of an external forward primer to those developed by Asmundsson and Rosenthal (2006) (see Table 3 for sequences).

PCR was conducted on 1.5µl of each DNA extraction sample with 5 µl of PCR Buffer (10X containing $MgCl_2$, Sigma), 5 µl of 2 mM dNTP mix (Fermentas), 50 pmol of each primer and 1.25-2.5U of Taq Polymerase (Sigma), with the total reaction volume reaching 50 µl. PCR amplification was conducted for 35 cycles and consisted of 94°C for 5 minutes, 94°C for 40 seconds, 58°C for 40 seconds, 72°C for 90 seconds and 72°C for 10 minutes. PCR amplification products were visualized on 0.8% agarose gels stained with GELRED dye (Biotium). Negative controls for the external and internal reactions consisted of molecular grade de-ionized water. All PCR products were purified using ExoSAP-IT (USB) according to the manufacturer's instructions and sequence confirmed with forward and/or reverse reads by Rocky Mountain Laboratory Genomics Unit DNA Sequencing Center, Division of Intramural Research, Hamilton, Montana.

2.3 Sequence analyses

Sequences were visualized using FinchTV software and analyzed using the Seqman application of the Lasergene software suite. Nucleotide and protein sequence alignments were created with ClustalW2 (Larkin et al., 2007). Closest *T. gondii* and *N. caninum* orthologs to the SnSAGs were assessed using BLASTp function available on ToxoDB (Gajria et al., 2008).

3. Results

3.1 Molecular tool development and isolate characterization

The specificity of each primer set was evaluated by testing against DNA from related parasites. At ITS-1 and RPOb, all coccidia tested amplified for both markers except RPOb in *S. cruzi* (Figure 5A) (RPOb data not shown). SnSAG1 and SnSAG1-5-6 primers were shown to be *S. neurona* specific. In contrast, SnSAG2, SnSAG3, SnSAG4, and Sn9 amplified both *S. neurona* and *S. falcatula* DNA (Figure 5A). DNA sequencing of the PCR products confirmed *S. neurona* was present in all specimens collected and enabled molecular characterization of *S. neurona* strains. Representative genomic DNA sequences for each *S. neurona* allele were deposited in GenBank (SnSAG1: GQ851951; SnSAG2: GQ851952; SnSAG3: GQ851954 and GQ851955; SnSAG4: GQ851957 and GQ851958; SnSAG5: GQ851960; RPOb: GQ851961; Sn9: GQ865624, GQ865625 and GQ865626). Results for each marker are discussed individually below.

3.2 Internal transcribed spacer region-1 (ITS-1) of the nuclear ribosomal gene array

The ITS-1 region was amplified with pan-tissue cyst coccidian-specific primers (Miller et al., In Prep). Size polymorphisms within the ITS-1 region distinguish *S. neurona* and *S. falcatula* from other tissue cyst-forming coccidia known to infect sea otters and other animals investigated

in this report (Figure 5A). DNA sequencing of the PCR products identified only *S. neurona*; no animals were found to be infected with *S. falcatula* (data not shown).

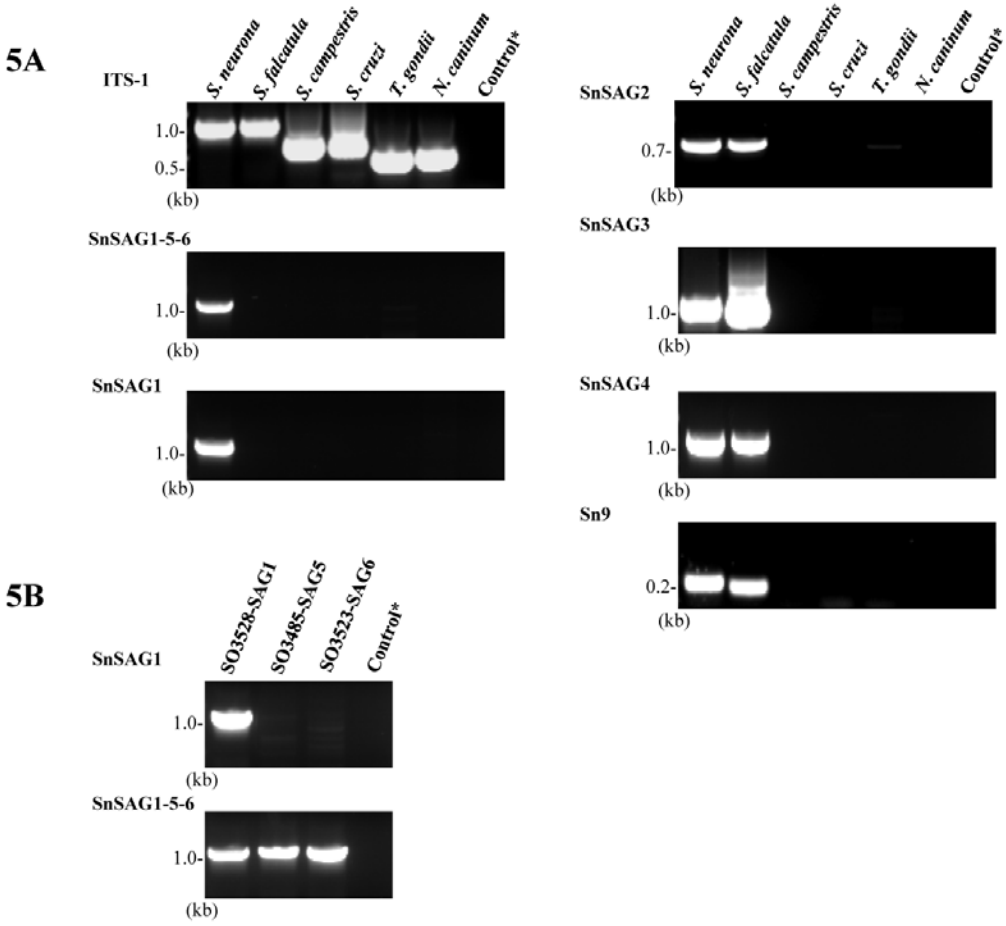


Figure 5. Specificity of genetic markers for *Sarcocystis neurona*. PCR amplicons for the primer sets ITS-1, SnSAG1-5-6, SnSAG1, SnSAG2, SnSAG3, SnSAG4, and Sn9 as visualized on 0.8% agarose gels stained with Gel-Red dye. **A.** ITS-1 allows differentiation of *S. neurona* and *S. falcatula* from other tissue cyst-forming coccidia due to size polymorphisms. All other genetic markers failed to amplify DNA from related Apicomplexan parasites including *Sarcocystis cruzi, Sarcocystis campestris, Neospora caninum* and *Toxoplasma gondii. Toxoplasma gondii* exhibited a weak band at SnSAG2 that was sequence negative and likely a spurious amplification. *Sarcocystis falcatula* amplified at SAG2, SAG3, SAG4, and Sn9. **B.** SnSAG1-5-6 primers could amplify either of the mutually exclusive surface antigens, SnSAG1 and SnSAG5, or putative surface antigen SnSAG6. Pictured are PCR amplicons from DNA of representative isolates positive for SnSAG1 (SO3528), SnSAG5 (SO3485), and SnSAG6 (SO3523). SnSAG1 primers amplified only strains positive for SnSAG1 by SnSAG1-5-6 primers. *Controls were molecular grade de-ionized water.

3.3 Mitochondrial cycloxygenase 1 (CO1)

Resolution of the CO1 sequence was limited to samples from which live *S. neurona* parasites were isolated in cell culture, primarily because contaminating host DNA from the infected tissues will also amplify using these primers. All *S. neurona* isolates that did amplify gave one unambiguous CO1 sequence that did not differ from previously reported sequences (Peat, 2005) (data not shown).

3.4 Apicoplast RNA polymerase beta-subunit (RPOb)

RPOb primers amplified ~700 bp product from all coccidia tested except *S. cruzi*. All *S. neurona* RPOb sequences in this study were identical and differed at four polymorphic sites from the only other *S. neurona* RPOb sequence published, which was a South American isolate (Dubey et al., 2003a). Interestingly, the sequences from this study possessed only a single nucleotide polymorphism from a sea otter isolate sequence originally described as an unidentified *Sarcocystis sp.* (Dubey et al., 2003a). The North American *S. falcatula* sequence identified in this study (GenBank Acc. Number GQ851962) also showed polymorphisms compared to previously published South American *S. falcatula* isolates (Dubey et al., 2003a). The *T. gondii* RPOb gene was amplified from SO4167 tissues and this sequence did not differ from those previously published (data not shown). This confirmed that SO4167 was dually infected with both *S. neurona* and *T. gondii*. When applied against *Sarcocystis campestris* DNA, the RPOb primers yielded a single sequence that was 95% homologous to the *S. neurona* sequences and represents the first sequence reported for this species (GenBank Acc. Number GQ851963).

3.5 SnSAG genes

3.5.1 SnSAG2, SnSAG3, SnSAG4

The *S. neurona* isolates in this study were PCR and sequence positive for SnSAG2, SnSAG3, and SnSAG4. All SnSAG2 sequences identified in this study were identical. SnSAG3 and SnSAG4 sequences identified two alleles for each of these loci. For SnSAG3, alleles differed at two nucleotide positions and one polymorphism was non-synonymous, resulting in a proline for alanine substitution at amino acid 87. SnSAG4 alleles differed by a single nucleotide polymorphism located within in the intron.

Highly conserved orthologs to SnSAG2, 3 and 4 genes were also identified with the same primer sets in the closely related species, *S. falcatula*. Nucleotide sequences for *S. falcatula* SAG2 and SAG4 shared 95% or greater identity with *S. neurona*. The SAG3 *S. falcatula* sequence possessed a 90 base pair indel in its intron and shared 95% identity with *S. neurona* in the coding region of the gene. GenBank accession numbers for SfSAG2, SfSAG3, and SfSAG4 are GQ851953, GQ851956, and GQ851959, respectively.

3.5.2 SnSAG1-5-6

SnSAG1 primers yielded amplification products from 11 of the 30 SnSAG1-5-6 positive samples. DNA sequencing with both primer sets yielded one allele and confirmed that the 11 SnSAG1 positive samples possessed the SnSAG1 gene. Sequencing of the remaining 20 SnSAG1-5-6 positive PCR amplicons identified two sequences. Eighteen samples possessed an identical sequence identified as SnSAG5. The SnSAG5 allele identified in this study was identical to that reported by Crowdus et al. (2008) but differed from the SnSAG5 gene reported for the isolate SnMU1 (Hyun et al., 2003) by six nucleotide polymorphisms and two indels.

The remaining two samples (SO3523 and SO3639) possessed a unique ~1kb nucleotide sequence that shared 71% and 83% identity with SnSAG1 and SnSAG5 respectively and had a predicted protein sequence of 281 amino acids (GenBank Acc. Number GQ851950). It is presumably a new, previously undescribed surface antigen that we refer to as SnSAG6.

Nucleotides 155-700 of this sequence were identical to a partial sequence previously reported from a sea otter *S. neurona* isolate (Crowdus et al., 2008). All samples that were negative at the SnSAG1 locus were positive for either SnSAG5 or SnSAG6, and no isolate was sequence positive at more than one of these loci (Figure 5B).

3.5.3 SnSAG orthologs

To examine whether SAG1-6 genes had sequence correspondence with their counterparts in related apicomplexan parasites, closest-orthologs to SnSAG antigens were identified in *N. caninum* and *T. gondii* using the BLASTp function available on ToxoDB (Gajria et al., 2008). The closest *T. gondii* ortholog to SnSAG1, 3, 4, 5 and 6 was TgSRS28, while the closest hit for SnSAG2 was TgSRS51. *Neospora caninum* orthologs consisted of hypothetical or putative SRS domain containing proteins. These analyses reveal the important point that the similarity of nomenclature of SAG1-6 genes in *S. neurona* is not related directly to sequence similarity to their counterparts in related Apicomplexan parasites. Results are summarized in Table 4.

Table 4

Closest *Toxoplasma gondii* and *Neospora caninum* orthlogs to SnSAG proteins

	SN protein					
	SnSAG1	SnSAG2	SnSAG3	SnSAG4	SnSAG5	SnSAG6
Acc. number*	GQ851951	GQ851952	GQ851954	GQ851957	GQ851960	GQ851950
Closest Tg ortholog	TgSRS28	TgSRS51	TgSRS28	TgSRS28	TgSRS28	TgSRS28
Acc. number**	TGGT1_011650	TGME49_108840	TGGT1_011650	TGGT1_011650	TGGT1_011650	TGGT1_011650
e-score†	1.2E-09	0.0017	1.1E-10	1.7E-11	0.00000043	0.00000015
Closest Nc ortholog	putative SRS protein	putative SRS protein	putative SRS protein	putative SRS protein	putative SRS protein	hypothetical protein
Acc. number**	NC_LIV_002120	NC_LIV_001820	NC_LIV_001380	NC_LIV_001710	NC_LIV_113970	NC_LIV_002120
e-score†	1.9E-09	0.012	0.000043	7.1E-09	0.000023	0.0000016

Orthologous proteins to the SnSAG antigens were identified using the BLASTp function available on Toxodb.org. *Accession numbers reference GenBank. **Accession numbers reference Toxodb. †e-score describes the expectation that the similarity between sequences noted would be due to chance alone.

3.6 Sn9 microsatellite

Sn9 showed the greatest number of *S. neurona* alleles (3) among the genotyping markers utilized in this study. Alleles were assigned by direct DNA sequencing and were based on visual inspection of the number of GT repeats detected in the sequence reads. The three alleles identified were of 14, 17, and 18 repeats at frequencies of 6/30, 13/30, and 10/30 isolates respectively (Table 2). The isolate from harbor seal, HS2224, was negative at this locus. The Sn9 allele amplified from *S. falcatula* (8 repeats) could also be resolved based on a species-specific polymorphism seven nucleotides downstream from the repeat region (GenBank Acc. number GQ865627).

3.7 Isolate diversity

Sequence analysis among the antigen-coding loci (SnSAG1, 3, 4, 5 and 6) allowed for the detection of five distinct multilocus sequence types among the *S. neurona* isolates. The genotypes were assigned based on the presence of SnSAG1, 5, or 6 genes and the inheritance pattern of alleles present at SnSAG3 and 4. With the addition of the single microsatellite marker, the number of genotypes distinguished increased to six (Table 2). The two most abundant genotypes isolated from the sea otters appeared to cluster geographically with genotype II found exclusively in northern locations whereas genotype I was more prevalent along the south-central California coast (Table 2). Also of interest was the finding that the isolates from raccoons (R1 and R2) from Wisconsin had an identical genotype to a harbor seal (HS1423) isolate from California (Table 2).

4. Discussion

The typing scheme developed in this study revealed minimal genetic diversity in marine *S. neurona* isolates at the loci examined. A lack of diversity at the conserved ITS-1, CO1 and RPOb loci among *S. neurona* isolates was expected as the utility of these markers is to confirm

infection by *S. neurona*. Size and sequence polymorphisms within the ITS-1 proved useful for discriminating *S. neurona* from other parasites in tissues collected and necropsy. A lack of allelic diversity and cross-reactivity with host DNA hampered the ability of CO1 to characterize strains and predict maternal inheritance patterns. However, this locus did facilitate confirmation of species identity for those strains where tissue culture derived merozoite DNA could be amplified as well as some infected tissue samples. Polymorphisms noted in the RPOb sequence between North American *S. neurona* and *S. falcatula* strains described in this study compared to previously published sequences from South American isolates (Dubey et al., 2003a), may be indicative of sustained geographic isolation. Intra- and inter-specific polymorphisms at this locus could prove useful for global studies of phylogenetics and the population genetic structure of *S. neurona* and other *Sarcocystis spp.*, though more South American samples are needed for an accurate assessment. The high similarity of the RPOb sequence from an unidentified *Sarcocystis sp*. (Dubey et al., 2003a) with RPOb sequences recovered from sea otters in this study may suggest that either the sea otter was infected with a closely related *Sarcocystis spp.* or was infected with *S. neurona* harboring a novel RPOb allele, or both. Further genetic characterization is needed to distinguish amongst these scenarios.

This study showed that known SnSAG antigens, SnSAG1-5, as well as SnSAG6, possessed a limited number of alleles among the *S. neurona* strains examined. Only one allele was identified for the SnSAG1, SnSAG2, SnSAG5 and SnSAG6 loci. This lack of allelic diversity within the SnSAGs was unexpected, especially considering that orthologous loci have been shown to be highly informative genotyping markers among clonal strains of the related parasite, *T. gondii* (numerous citations, initial studies include: Grigg et al., 2001b; Howe and Sibley, 1995; Parmley et al., 1994; Sibley and Boothroyd, 1992b). However, the allele at SnSAG5 did differ from a single, previously published isolate, Sn-MU1 (Hyun et al., 2003), by six nucleotide polymorphisms and two insertion/deletions, all of which resulted in a single,

conservative amino acid change. SnSAG3 and SnSAG4 also identified two alleles: two nucleotide polymorphisms resulted in one amino acid change for SnSAG3; and a single nucleotide polymorphism was detected in the SnSAG4 intron. These findings suggest that the diversity previously reported at the SnSAG4 locus, which was based on western blot analysis, was due to cross reactions of polyclonal antibodies to conserved regions of other proteins (Howe et al., 2008). This study also identified highly conserved (>90% identity for SnSAG2 and SnSAG4, >80% identity for SnSAG3) orthologs to SnSAG2, 3, and 4 in the closely related species *S. falcatula*, a finding that has implications for the specificity of diagnostic tests based on PCR and antibodies for these proteins (Ellison et al., 2003; Hoane et al., 2005).

The minimal diversity of SnSAG2-4 both within and between species might suggest that the function of these proteins is essential to parasite growth and survival and warrants further study. The mutual exclusiveness of SnSAG1, 5, and 6 presents an intriguing phenomenon that has yet to be explained. Whether they represent highly diverged alleles of the same antigen (Elsheikha and Mansfield, 2004) or are the result of a gene duplication event, similar to that described for SAG5B and SAG5C in *T. gondii* (Jung et al., 2004), cannot be determined until more sequence data are available. As research proceeds in discovering the function of these proteins, taking a comparative approach that accounts for loci conserved in related tissue cyst-forming coccidia will likely produce revealing insights into the biology of these important parasites.

The molecular characterization based on differential segregation of SnSAG alleles and a single microsatellite marker did not resolve *S. neurona* strains infecting marine mammals from terrestrial isolates. The overall genetic diversity among *S. neurona* strains examined was surprisingly minimal, and just a few multilocus genotypes were identified. This result could simply reflect the sample set used in this study, since it was composed predominantly of strains isolated from diseased animals, the majority of which were sea otters. It is also possible that

certain strains found infecting only sea otters may represent marine-adapted strains, but to resolve this question, additional *S. neurona* isolates from terrestrial animals will need to be studied. Focusing on the opossum definitive host will likely determine the true diversity present in the *S. neurona* population and should allow for specific questions of *S. neurona* biology to be addressed, such as the biological plausibility and potential source(s) of land-to-sea flow of this pathogen. This is especially relevant since the two genotypes identified in infected horses and raccoons in this study were also found in the marine environment.

The application of the robust, highly sensitive, new genetic markers capable of amplifying parasite DNA from infected tissue samples supported previous results (Sundar et al., 2008a) indicating that *S. neurona* genotypes clustered geographically along the California coast. Future studies should be focused on whether the apparent geographic dominance of certain strains in sea otters reflects the prevalence of these same strains in the adjacent terrestrial environment. The localized clonal dominance along the California coast, combined with the finding that the same genotype infected raccoons from Wisconsin and a harbor seal from California, raises intriguing questions about the transmission dynamics and life history traits of this organism. Determining a mechanism for how one strain can dominate on a local or global level will provide key insights to epidemiological phenomena of this parasite. Development and application of additional high resolution genetic typing markers should prove a valuable tool for future studies in this regard.

In addition, isolates from asymptomatic hosts will be needed to discern associations between the infecting genotype and disease outcome. The development and utilization of robust typing methods will likely be necessary to allow researchers to ask whether strain "type" is a predictor of severity of disease. A previous study found no association of microsatellite markers and neurologic disease (Asmundsson et al., 2006). However, this and other studies of microsatellite markers (Asmundsson et al., 2006; Sundar et al., 2008a) have relied on differences

in gel migration to determine size polymorphisms and type isolates; a method subject to individual bias and potential for inconsistency. Also, the study by Asmundsson et al. (2006) compared disease-causing strains isolated from intermediate/aberrant hosts (4 horses and 1 sea otter) to strains isolated from presumably asymptomatic opossums. It is highly likely that a strain that causes severe disease in an intermediate or aberrant host, such as a sea otter or horse, may cause no disease in the opossum since it is the definitive host. To truly comment on the virulence or disease causing potential of a strain infecting any susceptible host, comparisons should be made between strains isolated from symptomatic and asymptomatic infections of that same susceptible host. The method developed in the current study, that utilized visual counting of sequence reads to determine microsatellite size polymorphisms, presents a new, consistent way to apply these markers that may produce more definitive results. Likewise, a combination of the robust strain-level resolution provided by microsatellite markers and allelic variation at key virulence loci could prove an important tool in this regard, supporting further development of the typing scheme described in this study.

Previous studies have concluded that *S. neurona* possesses an intermediate population structure that exhibits both clonal propagation and recombination (Asmundsson et al., 2006; Elsheikha et al., 2006; Sundar et al., 2008a). Analysis of markers in the current study supports these conclusions. The differential segregation of SnSAG alleles indicates past recombination events, and the over-representation of certain strains provides evidence of clonal propagation. This parallels the current understanding of *T. gondii* population structure where sexual recombination provides the driving force for the emergence of successful strains that sweep clonally (Grigg and Sundar, 2009). This intriguing result begs the question whether a single, highly successful strain of this heteroxenous parasite can expand clonally in definitive or intermediate hosts, despite the genetic recombination that occurs during the sexual life cycle stage. Answers to this question are likely, again, to be similar to the closely related species, *T.*

gondii, where clonal propagation is explained by 1) its ability to be orally transmitted via carnivorism among intermediate hosts (Su et al., 2003) or 2) by the ability of a single clone to differentiate into both male and female gametes and self-mate in the intestine of the definitive host (Cornelissen and Overdulve, 1985; Pfefferkorn et al., 1977).

5. Conclusion

The multi-locus, DNA sequence level typing methods used in this study revealed spatial clustering superimposed on a background of minimal genetic diversity among *S. neurona* strains infecting sea otters and other marine and terrestrial mammals. The major conclusions of this study are that the conserved markers, ITS-1 and RPOb are useful for genus and species level resolution of tissue cyst coccidia, aiding in the identification of *S. neurona* infection. Examination of allelic segregation at six surface antigen genes (SnSAG1-SnSAG6) and microsatellite marker Sn9 can distinguish genotypes among *S. neurona* isolates, though not at a level of resolution sufficient to discriminate terrestrial isolates from *S. neurona* strains infecting sea otters and other marine mammals. As the microsatellite marker Sn9 identified the greatest number of alleles of the markers analyzed, it is likely that additional microsatellites will further develop this typing scheme to increase resolution. The limited genetic diversity detected in this study, composed of just a few recombinant genotypes, further supports the hypothesis of an intermediate genetic population structure for *S. neurona* in general, characterized by both clonal and sexual propagation. Overall, the evidence for clonality in this heterogamous parasite suggests the potential for self-mating or oral transmission between intermediate hosts, characteristics that were previously thought to be unique to *T. gondii*.

6. Acknowledgements

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CHAPTER IV

SELF-MATING IN THE DEFINITIVE HOST POTENTIATES CLONAL OUTBREAKS OF THE APICOMPLEXAN PARASITES *SARCOCYSTIS NEURONA* AND *TOXOPLASMA GONDII*

Preface

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Tissue-encysting coccidia, including *Toxoplasma gondii* and *Sarcocystis neurona*, are heterogamous parasites with sexual and asexual life stages in definitive and intermediate hosts, respectively. During its sexual life stage, *T. gondii* reproduces either by genetic out-crossing or via clonal amplification of a single strain through self-mating. Out-crossing has been experimentally verified as a potent mechanism capable of producing offspring possessing a range of adaptive and virulence potentials. In contrast, selfing and other life history traits, such as asexual expansion of tissue-cysts by oral transmission among intermediate hosts, have been proposed to explain the genetic basis for the clonal population structure of *T. gondii*. In this study, we investigated the contributing roles self-mating and sexual recombination play in nature to maintain clonal population structures and produce or expand parasite clones capable of causing disease epidemics for two tissue encysting parasites. We applied high-resolution genotyping

against strains isolated from a *T. gondii* waterborne outbreak that caused symptomatic disease in 155 immune-competent people in Brazil and a *S. neurona* outbreak that resulted in a mass mortality event in Southern sea otters. In both cases, a single, genetically distinct clone was found infecting outbreak-exposed individuals. Furthermore, the *T. gondii* outbreak clone was one of several apparently recombinant progeny recovered from the local environment. Since oocysts or sporocysts were the infectious form implicated in each outbreak, the expansion of the epidemic clone can be explained by self-mating. The results also show that out-crossing preceded selfing to produce the virulent *T. gondii* clone. For the tissue encysting coccidia, self-mating exists as a key adaptation potentiating the epidemic expansion and transmission of newly emerged parasite clones that can profoundly shape parasite population genetic structures or cause devastating disease outbreaks.

Author Summary

The parasites *Toxoplasma gondii* and *Sarcocystis neurona* have lifecycles that include a sexual stage in a definitive host and an asexual stage in intermediate hosts. For *T. gondii*, laboratory studies have demonstrated that the sexual stage can serve the dual purpose of producing new, virulent genotypes through recombination and promoting expansion of single clones via self-mating. Self-mating and other life history traits of *T. gondii*, including transmission of asexual stages among intermediate hosts, are assumed to account for the clonal population genetic structure of this organism. However, the relative contributions of sexual recombination and self-mating verses other life history traits in causing disease outbreaks or shaping *Toxoplasma's* population genetic structure have not been verified in nature, nor have these traits been extensively examined in related parasites. To address this knowledge gap, we conducted population genetic analyses on *T. gondii* and *S. neurona* strains isolated from naturally-occurring outbreaks affecting humans and sea otters, respectively. Our results identify self-mating as a key trait potentiating disease outbreaks through the rapid amplification of a

single clone into millions of infectious units. Selfing is likely a key adaptation for enhancing transmission of recently-emerged, recombinant clones and reshaping population genetic structures among the tissue-cyst coccidia.

1. Introduction

Population genetic studies of pathogenic microbes have been paramount to our understanding of disease resulting from emerging and re-emerging infectious organisms (Li et al., 2009). Studies performed to determine the relative contributions of drift and recombination in the production of genetic diversity have identified that most pathogens have methods to alter, exchange and acquire genetic material that are intimately associated with pathogenicity (Feil and Spratt, 2001; Li et al., 2009). For viral pathogens, enhanced levels of drift, genomic reassortment (Smith et al., 2009), and incorporation of host genes (Powers et al., 2008) have all been linked to emergence of virulence. Likewise, horizontal gene transfer between bacterial species has facilitated assimilation of pathogenicity islands, plasmids, prophages, and other insertional elements essential for disease and drug resistance phenotypes (Aires-de-Sousa et al., 2008; Amorim et al., 2007; Fraser et al., 2005a; Ogura et al., 2009; Reid et al., 2000). For eukaryotic pathogens, meiotic sex serves an analogous purpose functioning to alter the genetic make-up, and therefore the biologic and virulence potential of strains (Akopyants et al., 2009; Aly et al., 2009; Boyle et al., 2006; Byrnes et al.; Fraser et al., 2005b; Gaunt et al., 2003; Grigg et al., 2001a; Jenni et al., 1986). A general paradigm describing disease epidemics for many pathogens is that genetic diversification, complemented by the acquisition of traits that enhance relative fitness and facilitate clonal expansion, leads to the emergence of novel, virulent genotypes. Just as the life history traits for generating genetic diversity vary widely among pathogen types, it is often the case that the mechanistic basis for subsequent clonal expansion of pathogenic strains is unique on a taxonomic level. Determining the mechanisms and contribution of these life history traits to

disease is important for focusing prevention and treatment strategies to the most relevant pathogen strains and life cycle stages.

For the cyst-forming coccidia, which comprise a diverse group of parasites belonging to the phylum Apicomplexa, complex lifecycles that include both sexual and asexual stages have led to unusual population genetic structures for several species. For the widespread zoonotic pathogen, *Toxoplasma gondii*, the majority of strains infecting birds and mammals throughout North America and Europe are comprised of just three clonal lineages which exist as successful clones from a genetic out-cross (Grigg et al., 2001a; Howe and Sibley, 1995). These three lineages have apparently emerged only recently due to an enhanced fitness that facilitated their ability to effectively outcompete other genotypes (Boyle et al., 2006; Grigg and Sundar, 2009; Sibley and Ajioka, 2008; Su et al., 2003). Likewise, the veterinary pathogen *Sarcocystis neurona* possesses a surprisingly simple population genetic structure punctuated by the dominance of a few clonal lines in North America (Asmundsson et al., 2006; Rejmanek et al., 2010; Sundar et al., 2008a; Wendte et al., 2010b). Similar clonal structures have been reported for other parasitic protozoa that possess sexual cycles (Tibayrenc and Ayala, 2002) but identifying the precise genetic mechanisms that have led to the emergence of distinct clones among the different species in nature remains enigmatic.

In combination with population genetic data, the contributions of sexual out-crossing and clonal expansion as factors governing the emergence and eventual dominance of distinct, disease-producing clones have largely been inferred from laboratory studies of *T. gondii* among the cyst forming coccidia. Prior experiments demonstrated that a sexual cross between mouse-avirulent strains can produce genotypes representing a range of virulence in the mouse model, including some progeny several logs more virulent than the parents (Grigg et al., 2001a). This study identified that natural out-crosses likely produce at least some virulent genotypes, which may subsequently have potential to emerge through clonal amplification to cause extensive disease

(Grigg and Sundar, 2009; Sibley and Ajioka, 2008). Clonal propagation is possible since *T. gondii* can effectively bypass the sexual stage in felid definitive hosts and cycle, presumably indefinitely, among intermediate hosts. This can occur horizontally via oral transmission through carnivory among intermediate hosts (Grigg and Sundar, 2009; Su et al., 2003) or vertically by transplacental transmission (Dubey, 2009; Hide et al., 2009; Innes et al., 2009; Miller et al., 2008a). *Toxoplasma gondii* can also functionally bypass genetic diversification during the sexual stage by self-mating in the definitive host. Self-mating (also termed selfing, uni-parental mating, or self-fertilization) occurs when a single parasite clone can give rise to both male and female gametes capable of undergoing fertilization and producing viable offspring (Cornelissen and Overdulve, 1985; Pfefferkorn et al., 1977). In other words, no predetermined mating types are apparent and the end result is effectively clonal expansion via sex and meiosis.

Despite these important laboratory studies, the implications of these life-history traits and their relative effects on population genetic structures, especially in the context of virulence and disease outbreaks, have not been extensively studied in *T. gondii* or other cyst forming coccidia in a natural setting. Parasite life stages that are most important for causing mass-morbidity and mortality may be revealed through review of past, large-scale *T. gondii*-associated human outbreaks. For eleven reports of *T. gondii*-associated disease outbreaks in immune-competent people, eight events, including the four most devastating that caused disease or death in hundreds of individuals, were attributed to the oocyst form of the parasite, which is only produced during the sexual life cycle stage in the definitive feline host (Grigg and Sundar, 2009). Furthermore, an outbreak of the related veterinary pathogen *Sarcocystis neurona* that resulted in the death of nearly 1.5% of the threatened Southern sea otter population over the course of a single month is thought to have resulted from exposure to infectious sporocysts originating in the definitive opossum host (Miller et al., 2010). Circumstantial evidence, such as a complete lack (Wallace et al., 1972) or much reduced (Dubey et al., 1997a; Munday, 1972; Wallace, 1969) prevalence of *T.*

gondii in certain island environments without cats, also gives weight to the importance of the definitive host stage in the parasite life cycle. Similarly, *S. neurona* has not been identified outside of its definitive host range in the Americas. The apparently profound importance of this stage in the lifecycle of not just *T. gondii*, but other related parasites, warrants further study to determine the influence it could impart to shaping parasite population genetic structures and which genetic mechanisms inherent to this life stage (i.e. selfing or out-crossing) are more likely to precede a disease outbreak in nature.

To determine the genetic basis governing the exposure, evolution, and emergence of virulent genotypes during natural outbreaks linked to sexual stages of these parasitic protozoa, we tested whether epidemic isolates exist as: 1. a diverse array of multiple, novel genotypes that are the products of an out-crossing event in the definitive host, or 2. epidemic clones of a single genotype derived via selfing in the definitive host. To distinguish between these two possibilities, high resolution genetic typing was used to characterize parasite strains associated with a *T. gondii* outbreak in humans (de Moura et al., 2006) and a *S. neurona* outbreak in sea otters (Miller et al., 2010), both of which were associated with unusually high levels of morbidity and mortality. The population level genetic studies presented here argue that selfing in the definitive host plays a central role in the epidemic expansion of newly emerged, recombinant parasite strains, thus potentiating clonal outbreaks caused by tissue cyst-forming coccidia.

2. Results/Discussion

2.1 An outbreak linked to T. gondii *oocyst ingestion was associated with a single parasite genotype*

A microsatellite-based typing scheme using the markers B17, B18, TgMa, TUB2, W35 (Ajzenberg et al., 2005), and M95 (Blackston et al., 2001) was applied to determine the molecular genotypes of *T. gondii* isolates associated with a human water-borne outbreak in Brazil. This

outbreak, which occurred over a short time span in 2001, was linked to oocyst-contamination of a municipal water supply in the town of Santa Isabel do Ivai and resulted in infection and symptomatic disease in hundreds of people (de Moura et al., 2006). Initial genetic typing analyses performed on two *T. gondii* strains isolated from the water cistern implicated as the source of the outbreak (de Moura et al., 2006), as well as isolates from chickens (Dubey et al., 2003b) and cats (Dubey et al., 2004) from the immediate environment were limited to PCR-RFLP at a single locus, SAG2, leading to the conclusion that the outbreak strain was a canonical Type I strain (see below). Later, more extensive analysis by PCR-RFLP (Dubey et al., 2008d) and DNA sequencing on a limited set of markers (Vaudaux et al., 2010) showed that the outbreak-associated strains from the water cistern were clonal and non-archetypal. The majority of people who seroconverted during the outbreak also possessed a serologic profile consistent with infection by the outbreak clone, and the outbreak genotype appeared to be highly prevalent in the surrounding environment immediately following the outbreak event, infecting 4/11 chickens (TgBrCk98, TgBrCk101-103) and 1 cat (TgCatBr85) (Vaudaux et al., 2010) (Table 5)

To determine the extent of genetic relatedness among the outbreak-associated strains, high resolution MS typing and DNA sequencing using markers distributed on 11 of the 14 chromosomes was applied. This dataset distinguished the two water cistern, outbreak-associated strains at the genetic level from all others present in the environment, except for one chicken isolate (TgCkBr103) (Table 5). Unfortunately, insufficient DNA remained from the cat isolate, TgCatBr85, which precluded testing whether it was genetically identical to the cistern isolates.

Utilizing the MS typing scheme confirmed the conclusion that the causal agent was a unique, emergent *T. gondii* strain with a potential for enhanced virulence. The additional typing provided in the current study refined the conclusions of previous studies in two key aspects.

			Genetic Marker*													MS (sequence analysis)**					
		Sero-type	DNA sequence																		
			B1	GRA6	GRA7	SAG1	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	B17 TC_n	B18 CA_n	TgMA TG_n	TUB2 TG_n	W35 TG_n	M95 allele
Sample	Origin																				
Outbreak 1	WC	I/III	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	10a	10	7a	8	10	1
Outbreak 2	WC	na	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	10a	10	7a	8	10	1
						PCR RFLP															
TgCatBr85	Cat	na	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	na	na	na	na	na	na
TgCkBr 93	Ch	na	U_I	II	na	I	III	III	III	II	I	III	I	II	I	10b	10	8a	8	7a	2
TgCkBr 94	Ch	II	U_I	II	na	I	III	III	III	II	I	III	I	II	I	10b	10	8a	8	7a	2
TgCkBr 95	Ch	na	U_I	u-2	U_{III}	I	III	III	III	III	I	I	I	III	III	19	10	8b	8	7a	3
TgCkBr 96	Ch	na	U_I	u-2	III	u-1	II	III	III	III	II	I	I	III	I	22	11	7b	8	7a	2
TgCkBr 97	Ch	I/III	U_I	u-3	U_{III}	I	II	III	III	III	I	III	I	II	III	10b	10	7a	8	7b	4
TgCkBr 98	Ch	I/III	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	10a	10	7a	7	10	1
TgCkBr 99	Ch	na	U_I	u-1	U_{III}	I	III	III	III	II	u-1	I	I	II	I	8	na	8a	8	7a	2
TgCkBr 100	Ch	II	U_I	II	U_{III}	I	III	III	III	II	u-1	I	I	II	I	8	10	8a	8	7a	2
TgCkBr 101	Ch	na	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	8	10	7a	8	7a	1
TgCkBr 102	Ch	na	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	8	10	7a	8	7a	1
TgCkBr 103	Ch	na	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	10a	10	7a	8	10	1
CEP	Lab	na	III	III	III	III	III	III	III	III	III	III	III	III	III	7	10	7a	7	6	3

Table 5. Genotype analysis of *Toxoplasma gondii* strains associated with an outbreak in Santa Isabel do Ivai, Brazil. All *T. gondii* isolates were analyzed directly by sequencing at microsatellite (MS) loci and PCR-RFLP at the remaining loci except for Outbreak 1 and Outbreak 2 which were directly sequenced at all loci. Outbreak 1, Outbreak 2, TgCatBr85, and TgCkBr98-103 all possess one of two alleles at each locus, suggesting they are sibling progeny from a recent outcross. Outbreak 1 and Outbreak 2 were oocyst samples isolated from two separate water filters from water supplies implicated in the outbreak and possess identical genotypes indicative of a clonal outbreak. This suggests an outcross preceded the outbreak and was followed by a selfing event in the definitive host that enhanced the clonal expansion and transmission of the newly emerged, recombinant outbreak genotype. Shaded alleles indicate those which are identical to the Outbreak genotype. *Serotype, DNA sequence, and PCR RFLP data from Vaudaux et al. [44]; **Numbers indicate dinucleotide repeat count and letters indicate distinguishing SNPs surrounding the repeat region; MS: microsatellite; WC: water cistern; Ch: chicken; Lab: laboratory strain; na: not available.

First, the much higher level of resolution provided by the markers used and the sequence level analysis imparts a higher level of confidence to the conclusion that the outbreak was in fact clonal. The possibility that the outbreak-associated clones are not genetically identical in lieu of additional typing cannot be excluded, but several facts strongly argue against this: 1. The 18 markers were distributed across all but three of the 14 chromosomes; 2. MS markers are prone to rapid evolution and therefore provide high resolution; 3. Strains from Brazil are genetically divergent from archetypal lines, as evidenced by the segregation of alleles amongst strains in Table 5, and hence, less prone to linkage disequilibrium effects. Furthermore, only a single, oocyst-derived clonotype was isolated from independent filters collected from two different water-holding tanks providing additional evidence that these isolates resulted from self-mating rather than a genetic out-cross.

Second, this study refines previous work on the Santa Isabel outbreak by showing that the outbreak strain was actually rare in the surrounding environment, opposed to the high prevalence reported previously (Vaudaux et al., 2010). Moreover, close examination of the environmental isolates reveals that many of them, including those previously identified as the outbreak clone, and the outbreak clone itself, resemble recombinant progeny; only two allelic types are present that segregate independently across the loci examined (see TgCkBr98, 99, 100, 101, 102, 103, TgCatBr85 and Outbreak 1 and 2 in Table 5). These data argue that prior to the outbreak, the epidemic clone was produced by a genetic out-cross and was subsequently expanded by self-mating. This confirms that the more extensive resolution provided by the current study was necessary to truly distinguish an epidemic clone in a region known to contain a diverse array of *T. gondii* genotypes, including many that are apparently siblings of this strain (Vaudaux et al., 2010). This result also speaks to the important role selfing in the definitive host can play; allowing a single, emergent genotype of low environmental prevalence to rapidly rise to dominance in the surrounding population by infecting several hundreds of hosts over a short time span.

Collectively these data support high-resolution genotyping schemes as important tools for detecting informative genetic signatures in this parasite species. Initial population genetic studies showed that *T. gondii* strain diversity was comprised of three main clonal groups: Type I, II, and III (Howe and Sibley, 1995). As a result of these early studies, many broader population genetic studies have since relied on typing at only one or just a few loci to classify strains as type I, II, or III. However, it is now apparent that strains from diverse geographic locales and host species are more often infected with strains bearing unique alleles or allelic combinations, so relying on a few markers is insufficient for robust conclusions (Grigg and Sundar, 2009). The first quantitative analysis testing the accuracy of single locus typing found a very low predictive value for the loci analyzed to correctly identify strain genotype (Lehmann et al., 2004). Indeed, results

presented in the current study, when compared with results from more limited genetic studies of the same strains conducted previously (de Moura et al., 2006; Dubey et al., 2003b; Dubey et al., 2004; Dubey et al., 2008d; Vaudaux et al., 2010), provide a clear illustration of the value more extensive genetic typing can have in refining conclusions. This is especially relevant in outbreak investigations where variations in parasite genotype can be highly informative for explaining disease manifestation. High-resolution genetic typing appears to be critical for eliminating preconceived biases in epidemiologic investigations to ensure accurate discernment of disease-associated *T. gondii* strains and to recognize clonal outbreaks.

These results validate the utility of testing for epidemic clones from prospective and retrospective studies of *T. gondii* disease outbreaks (Grigg and Sundar, 2009). In support of this, Dumar and colleagues applied a similar typing scheme to a *T. gondii* outbreak in Suriname and discovered that all five patients from whom they isolated parasites were infected with the same, previously undiscovered genotype (Demar et al., 2007). Importantly, the outbreak in Suriname was another waterborne outbreak attributable to human exposure by infectious oocysts, further evidencing selfing in the definitive host as a key mechanism for allowing clonal expansion of virulent genotypes, ultimately resulting in disease epidemics.

2.2 Genetic typing of outbreak-strains of the related pathogen, Sarcocystis neurona

Since parasite genetic material from past *T. gondii* outbreaks in humans is in limited supply for the majority of cases, we sought to further assess the role of self-mating in disease outbreaks by examining an epizootic of the related veterinary pathogen, *Sarcocystis neurona*, infecting the Southern sea otter (*Enhydra lutris nereis*) of California. As a threatened species, the Southern sea otter population is well monitored and accounted for by conservation groups, creating a unique opportunity to investigate infectious disease in a natural setting. Sea otters are also aberrant hosts for many terrestrial pathogens that can be washed to sea and their high

susceptibility to many of these pathogens allows them to serve as a sentinel species for pathogens circulating in the adjacent terrestrial environment (Miller et al., 2008b). During April, 2004, the highest monthly mortality rate ever recorded in nearly 30 years of data collection occurred among Southern sea otters (Miller et al., 2010). Over the course of approximately one month, at least 40 sea otters stranded dead or dying along an 18 kilometer stretch of coast within the 500-600 kilometer Southern sea otter range. Sixteen otters were in sufficient condition to allow for complete post-mortem analysis inclusive of PCR assessment and microscopic examination of tissues. Among these otters, the major cause of death for 15 of the 16 examined animals was *S. neurona*-associated brain and/or systemic disease (Miller et al., 2010).

Preliminary genetic analysis using only four polymorphic markers against parasite strains infecting a subset of these otters (n=7) suggested they were genetically homogenous (Wendte et al., 2010b). However, the limited polymorphism present in the markers used, and lack of information about the population genetic structure of *S. neurona* in California prevented a confident conclusion that they represented an epidemic clone. The present study developed and applied a battery of higher resolution, polymorphic microsatellite and gene-coding markers to type *S. neurona* strains. Additional samples were included, encompassing 12 *S. neurona* strains from otters that died during the outbreak, as well as additional strains from other geographic locations and/or time periods. The high number of sea otter deaths associated with this epizootic provided a unique opportunity to test whether self-mating, as identified in the human *T. gondii* outbreaks, could explain the genetic origin for the *S. neurona* strains that caused the outbreak. In addition, genetic data from the current study was combined with *S. neurona* typing data reported by Rejmanek et al. (2010) to determine the population genetic structure of *S. neurona* in California spanning 15 years of study.

2.3 eBURST analysis reveals two main S. neurona *clonal complexes in California*

Sequence-level analysis of five surface antigen (Ag) genes (SnSAG1, 3, 4, 5, and 6) (Wendte et al., 2010b) and nine microsatellite (MS) markers (Sn2-Sn5, Sn7-Sn11) (Rejmanek et al., 2010; Wendte et al., 2010b) identified 12 Ag types and 33 MS types among 87 *S. neurona*-infected samples based on the allele combinations detected at each locus (Table 6; See Table 7 for complete strain and typing information). Seventy-four of the 87 samples were from mammals in California; other states represented include Georgia (n=2), Illinois (n=1), Missouri (n=3), Washington (n=5), and Wisconsin (n=2). Combining Ag and MS alleles could distinguish 35 total genotypes, but for this study these typing schemes were analyzed independently because of

Table 6 *Sarcocystis neurona* genotyping data summary

Data Set	Host	Samples	Antigen types	MS types	total genotypes	eBurst complexes (MS)	eBurst singletons (MS)	Proportion complex 1	Proportion complex 2	Total proportion complex 1/2
Overall	Sea otter	57	9	20	20	5	2	0.47	0.32	0.79
	Harbor seal	6	2	6	6	nd	nd	0	0.17	0.17
	Racoon	2	1	2	2	nd	nd	0	0	0
	Opossum	13	6	7	9	1	5	0	0.31	0.31
	Horse	7	3	4	4	nd	nd	0	0.57	0.57
	Porpise	1	1	1	1	nd	nd	0	1.00	1.00
	Cat	1	1	1	1	nd	nd	0	1.00	1.00
	Total	87	12	33	35	8	8	0.31	0.34	0.65
Monterey, CA (ATOS 1-400)	Sea otter	30	9	12	12	4	2	0.07	0.63	0.70
	Opossum	10	5	5	6	1	3	0	0.40	0.40
	Horse	4	2	2	2	nd	nd	0	0.75	0.75
	Porpise	1	1	1	1	nd	nd	0	1	1.00
	Total	45	11	15	16	4	4	0.07	0.60	0.64
Morro Bay, CA (ATOS 800-1200)	Sea otter	27	3	9	9	1	3	0.93	0	0.93
	Total	27	3	9	9	1	3	0.93	0	0.93

the likelihood that these parts of the genome are under different selection pressures and subject to differing evolutionary processes (Feil and Spratt, 2001). The majority (56/87) of *S. neurona* strains were classified as either Ag type I or Ag type II (Figure 6A). Certain MS types were also

over represented in the sample set, with MS types ‘a’, ‘c’, and ‘g’ accounting for 47/87 samples (Figure 6B). Importantly, 11/12 *S. neurona* strains from sea otters stranding during the mortality event in 2004 were an exact genetic clone at each marker analyzed (Ag type I, MS type ‘c’). The remaining outbreak sample (Ag type I, MS type ‘d’) differed from the other outbreak strains by only a single stepwise mutation at MS marker Sn4 (Table 7).

Since this and all previous studies of *S. neurona* have found a high level of sequence homology among strains (Asmundsson et al., 2006; Elsheikha et al., 2006; Rejmanek et al., 2010; Sundar et al., 2008a; Wendte et al., 2010b), we chose to analyze strain relatedness with the eBURST algorithm (Feil et al., 2004; Spratt et al., 2004). This program helps eliminate confounding effects that low sequence diversity and moderate levels of recombination can have on other methods of intra-specific sequence analysis, such as clustering, dendrograms, and phylogenetic trees, as demonstrated in (Asmundsson et al., 2006; Rejmanek et al., 2010; Sundar et al., 2008a), by only focusing on single clones and their most recent descendents (Feil et al., 2004; Spratt et al., 2004; Turner et al., 2007). We adapted the MS data for the nine markers that permit simultaneous comparison of all strains (Sn2-Sn5, Sn7-Sn11) to serve as a multi-locus typing scheme. This typing scheme, which is based on the number of repeats at each locus, was amenable to use with this program.

Using the default settings, which group isolates based on the premise that they are single locus variants (SLVs), or share 8 out of 9 alleles, we identified 8 clonal complexes (CC1-8), only 3 of which contained more than two genotypes, and 8 singletons (genotypes differing by 2 or more alleles from all others) (Table 6; Figure 7). Intriguingly, just two clonal complexes, CC1 and CC2, accounted for almost 64% (56/87) of the strains analyzed in this study (Figure 2C). This result held true even when correcting for bias introduced by the outbreak event by removing these samples from the data set, as 44/75 samples (59%) still belonged to CC1 or CC2. All SLVs identified in this study differed by a single stepwise (i.e. a single di-nucleotide repeat) mutation,

which supports the assumption that the eBURST groupings represent clonal complexes in which allelic variation is a result of mutation/drift and not recombination (Table 7) (Turner et al., 2007). The only exceptions to this were SLVs 'l' and 'o', members of CC3, that differed by 3 di-nucleotide repeats at MS Sn11. These isolates were from a sea otter in California and a horse from Missouri so the greater number of stepwise mutations detected may be a result of extended geographic isolation, thus allowing time for more drift to occur (Table 7). A single mutation event that resulted in multiple stepwise mutations is also plausible.

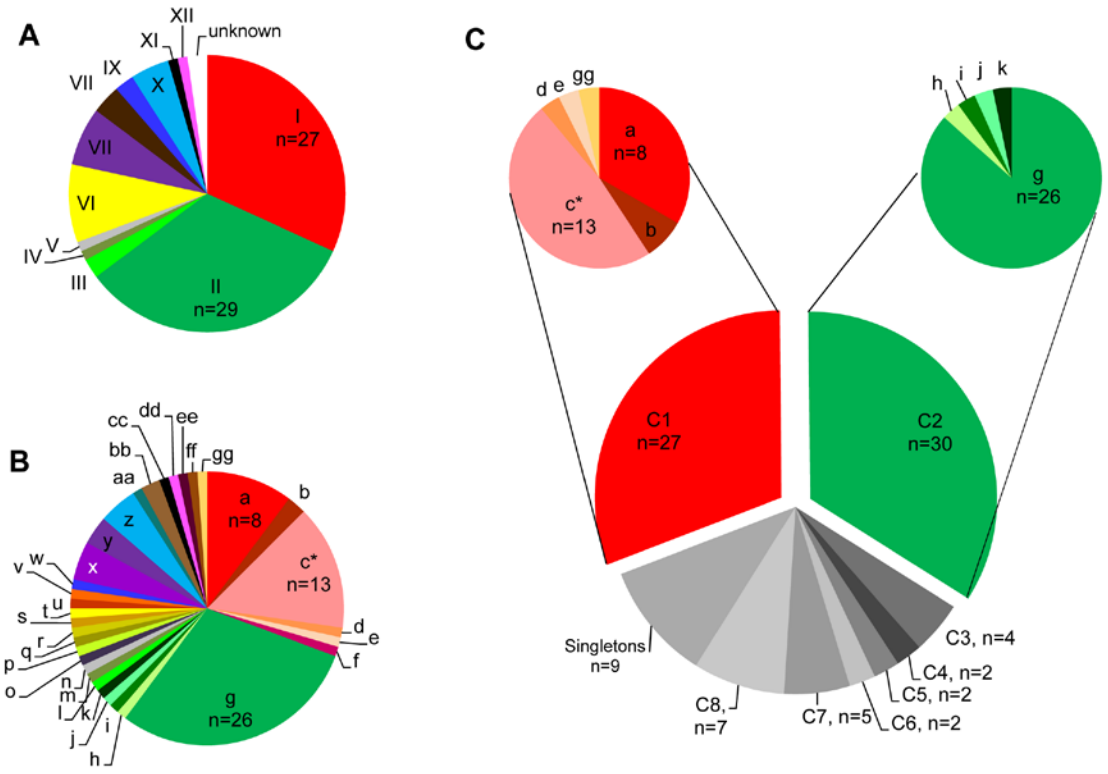

Figure 6. ***Sarocystis neurona*** **genotyping results.** Distribution of the 12 Ag types (**A**) and 33 MS types (**B**) identified among all *Sarcocystis neurona* samples studied (n=87). Ag type I and II accounted for the majority of all samples with 27 and 29 samples, respectively. The most numerous MS type identified was type g, accounting for 26 total samples. **C**) Further analysis of MS types using the eBURST program on default settings for 9 loci (Sn2-Sn5, Sn7-Sn11), revealed that 64% of all isolates belonged to two clonal complexes. Clonal complex 1 (CC1) was comprised of MS types a, b, c, d, e, and gg and CC2 of types g, h, i, j, and k. All MS types in CC1 possessed Ag type I. MS types g, h, i, and j of CC2 possessed Ag type II, whereas MS type k possessed Ag type III. *MS type c was found in 11/12 examined *S. neurona* strains from sea otters that died during the 2004 epizootic.

Table 7

Sarcocystis neurona genotypes and sample source information‡

						Genetic Marker																		
						SnSAG1-5-6	SnSAG3*				SnSAG4†	Sn2 (GT)n	Sn3 (AT)n	Sn4 (CA)n	Sn5 (CA)n	Sn7 (CA)n	Sn8 (CA)n	Sn9 (GT)n	Sn10 (AT)n	Sn11 (CA)n	Sn1520 (CTA)n	Sn1863 (AC)n	Sn515 complex	
							239	503	504	1057	592													
Sample	ATOS/Geo. Loc.	Date collected	Source#	Ag Type	MS Type		C	A	T	C	C													NT count
SO3106	318	Apr-99	Wendte et al.	II	j	1	G	·	·	T	·	9	12	12	9	17	10	17	11	13	9	17	120	
SO3339	46	Apr-00	This study	V	n	5	G	·	·	T	·	9	11	12	9	18	10	16	9	13	9	15	81	
SO3483	820	Mar-01	Wendte et al.	I	a	5	·	·	·	·	·	10	11	13	9	22	10	17	9	14	10	16	81	
SO3485	827	Mar-01	Wendte et al.	I	a	5	·	·	·	·	·	10	11	13	9	22	10	17	9	14	10	16	81	
SO3501	915	Apr-01	Wendte et al.	I	a	5	·	·	·	·	·	10	11	13	9	22	10	17	9	14	10	16	81	
SO3508	827	Apr-01	This study	I	b	5	·	·	·	·	·	10	11	13	9	21	10	17	9	14	10	16	81	
SO3523	141	May-01	Wendte et al.	VII	y	6	·	·	·	·	G	9	11	13	9	19	10	14	9	15	10	14	81	
SO3528	292	May-01	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO3629	276	Dec-01	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO3634	258	2001	This study	X	z	6	·	·	·	·	·	9	10	13	10	20	10	14	9	14	10	14	81	
SO3639	299	Dec-01	Wendte et al.	VII	x	6	·	·	·	·	G	9	11	13	9	20	10	14	9	15	10	14	81	
SO3660	384	2002	This study	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO3866	309	Apr-03	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO3892	917	Apr-03	Wendte et al.	I	a	5	·	·	·	·	·	10	11	13	9	22	10	17	9	14	10	16	81	
SO4135	824	Mar-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4151	825	Apr-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4166	827	Apr-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4167	827	Apr-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4168	832	Apr-04	Wendte et al.	I	d	5	·	·	·	·	·	10	11	12	9	21	10	17	9	13	10	16	81	
SO4169	819	Apr-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4171	819	Apr-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4177	925	Apr-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4183	919	Apr-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4189	808	Apr-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4195	818	Apr-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4202	925	Apr-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4174	1137	Apr-04	This study	I	b	5	·	·	·	·	·	10	11	13	9	21	10	17	9	14	10	16	81	
SO4178	1135	Apr-04	Wendte et al.	I	f	5	·	·	·	·	·	10	11	13	9	22	10	18	9	13	11	16	81	
SO4181	303	Apr-04	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO4194	21	Apr-04	Wendte et al.	IX	w	1	·	·	·	·	·	9	10	13	9	20	10	14	9	14	10	14	81	
SO4240	261	Jun-04	This study	X	z	6	·	·	·	·	·	9	10	13	10	20	10	14	9	14	10	14	81	
SO4285	806	Aug-04	Wendte et al.	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4289	827	Aug-04	This study	I	c	5	·	·	·	·	·	10	11	13	9	21	10	17	9	13	10	16	81	
SO4413	920	Feb-05	This study	III	i	5	G	·	·	T	G	9	11	12	9	18	10	17	10	13	10	15	81	
SO4529	396	Jul-05	This study	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO4387	337	2005	This study	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
SO4530	924	2005	Rejmanek et al.	I	e	na	·	·	·	·	·	10	11	12	9	21	10	16	9	14				

Table 7 Continued

Sarcocystis neurona genotypes and sample source information

						Genetic Marker																		
						SnSAG1-5-6	SnSAG3*				SnSAG4†	Sn2 (GT)n	Sn3 (AT)n	Sn4 (CA)n	Sn5 (CA)n	Sn7 (CA)n	Sn8 (CA)n	Sn9 (GT)n	Sn10 (AT)n	Sn11 (CA)n	Sn1520 (CTA)n	Sn1863 (AC)n	Sn515 complex	
							239	503	504	1057	592													
Sample	ATOS/Geo. Loc.	Date collected	Source	Ag Type	MS Type		C	A	T	C	C													NT count
SO4653	258	2006	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4697	321	2006	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4711	321	2006	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4725	321	2006	Rejmanek et al.	VI/VII	u	na	·	·	·	·	G	10	10	13	9	18	10	14	9	14				
SO4755	808	2006	Rejmanek et al.	I	a	na	·	·	·	·	·	10	11	13	9	22	10	17	9	14				
SO4786	370	2006	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4834	260	2006	Rejmanek et al.	II/V	h	na	G	·	·	T	·	9	12	12	9	16	10	18	11	13				
SO4928	260	2007	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4970	260	2007	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO4972	812	2007	Rejmanek et al.	VI/VII	y	na	·	·	·	·	G	9	11	13	9	19	10	14	9	15				
SO5002	259	2007	Rejmanek et al.	VI/VII	y	na	·	·	·	·	G	9	11	13	9	19	10	14	9	15				
SO5073	261	2007	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO5110	323	2007	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
SO5226	289	2008	Rejmanek et al.	III	k	na	G	·	·	T	G	9	13	12	9	17	10	18	11	13				
SO5259	827	2008	Rejmanek et al.	I	a	na	·	·	·	·	·	10	11	13	9	22	10	17	9	14				
SO5263	833	2008	Rejmanek et al.	I	gg	na	·	·	·	·	·	10	11	12	9	21	10	17	9	14				
SO5274	321	2008	Rejmanek et al.	I	a	na	·	·	·	·	·	10	11	13	9	22	10	17	9	14				
SO5278	321	2008	Rejmanek et al.	I	a	na	·	·	·	·	·	10	11	13	9	22	10	17	9	14				
SO5283	330	2008	Rejmanek et al.	IV	m	na	G	-	-	T	·	9	11	12	9	18	10	17	9	13				
SO5296	318	2008	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
HS1423	CA	Jul-99	Wendte et al.	VI	t	5	·	·	·	·	G	10	10	13	9	17	10	14	9	14	9	15	81	
HS1531	CA	Jul-99	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
HS0604	WA	Jun-04	This study	VI	r	5	·	·	·	·	G	10	11	13	9	18	10	14	10	13	14	16	81	
HS0604-03	WA	Jun-04	This study	?	ee	na	na	na	na	na	na	9	10	15	9	19	10	16	8	14	na	16	81	
HS0606-06	WA	Jun-06	This study	VI/VII	s	na	·	·	·	·	G	10	11	13	9	19	10	16	10	14	14	16	81	
HSGI07-12	WA	2007	This study	VI	ff	5	·	·	·	·	G	10	11	13	9	19	10	16	10	13	14	16	81	
Porp	Monterey, CA	2006	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
H1	Santa Rosa, CA	1994	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17		
H2	Santa Rosa, CA	1994	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17		
H3	CA	1995	Wendte et al.	II	g	1	G	·	·	T	·	9	12	12	9	17	10	18	11	13	9	17	120	
H4	MO	1999	Rejmanek et al.	XII	dd	na	·	·	·	G	G	10	16	13	9	19	11	14	10	14				
H5	MO	1999	Rejmanek et al.	I/IX/X/?	o	na	·	·	·	·	·	9	11	12	9	18	10	17	10	16				
H6	Berkeley, CA	2009	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13				
H7	Berkeley, CA	2009	Rejmanek et al.	XI	cc	na	·	-	-	·	G	9	13	13	10	16	10	14	9	14				
O1	Monterey, CA	2005	Rejmanek et al.	X	z	na	·	·	·	·	·	9	10	13	10	20	10	14	9	14				
O2	Monterey, CA	2006	Rejmanek et al.	X	z	na	·	·	·	·	·	9	10	13	10	20	10	14	9	14				
O3	Monterey, CA	2005	Rejmanek et al.	VIII	x	na	·	-	-	·	G	9	11	13	9	20	10	14	9	15				
O4	Rio vista, CA	2007	Rejmanek et al.	II/V	j	na	G	·	·	T	·	9	12	12	9	17	10	18	10	13				

Table 7 Continued

Sarcocystis neurona genotypes and sample source information

						Genetic Marker																				
						SnSAG1-5-6	SnSAG3*				SnSAG4†	Sn2 (GT)n	Sn3 (AT)n	Sn4 (CA)n	Sn5 (CA)n	Sn7 (CA)n	Sn8 (CA)n	Sn9 (GT)n	Sn10 (AT)n	Sn11 (CA)n	Sn1520 (CTA)n	Sn1863 (AC)n	Sn515 complex	
							239	503	504	1057	592														
Sample	ATOS/Geo. Loc.	Date collected	Source	Ag Type	MS Type		C	A	T	C	C														NT count
O5	Monterey, CA	2007	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13					
O6	Monterey, CA	2008	Rejmanek et al.	VI/VII	x	na	·	·	·	·	G	9	11	13	9	20	10	14	9	15					
O7	Monterey, CA	2008	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13					
O8	Monterey, CA	2008	Rejmanek et al.	VIII	x	na	·	-	-	·	G	9	11	13	9	20	10	14	9	15					
O9	Monterey, CA	2008	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13					
O10	Rio vista, CA	2007	Rejmanek et al.	IX	aa	na	·	·	·	·	·	9	10	13	9	17	10	18	9	13					
O11	GA	2008	Rejmanek et al.	VI/VII	bb	na	·	·	·	·	G	10	12	13	9	23	10	14	11	14					
O12	GA	2008	Rejmanek et al.	VIII	bb	na	·	-	-	·	G	10	12	13	9	23	10	14	11	14					
O13	IL	2008	Rejmanek et al.	VI/VII	v	na	·	·	·	·	G	10	11	13	10	16	10	14	10	14					
R1	WI	2006	Wendte et al.	VI	q	5	·	·	·	·	G	10	11	14	9	17	10	14	10	14	15	16	81		
R2	WI	2006	Wendte et al.	VI	p	5	·	·	·	·	G	10	11	13	9	17	10	14	10	14	15	16	81		
Cat	MO	2000	Rejmanek et al.	II/V	g	na	G	·	·	T	·	9	12	12	9	17	10	18	11	13					

ATOS: The 'As The Otter Swims' number refers to each sea otter's stranding location, based upon defined and sequential 0.5 kilometer segments of the California coastline, starting with zero (0) just north of San Francisco and increasing numerically from north to south.

SO: Sea Otter; HS: Harbor Seal; Porp: Porpoise; H: Horse; O: Opossum; R: Raccoon

CA: California; WA: Washington; MO: Missouri; GA: Georgia; IL: Illinois; WI: Wisconsin

NT: nucleotide

na: Not Available

*numbers refer to nucleotide position on reference sequence: GQ851954

†numbers refer to nucleotide position on reference sequence: GQ851957

‡Samples are organized and color coded by host species in the left columns (Blue: sea otter; Dark Blue: sea otters stranded in the 2004 epizootic; Sea Green: marine mammals other than sea otters; Brown: terrestrial mammals) and then color coded by antigen (Ag) and Microsatellite (MS) type in the remaining columns

#Samples from Wendte et al. were additionally typed at MS markers Sn2-Sn5, Sn7, Sn8, Sn10, Sn11, Sn1520, Sn1863, Sn515 for the current study

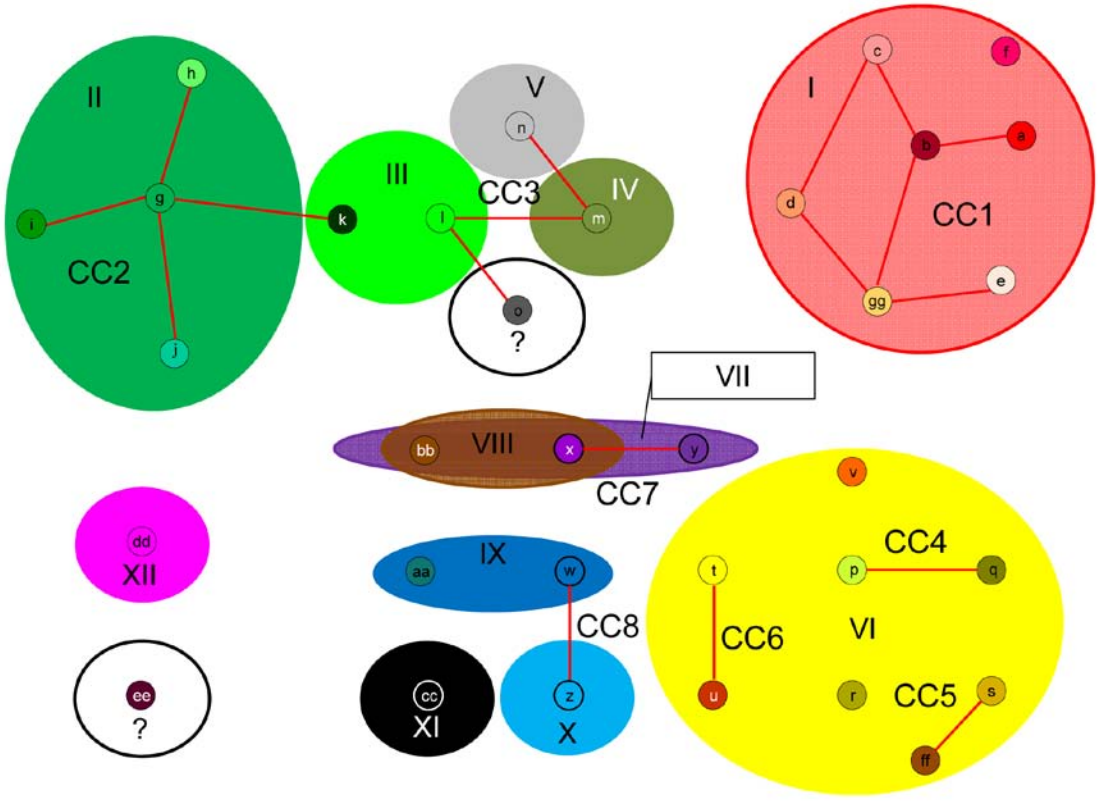

Figure 7. Modified eBURST analysis output. Default eBURST settings were used to analyze *Sarcocystis neurona* sequence types based on MS markers Sn2-Sn5 and Sn7-Sn11. MS types identified are represented as small circles and designated by lowercase letters. Lines connect MS types that are identical at 8 out of 9 MS loci and are therefore considered part of a clonal complex (CC). eBURST identified 8 clonal complexes (designated CC1-CC8) and 8 singletons. Large colored ovals are overlain to indicate the Ag type (Ag types I-XII) that characterizes each MS type identified by eBURST. MS and Ag type color schemes refer to those described in Table 7. Results support an intermediate population structure with both clonal propagation and sexual recombination. All members of CC1 possess an identical Ag type (Ag type I). MS types x and bb were found in samples with different Ag types (VII and VIII). Ag types VII and VIII differ by a single di-nucleotide indel at Ag marker SnSAG3, likely representative of drift rather than recombination as a mechanism to account for allele differences in this case. In contrast, MS type k has a markedly different Ag type (III) compared to other members of the CC2, which all possess Ag type II. Ag types II and III have different alleles at all Ag loci examined, making a recombination event the most parsimonious explanation for the difference between MS type k and other members of CC2 rather than genetic drift.

Since recombination appeared to be rare between clonal complexes based on MS markers, we decided to overlay the results of the Ag typing analysis on the eBURST output

(Figure 7). The results were consistent with previous claims of an intermediate population structure for *S. neurona* (Asmundsson et al., 2006; Elsheikha et al., 2006; Rejmanek et al., 2010; Sundar et al., 2008a; Wendte et al., 2010b) in that both clonal propagation and sexual recombination were supported. All members of CC1 and 29/30 members of CC2 possessed an identical Ag type (Ag types I and II, respectively). In contrast, all MS types in CC3 and CC8 possessed a distinct Ag type. There were also two cases (MS types 'x' and 'bb') where the same MS type was identified with two distinct Ag types (Ag types VII and VIII) and the reverse scenario also occurred where the same Ag type (VI) characterized three clonal complexes based on MS types (CC4, CC5, CC6), all of which could potentially indicate recombination events (Figure 7).

Overall, these data support a population structure that is highly clonal, though evidence for recombination is present as well. This intermediate population structure is similar to that described for *T. gondii*, though definitive conclusions will require a sample set less biased towards diseased animals (Feil and Spratt, 2001). It is worth noting here that the population structure of the organisms described in this study is, like all population genetic structures, only as resolved as the markers allow. For example, finer resolution can be achieved by applying the marker SnD2 from Rejmanek et al. (2010) to SO4711, SO4786 and O7 to show that they are different strains. What this does not change, though, is that these strains are members of the same clonal complex and that resolution at this level is sufficient to identify an outbreak clone and to document geographic partitioning of strains along the California coastline (see below). This level of resolution is more robust to the possibility of strand slippage and evolution of new alleles during PCR that could make identical clones appear distinct with finer levels of resolution. An example of this may have occurred with SO4387, identified in this study as MS type 'g,' but by Rejmanek et al. (2010) as MS type 'i.' These types differ by a single repeat at MS Sn9 (Table 7). It is also possible that this otter was co-infected with two closely related strains. Consistent

identification of SLVs in many samples increases the confidence that they represent truly different strains. The outstanding potential these microsatellite markers have for more robust strain resolution, if interpreted cautiously, can facilitate addressing more specific questions, such as the identity and point source of an epidemic clone.

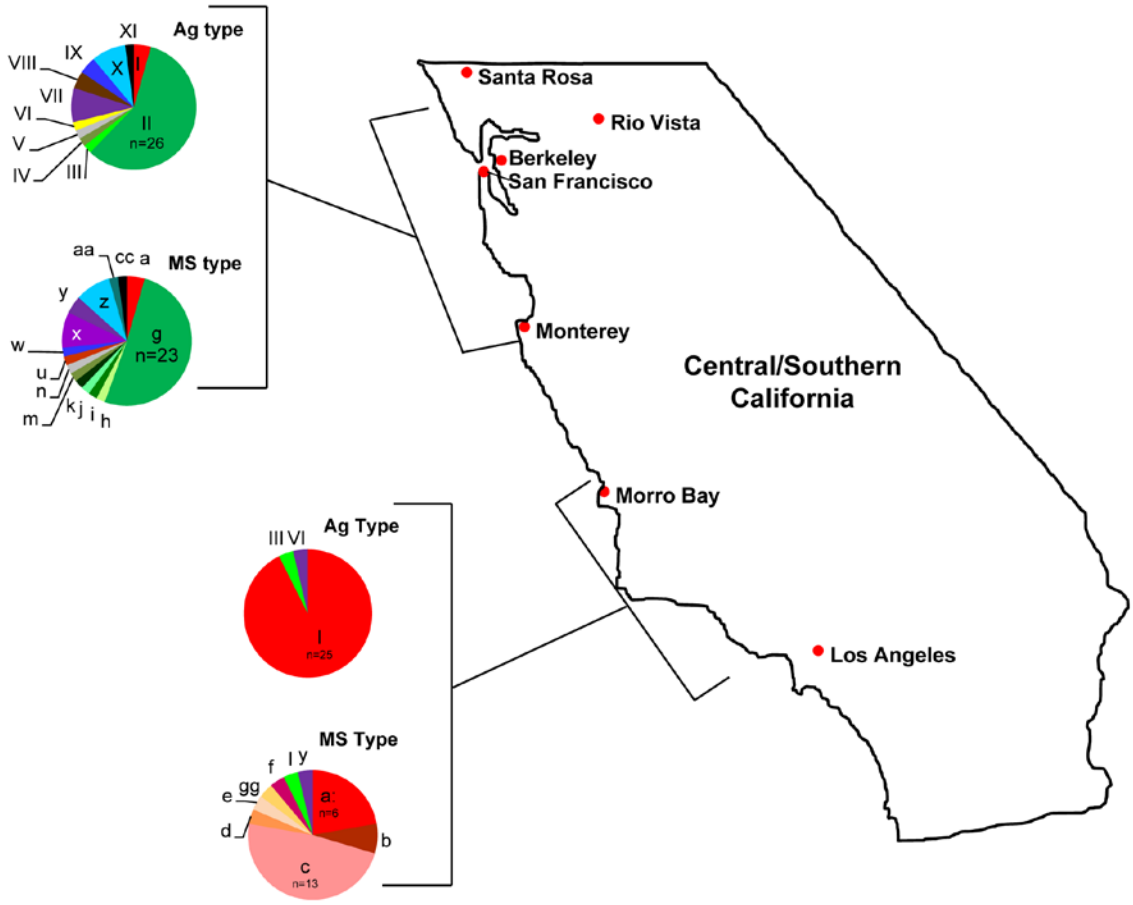

Figure 8. Geographic distribution of *Sarcocystis neurona* Ag and MS types in California. All sea otter samples were collected in two distinct, ~200km stretches along the California coast: one in central California from just north of San Francisco Bay to just south of Monterey Bay, and one to the south from just north of Morro Bay to just north of Los Angeles. Nearly all (93%) of the 27 samples from the southern region belonged to eBURST defined clonal complex (CC) 1 and none were identified as CC2. In the north, 63% of 45 samples belong to CC2 and only two representatives of CC1 were found. Terrestrial isolates from California were from 10 opossums and 4 horses. These, along with one sample from a porpoise, were from the northern range and included as such. The majority of sea otter samples were from two small areas of coastline: one near Monterey Bay in the north and the other near Morro Bay in the south (see Table S1 for details).

2.4 Temporal stability, geographic and host distribution of strains in California

The majority of strains (72/87; 83%) evaluated in this study were collected from two distinct 200km stretches along the California coast or the adjacent terrestrial environment (Table 7; Figure 8). As such, we utilized this subset of the data to examine the temporal stability of strains and their geographic and host distribution in central California.

The total time period covered by the strains analyzed in this study is 15 years (1994-2009). Sample sizes were not evenly distributed across each year and some years (1996-1998) had no representative samples, so it is likely that genotype life spans are underestimated. Despite this, at least one clonal complex, CC2, appears to be very stable in nature over time, exhibiting a lifespan encompassing the entire length of this study. CC2 was sampled during 12 of the 13 years for which a sample was collected (Table 8). Within this complex, Ag type II, MS type 'g' had a lifespan of the full time period examined (15 years) and was the longest lived of any Ag or MS type (Figure 9; Table 8). The other clonal complexes present in California, CC2, CC3, CC6-CC8, appeared to be stable as well, with life spans ranging from 5-8 years (Table 8). Collectively these data provide supporting evidence for *S. neurona*'s ability to propagate clonally. However, it will be important to test whether or not these allelic combinations appear more often than would be expected by chance to confirm clonal propagation as more sequencing data becomes available from strains collected from non-diseased animals and the position of the markers in the genome is identified (Smith et al., 1993). Interestingly, the genotype associated with the outbreak, Ag type I, MS type 'c', was only found during 2004 (Figure 9). These samples were all associated with otters dying during the epizootic in April, 2004, except for two samples that were obtained from sick otters in the same area four months after the event ended (Table 7). The implications these observations may have for strain virulence are discussed below.

Table 8 *Sarcocystis neurona* Genotype Presence Over Time in California

			Year															
Clonal Complex	Ag type	MS type	94	95	99	00	01	02	03	04	05	06	07	08	09	Total	ST Life span (years)	CC Life span (years)
1	I	a					3		1			1		3		8	7	7
1	I	b					1			1						2	3	
1	I	c								13						13	1	
1	I	d								1						1	1	
1	I	e									1					1	1	
1	I	gg												1		1	1	
2	II	g	2	1			2	1	1	1	2	6	5	3	1	24	15	15
2	II	i			1											1	1	
2	II	j											1			1	1	
2	III	k												1		1	1	
3	III	l									1					1	1	8
3	IV	m												1		1	1	
3	V	n				1										1	1	
6	VI	t			1											1	1	7
6	VI	u										1				1	1	
7	VII	x					1							1		2	7	7
7	VII	y					1						2			3	6	
7	VIII	x									1			1		2	3	
8	IX	w					1			1						2	3	5
8	X	z								1	1	1				3	3	
Singleton	I	f								1						1	1	
Singleton	IX	aa											1			1	1	
Singleton	XI	cc													1	1	1	
	Total		2	1	2	1	9	1	2	19	6	9	9	11	2			

Ag: Antigen gene

MS: Microsatellite

ST: Sequence Type (combination of Ag and MS type)

CC: Clonal Complex

On visual inspection, it appeared that the genetic composition of *S. neurona* strains from the Monterey Bay area was distinct from the southern strains obtained in or near Morro Bay (Figure 8; Table 6). We further tested this hypothesis by conducting χ^2 analysis on the proportion of the majority clonal complexes (CC1 and CC2) that comprised each population. There was a highly significant difference between northern and southern strains (Figure 10). Significance

remained when analysis was restricted to sea otter samples, in order to eliminate any confounding effects due to host species, because all southern strains were from sea otters (Figure 10). This conclusion is consistent with data reported previously on *S. neurona* strains from coastal California (Sundar et al., 2008a; Wendte et al., 2010b), but contrasts with the conclusions of Rejmanek et al. (2010).

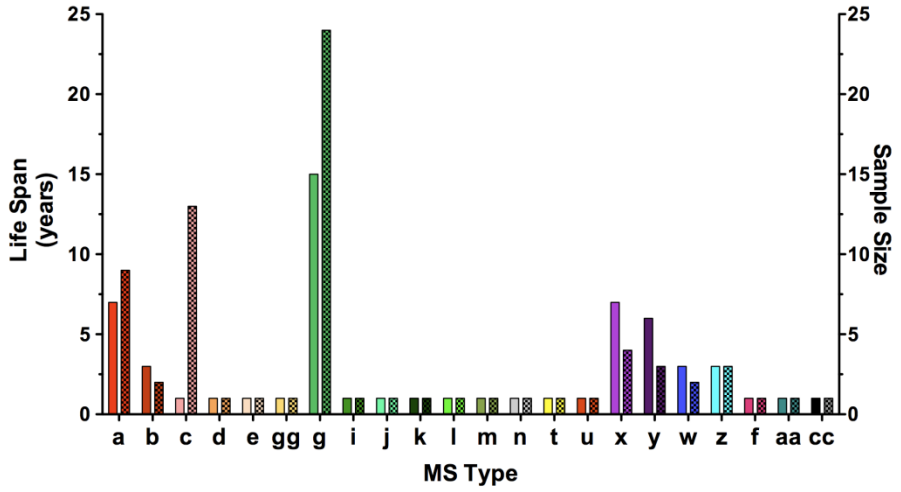

Figure 9. *Sarocystis neurona* MS type lifespan and sample size in California. Lifespans for microsatellite (MS) types (solid bars) were defined as the time period from identification the first representative sample to the last during the 15 years (1994-2009) encompassed in this study. Sample sizes are indicated by checkered bars. MS type 'g,' a member of eBURST defined clonal complex 2 (CC2), was the longest lived and most prevalent MS type, having a representative sample in all of the years for which samples were available. There were no *S. neurona* samples available for testing during 1996-1998. MS type 'c,' the genotype implicated in the sea otter epizootic, was only found in 2004 during the month of the outbreak and four months thereafter in the same region.

We also sought to identify a potential terrestrial source for *S. neurona* strains present in the marine environment. Experimental evidence for the model organism, *T. gondii*, supports a route of infection for sea otters through ingestion of *S. neurona* sporocysts that were washed to the ocean in contaminated fresh water and then concentrated in the otters' filter-feeding invertebrate prey (Arkush et al., 2003; Lindsay et al., 2004; Miller et al., 2008b). Implicating

opossums as the ultimate terrestrial source of infection is supported by comparing the prevalence of the majority clonal complexes (CC1 and CC2) in sea otters and opossums in the northern, Monterey Bay area study site (the only locale from which opossum samples were obtained). Strain prevalence differences between these groups were not statistically different, suggesting that monitoring strain types in coastal dwelling opossums will be predictive of genotypes infecting adjacent marine dwelling otters (Figure 10). Observational data from the outbreak noting an abundance of razor clams and evidence of sea otter movement into the area for feeding (i.e. accumulation of broken shells on the shore) just prior to the event, further support this model of land-to-sea parasite transfer (Miller et al., 2010). Sea otters very rarely consume known intermediate hosts of *S. neurona* (Ebert, 1968), leaving the ingestion of sporocysts as the most biologically plausible route for sea otter infection regardless of the land-to-sea transport mechanism, and strongly supporting the conclusion that this outbreak originated from a selfing event in the opossum host.

2.5 Parasite genotypes and virulence

Disease is a complex manifestation of the interplay between intrinsic pathogen factors (i.e. pathogen genotype) and numerous external factors, including dose, host immune status, and environmental conditions such as weather that can influence transmission. Delineating the relative contribution of each of these factors to a given disease outbreak is a difficult process, as is illustrated by the outbreaks described in this study. It is plausible that the *S. neurona* strain associated with the 2004 epizootic is intrinsically more virulent than other strains since it was only identified during the time period surrounding the outbreak and may have been too virulent for continued propagation. Also, the majority of otters infected died within 24-48 hours of stranding and had high IgM titers (Miller et al., 2010). The rapid rise and subsequent fall of a

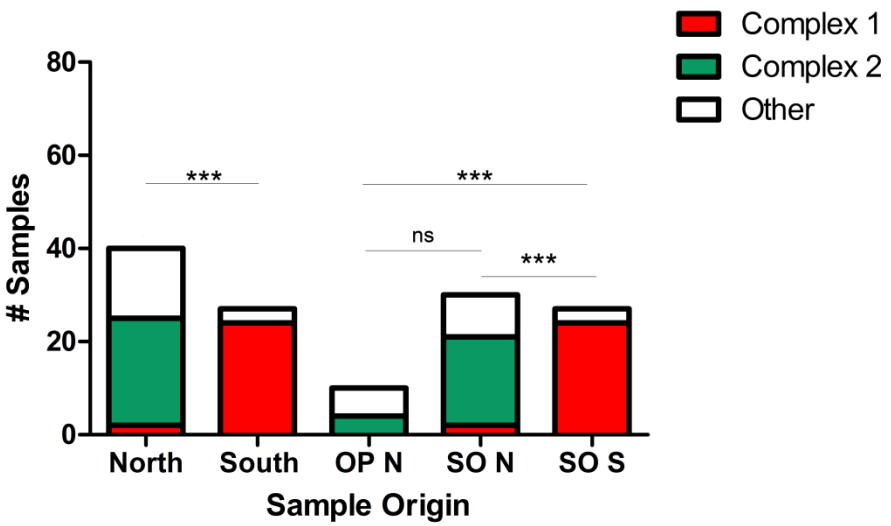

Figure 10. Geographic partitioning and host associations of *Sarcocystis neurona* strains. Distinct *S. neurona* populations as defined by the proportion of the population belonging to the dominant eBURST defined clonal complexes (CC) 1 or 2 were found infecting animals in the northern and southern ranges examined in California (see Figure 4). This difference remained significant by Chi-Square analysis when only sea otter samples were compared. When samples from sea otters from the northern range were compared to opossum samples from the adjacent terrestrial environment, no significant difference was found. There were no samples from terrestrial mammals in the southern range. OP N: opossum samples from the northern range; SO N: sea otter samples from the northern range; SO S: sea otter samples from the southern range; ns: not significant; ***$p<0.00001$.

virulent strain type is a phenomenon noted in many outbreaks of a diverse array of pathogens from viruses (e.g. Influenza virus (Smith et al., 2009)) to bacteria (e.g. *Leptospira interrogans* (Thaipadungpanit et al., 2007)) to fungi (e.g. *Coccidioides immitis* (Fisher et al., 2000)). However, this phenomenon may also be attributable to sampling biases (Feil and Spratt, 2001) or environmental factors (Fisher et al., 2000) making the assumption that the virulent genotype is not adaptive inaccurate. Equally in the case of the sea otter outbreak, numerous external factors, including concurrent infection with other pathogens and domoic acid poisoning, abundant food source with potential for contamination with sporocysts, and a large rainstorm preceding the event that could have increased sporocyst deposition, may have played a contributing role in conferring this *S. neurona* strain with a virulent phenotype (Miller et al., 2010).

Similarly, the *T. gondii* strain implicated in the 2001 Brazil outbreak appeared to rise in prevalence during the outbreak but then decline over time in the local environment (Vaudaux et al., 2010). This was also a unique, newly identified genotype that caused symptomatic disease in 155 immune-competent individuals—an unusual phenomenon for this normally asymptomatic parasite. Importantly, though, ~270 other individuals with access to the same water cistern seroconverted during this time with no overt signs of disease (Vaudaux et al., 2010), invoking a role for environmental and host factors in this outbreak.

A striking character of both these outbreak events is the key role self-mating in the definitive host served as a catalyst allowing virulent pathogen genotypes to rapidly reach high levels under the right conditions to precipitate a disease epidemic.

2.6 Self-mating potentiated the emergence of the S. neurona *and* T. gondii *epidemic clones*

Epidemic clonality associated with sporocyst or oocyst ingestion strongly suggests that self-mating in the definitive host was the key event leading to these outbreaks. Selfing in the definitive host has been confirmed experimentally for *T. gondii* (Cornelissen and Overdulve, 1985; Pfefferkorn et al., 1977) but only indirectly assumed for *S. neurona* (Butcher et al., 2002). Prior to this study, rigorous genetic characterization of selfing events in nature were lacking and the question as to whether a productive sexual out-cross or a selfing event precedes an outbreak linked to oocysts or sporocysts had not previously been tested.

Early population genetic studies using limited, poorly resolved markers identified a paucity of mixed strain *T. gondii* or *S. neurona* infections in nature and these data have previously been interpreted to suggest that most definitive host infections would be by a single strain and therefore out-crossing would be rare in nature (Sibley, 2003). However, more recent studies using unbiased, multi-locus typing schemes have consistently identified mixed strain infections among natural intermediate hosts suggesting that prey species of definitive hosts are more

frequently harboring mixed strain infections than previously realized (Aspinall et al., 2003; Boughattas et al., 2010; Dubey et al., 2007b; Dubey et al., 2009b; Dubey et al., 2006a; Dubey et al., 2006b; Dubey et al., 2006c; Dubey et al., 2005; Elbez-Rubinstein et al., 2009; Lindstrom et al., 2008; Parameswaran et al., 2010; Ragozo et al., 2010; Sundar et al., 2008b). Hence, the lack of mixed strain infections identified in earlier studies may simply reflect the techniques used, such as bioassay or limited genetic typing, that were biased toward certain strains and likely missed multiple infections and the true diversity of genotypes present.

As more high resolution, multilocus genetic markers are being applied against previously characterized strains of *T. gondii*, an increasing number are being re-classified as recombinants, defined as products of sexual out-crossing events, including strains previously linked to outbreaks (Grigg and Sundar, 2009). Given the virulent nature of the two outbreaks examined here, and the evidence that out-crossing between two avirulent, haploid parents can produce progeny with enhanced virulence (Grigg et al., 2001a), we originally hypothesized that out-crossing might explain the genetic origin and expansion of the outbreak strains, rather than self-mating. Intriguingly, close examination of the environmental isolates surrounding the *T. gondii* outbreak supported this hypothesis because the epidemic clone was one of many progeny produced by a local genetic out-cross. However, the available evidence indicated that, while out-crossing certainly preceded the outbreak, it was the subsequent selfing event that was responsible for the epidemic expansion and transmission of the virulent clone that caused the outbreak. Certainly this dataset argues that sex and self-mating combined to produce the *T. gondii* clonal outbreak. Further typing of additional outbreaks is warranted to examine whether or not an out-cross is independently sufficient to cause an epidemic attributable to multiple, recombinant progeny.

This two-step process of local epidemic expansion via a sexual out-cross followed by clonal propagation of a few progeny with enhanced adaptations or virulence is reminiscent of the process envisioned on a larger scale for the pandemic rise of the archetypal *T. gondii* clones

(Types I, II, and III), also found to be the progeny of an out-cross (Boyle et al., 2006; Grigg et al., 2001a). Documenting this process in real time at a local level has provided key insight into mechanisms that account for clonal propagation in nature. It was previously proposed based on laboratory studies that clonal dominance of archetypal *T. gondii* strains was attributable to an enhanced ability for oral transmission through carnivory, a hypothesis which certainly warrants further investigation in natural settings (Su et al., 2003). However, recent studies have since shown that this trait does not operate as originally proposed (Fux et al., 2007; Khan et al., 2007). These findings raised the possibility that other life history traits may likewise be important in perpetuating clones.

In this light, it is worth noting that all aspects of the parasite lifecycle that promote clonal propagation, namely selfing, oral transmission through carnivory, and transplacental transmission, contribute in part to clonality in the population structure. However, when considering their relative roles, the advantage in fecundity the sexual stage can impart during a selfing event to a single parasite genotype, as documented in this study, provides strong evidence this mechanism is likely the major contributor to localized or regional clonal dominance of certain strains. The basic reproductive number (R_0), or number of secondary infections a single infected individual will cause, is many orders of magnitude greater in the definitive host (which releases millions of environmentally stable, infectious propagules capable of waterborne or aerosolized transmission (Dubey, 2001)) compared to an intermediate host (in which the infectious units produced can only be passed to those directly feeding on tissues). Oocysts or sporocysts can also successfully infect intermediate hosts at much lower doses (even a single oocyst) than tissue cysts (Dubey, 2006; Dubey et al., 1996). Oocyst deposition therefore exists as a potent mechanism for causing widespread epidemics and establishes a plausible rationale for explaining how selective sweeps can occur among these heterogamous pathogens. Determining

what factors govern whether these sweeps occur on a local, and presumably more frequent, epidemic level or reach pandemic proportions are important subjects for future research.

Our results also confirm that fecal contamination of food and water sources represents a major threat to human and animal health, hence targeting the definitive host or the oocyst stage of these parasites is an excellent first-step strategy to disrupt transmission. This conclusion is further supported by studies showing the importance of the definitive host stage for maintaining continued transmission of this parasite in island communities (Dubey et al., 1997a; Munday, 1972; Wallace, 1969; Wallace et al., 1972) and how local vaccination of definitive feline hosts can significantly reduce *T. gondii* infection rates (Mateus-Pinilla et al., 1999) .

The scope of explanatory power for this selfing model can also be extended to other highly clonal, cyst forming parasites, including the clonal outbreak linked to *S. neurona* and likely other pathogenic *Sarcocystis spp.* and *Neospora spp*. This finding is significant since many aspects of the *T. gondii* life cycle have previously been proposed to be unique to this species among the tissue encysting coccidia, including its broad host range inclusive of nearly all warm-blooded vertebrates and its ability to be transmitted through carnivory among intermediate hosts (Costa et al., 2008;] (but also see: [Dubey et al., 2001a; Gondim et al., 2010; Grigg and Sundar, 2009; Mansfield et al., 2008; Miller et al., 2009; Sibley and Ajioka, 2008; Su et al., 2003)). Notably, selfing has also been demonstrated in more distantly related Apicomplexan parasites, including *Eimeria spp.* and *Plasmodium spp*. (Cornelissen and Overdulve, 1985). In addition, the processes of homothalism and same-sex mating identified in fungi serve the analogous purpose of clonal propagation via a mechanism more generally thought to serve in genetic recombination and out-crossing (Heitman, 2010). This suggests that selfing, as a genetic mechanism of clonal propagation, has potential to play a pivotal and previously under-recognized role for a diverse array of eukaryotic pathogens in the expansion of genotypes that cause disease epidemics and/or emerge as highly successful clonotypes to rapidly alter population genetic structures.

3. Materials and Methods

3.1 Ethics Statement:

Animal carcasses were gathered and samples processed in accordance with guidelines and with approval by the Washington Department of Fish and Wildlife, Marine Mammal Investigations as part of the Northwest Marine Mammal Stranding Network and authorized under 109(h) (16 U.S.C. 1379(h)) section of the Marine Mammal Protection Act (MMPA) and National Marine Fisheries Service (NMFS) MMPA Research permit 782-1702. Work in California was conducted under United States Fish and Wildlife Service (USFWS) permit MA 672724-9 issued to United States Geological Survey Biological Resource Discipline (USGS-BRD).

3.2 Sarcocystis neurona *and* Toxoplasma gondii *DNA and genetic typing markers*

Parasite DNA was obtained either from infected host tissues or parasite isolates maintained in tissue culture as described previously (Wendte et al., 2010b). Samples were analyzed using a typing scheme that included the surface antigen markers: SnSAG1, SnSAG3, SnSAG4, SnSAG5, SnSAG6 (Wendte et al., 2010b) and 9 microsatellite markers Sn2-Sn5 and Sn7-Sn11 originally described by Asmundsson and Rosenthal (Asmundsson and Rosenthal, 2006) but applied as modified in Wendte et al. (2010b) and Rejmanek et al. (2010). Three additional microsatellite markers were designed by the following method: Publically available *Sarcocystis neurona* expressed sequence tags (ESTs) were downloaded from the NCBI dbEST database (http://www.ncbi.nlm.nih.gov/dbEST) and the *S. neurona* Gene Index (maintained by the Computational Biology and Functional Genomics Laboratory at the Dana Farber Cancer Institute, http://compbio.dfci.harvard.edu/tgi/) databases. The downloaded ESTs were assembled into contigs using the SeqMan (Lasergene) application. Contig sequences were then processed with the MISA microsatellite identification program (http://pgrc.ipk-gatersleben.de/misa/) with the following repeat parameters: definition (unit size-minimum repeats): 2-12, 3-7, 4-5, 5-4, 6-3,

7-3, 8-2, 9-2, 10-2, 11-2, 12-2, 13-2, 14-2, 15-2; interruptions (maximum difference between 2 simple sequence repeats): 25.

Approximately 50 microsatellites of sufficient length and/or complexity were identified. Three (Sn1520, Sn1863 and Sn515) of these markers were not previously published and possessed sufficient non-redundant flanking sequence to allow for nested primer design and produced robust size-polymorphic PCR amplification products. Primers were validated as described (Wendte et al., 2010b) and found to be specific and sensitive for *S. neurona* DNA in tissues (data not shown). The primers designed are as follows: Sn1520 Fext-GGGGCAGAACCATCGTAGTA, Rext- GTGAAGCATTTCCCCTACGA, Fint-GGCGGTAGTCACTTGCTGA, Rint- GTGGGAGAAGACGGTCGTTA; Sn1863 Fext-CATGGCGTGCGTTAACTAAA, Rext- CGTACAAACACACGCTCCAC, Fint-CCATTCATCGACAGCGACTA, Rint- TGAGACAGCCGTCAAACACT; Sn515 Fext-CTTCTAGCGGCTGTTTCTCC, Rext- TCTGTGTGGGTGTGGAAGTC, Fint-GACCCCCTCTCTGCTTCTCT, Rint- ACGCAAATGCGAACATATCA. Representative sequences for each allele at each locus were placed in GenBank under the following accession numbers: Sn1520: HM851251, HM851252, HM851253, HM851254, HM851255; Sn1863: HM851256, HM851257, HM851258, HM851259; Sn515: HM851249, HM851250. PCR, DNA sequencing and analysis were conducted as described previously, except, to control for bias in scoring results, random sample IDs were assigned to samples before sequencing so that sequence analysis for some loci was blinded (Wendte et al., 2010b).

For this study, *S. neurona* DNA from 15 sea otters and 4 harbor seals was analyzed. Additionally, samples from 21 sea otters, 2 harbor seals, 3 horses, and 2 raccoons previously described by Wendte et al. (2010b) at the SnSAG antigen loci and MS Sn9, were further typed in this study at the remaining 10 MS loci. Finally, *S. neurona* DNA from 21 sea otters, 1 porpoise, 4 horses, 13 opossums, and 1 cat that was previously typed by Rejmanek et al. (2010) at

SnSAG3, SnSAG4, and MS markers Sn2-Sn5 and Sn7-Sn11 were combined with the data in this study for a total sample set that included 87 samples from 57 sea otters, 6 harbor seals, 2 raccoons, 13 opossums, 7 horses, 1 porpoise, and 1 cat. In all, 75 of the 87 samples were from California. Other states represented include Georgia (n=2 samples), Illinois (n=1), Missouri (n=3), Washington (n=4), and Wisconsin (n=2). Some overlap existed between the samples typed in this study and those reported by Rejmanek et al.: samples SO4387, SO4413, H1, H2, and H3 in this study are reported as SO1, SO2, Horse 1, Horse 2, and Horse 3 in Rejmanek et al. (2010), respectively. Complete information about the sample origins is found in Table 7.

Toxoplasma gondii isolates from a water cistern (n=2), chickens (n=11), and one cat associated with a human waterborne toxoplasmosis outbreak (Vaudaux et al., 2010), as well as laboratory strain CEP were typed at microsatellite loci B17, B18, TgMA, TUB2, W35 (Ajzenberg et al., 2005) and M95 (Blackston et al., 2001). Markers were PCR amplified and sequenced to assign alleles as for *S. neurona* markers (Wendte et al., 2010b). Representatives of each microsatellite allele at each locus were placed in Genbank under accession numbers: B17: HM851260-67; TgMA: HM851268-73; W35: HM851274-77; M95: HM851278-81.

3.3 Genotyping and eBURST analysis

Because different parts of the genome are likely under different selective pressures, all *S. neurona* samples were categorized by an antigen (Ag) type designated by roman numerals and a microsatellite (MS) type indicated by a lowercase letter designation. Ag types were defined by the presence/absence of mutually exclusive antigen genes (SnSAG1, SnSAG5, or SnSAG6) and the inheritance pattern of alleles at SnSAG3 and SnSAG4 (Rejmanek et al., 2010; Wendte et al., 2010b). MS types were assigned on the basis of allele combinations defined by the number of di- or tri- nucleotide repeats at each locus (Sn2-Sn5 and Sn7-Sn11, Sn1520, Sn1863). Sn515 was a complex repeat in which each isolate possessed one of two alleles. Samples from the study by

Rejmanek et al. (2010) were not typed at the SnSAG1-5-6 loci, but were placed into Ag groups based on the allelic profile at SnSAG3 and SnSAG4 and by the Ag group their MS type was associated with in samples typed at all markers. For example, based on the alleles at SnSAG3 and SnSAG4, sample SO4 (Table 7) could be placed either in Ag type II or V, but its MS type was only found associated with Ag type II in samples where all markers were typed, making this the most likely, though not definitive, Ag type designation. The *S. neurona* strains assessed by Rejmanek et al. (2010) were also not typed at MS markers Sn1520, Sn1863, and Sn515. Presumptively classifying these samples into MS types based on alleles at Sn2-Sn5 and Sn7-Sn11 is likely accurate, though, since these three markers did not provide additional resolution to MS types for the 46 additional *S. neurona* strains described in this study.

The alleles present at MS markers Sn2-Sn5 and Sn7-Sn11 were used for creation of a multi-locus sequence typing scheme by which all isolates could be compared. The numerical designation of alleles allowed the detection of which MS types formed clonal complexes using the eBURST program (Feil et al., 2004). Default settings were used which grouped MS types on the basis of sharing alleles at 8 of the 9 markers analyzed.

To assess *T. gondii* isolates for clonality, MS alleles were combined with previously published DNA sequence analysis at three genetic loci, PCR-RFLP or DNA sequencing at 10 loci, and serologic analysis as described by Vaudaux et al. (2010).

3.4 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 and χ^2 values were considered significant at P=0.05.

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CHAPTER V

OVERALL CONCLUSIONS

The conclusions of the preceding chapters are based on what amounts to at most a tiny fraction of the potentially informative genetic information possessed by an individual parasite. This limitation makes some of the inferences discussed here, especially those pertaining to intra- and inter-strain genetic diversity and relatedness, tentative pending further, more robust analyses. The results discussed in Chapter IV lead to the conclusion that self-mating in the definitive host is a key mechanism a single parasite genotype can utilize to create high numbers of genetically identical offspring and rapidly be transmitted. This conclusion is valid within the limits of the data generated. However, the typing scheme applied is highly unlikely to detect more obscure mechanisms of genetic diversification that can occur even when dividing clonally, thus making the conclusion that the offspring are exact genetic clones potentially inaccurate. For the related parasite, *Plasmodium falciparum*, mitotic and meiotic cell division can both lead to genetic diversity among clones through the generation of karyotypic alterations including aneuploidy (Corcoran et al., 1988; Van der Ploeg et al., 1985; Wellems et al., 1987). While only minimally tested for in *T. gondii*, the presence of karyotypic diversity among *T. gondii* strains (Sibley and Boothroyd, 1992a) suggests large chromosomal deletions/insertions/duplications, that would only rarely be detected by PCR based typing schemes, may represent an important mechanism for generating genetic diversity even when the parasite is replicating clonally, such as through self-mating.

Another possibility for creating genetic alterations during clonal propagation is through the process of gene conversion. While it is unclear whether this process will occur more frequently than random mutation, there is some evidence that it is an important process in the evolution of tandemly duplicated genes (Colbourne et al., 2011). Notably, many genes associated with virulence and immune induction for *T. gondii*, including SAG and ROP genes, are located in the genome as tandem arrays, presumably the result of gene duplication events (Jung et al., 2004; Peixoto et al., 2010). Moreover, the phenotypic effects of at least a one of these genes (Khan et al., 2009) has been shown to be highly dependent on expression levels. Generation of gene copy number variation is a major mechanism by which both gene conversion and alterations in ploidy affect genetic diversity and increases or decreases in copy number via these processes have been linked directly to expression and phenotypic level changes (Pavelka et al., 2010). Therefore, since it is possible that they could play an important role in altering the virulence phenotype of apparently clonal strains, it is pertinent that these mechanisms of genetic diversification that are undetectable with the genetic analyses presented in this study be addressed in future studies. Fortunately, the feasibility of whole genome level analyses for multiple strains is now a reality, making answers to these questions an obtainable goal.

Despite the potential caveats of inherent limitations in the data, one conclusion that will likely remain valid is that attesting to the importance of the definitive host stage as a potentiator of transmission and disease for these parasites. Many studies, in addition to those discussed in Chapter IV (see above), have supported this conclusion as well (Arenas et al., 2010; Mateus-Pinilla et al., 2002). This suggests that the most potent solution to disease may actually be highly practical and immediately applicable, at least for *T. gondii*. Evidence provided thus far predicts that institution of mandatory vaccination for cats will lead to major reductions of infection and disease in both human and animal hosts. This strategy has been successfully tested empirically on a local level with an experimental vaccine (Mateus-Pinilla et al., 1999) and time will tell

whether it can be applied regionally, or even globally, and extended to related parasites, such as *S. neurona*. It is critical that such practical, though often under pursued, solutions to stopping disease be given strong consideration for distribution of research efforts and resources.

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VITA

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Master of Science

Thesis: LIFE HISTORY PERSPECTIVES ON PARASITE GENOTYPES, DISEASE OUTBREAKS, AND WILDLIFE HOSTS FOR *TOXOPLASMA GONDII* AND *SARCOCYSTIS NEURONA*

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Title of Study: LIFE HISTORY PERSPECTIVES ON PARASITE GENOTYPES, DISEASE OUTBREAKS, AND WILDLIFE HOSTS FOR *TOXOPLASMA GONDII* AND *SARCOCYSTIS NEURONA*

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Major Field: Veterinary Biomedical Science

Scope and Method of Study:

The objectives of this study were to generate and analyze molecular genotyping data for the parasites, *Sarcocystis neurona* and *Toxoplasma gondii*, in an effort to: 1. Define the spectrum of parasite genotypes infecting wildlife hosts, 2. Identify evidence for genetic exchange (recombination) among parasite isolates, 3. Identify parasite genotypes responsible for disease outbreaks, and 4. Describe a causal link between parasite life history events and outbreak occurrence. Methods applied include evidence synthesis of previously published parasite molecular genotyping data and development and application of new parasite typing schemes to parasite genetic material recovered from outbreak events.

Findings and Conclusions:

Polymerase chain reaction and DNA sequencing based typing schemes were successfully developed for *Sarcocystis neurona* and *Toxoplasma gondii*. Findings revealed a diverse parasite gene pool circulating among wildlife hosts with evidence of genetic recombination among strains. Genetic typing of strains associated with disease outbreaks of both *T. gondii* and *S. neurona* revealed clonal outbreaks linked to water contamination by definitive host fecal shedding following the parasite sexual life cycle stage. Furthermore, the *T. gondii* outbreak clone was apparently a recently emerged recombinant strain. This suggests that the parasite sexual stage potentiates disease outbreaks by serving the dual roles of emerging new lines through recombination and rapidly amplifying and transmitting virulent strains through self-mating and fecal shedding. These findings indicate the definitive host stage is an essential target for disease prevention strategies.

ADVISER'S APPROVAL: Dr. Michael E. Grigg