Toxoplasma gondii: Antibody Prevalence and Risk Factors in CU Boulder Students

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Abstract

Worldwide parasite prevalence presents a considerable burden to human health and prosperity. As such, human parasitology is a pertinent research area to promote global public health. *Toxoplasma gondii*, a human parasite of public health concern, is present in many populations in most countries, including the United States. During the winter of 2017, we collected salivary samples and survey data from 400 undergraduate student volunteers at the University of Colorado Boulder to investigate: 1) if human saliva examination could be used as a valid method for determining *T. gondii* antibody presence; 2) the prevalence of *T. gondii* infection within the tested population; and 3) if any behavioral factors such as dietary preferences, water source and hygiene (environmental factors), or cat affinity (as reported by surveys) could be used to predict *T. gondii* infection exposure. The association between behavioral variables and *T. gondii* antibody presence was determined by chi-squared analysis and the creation of three generalized linear models (GLMs) that outlined the major known infection routes. Our results indicated that salivary examination for *T. gondii* antibodies using an enzyme-linked immunosorbent assay test was an accurate and non-invasive method of detecting *T. gondii* exposure (sensitivity = 94.2%, specificity = 100%). We saw an antibody prevalence of 27% in our sample population, which is considerably higher than the national average of 11% as reported by the Centers for Disease Control and Prevention. Chi-squared analysis showed a significant association between field of study and *T. gondii* antibody presence ($P = 0.0482$). Among the GLMs, we found that dietary choice best predicted *T. gondii* antibody presence. These results support salivary examination as a valid method of testing *T. gondii* seroprevalence, field of study is significantly related to antibody presence, and suggest that variation in diet is likely an important exposure pathway, despite the traditional focus on cat-related variables.

Introduction

National public health has become an increasingly important field of study, especially concerning parasites, over the past few decades as research has progressed and outbreaks have become better documented. Parasites, or organisms who must inhabit hosts to survive, have come to the forefront of both national and global attention as relief efforts for high profile parasites such as malaria and tropical helminths (worms) have become increasingly population. Parasites such as malaria capture global attention with astounding infection statistics and increasing numbers of outbreaks reported by the World Health Organization (WHO). For example, the WHO reported 216 million cases of malaria in 2016, 90% of which were concentrated in Africa (WHO 2017). When people living in the United States think of parasitic infections, they normally think of infections such as malaria that occur primarily outside of the country. While, the United States population tends to dissociate itself from parasites, according to the Centers for Disease Control and Prevention (CDC) parasites infect tens of millions of Americans each year (CDC: Parasites – Neglected Parasitic Infections 2017). Human parasites are commonly spread through contaminated water or soil, consuming undercooked meats, or contact with other contaminated individuals (Anderson et al. 1992; Bowie et al. 1997). With the recent rise of parasite infections in the United States, the CDC describes five "Neglected Parasitic Infections" that are targets for increased awareness measures and public health action. Among these parasites, *Toxoplasma gondii*, a protozoan parasite, exhibits the highest infection rate at over 60 million new cases per year (CDC: Neglected Parasitic Infections 2017).

Toxoplasmosis, caused by the parasite, *T. gondii* (CDC: Parasites – Toxoplasmosis 2017) is one of the most prevalent parasitic infections in the United States. *Toxoplasma gondii* is the leading cause of foodborne illness and death in the United States, and as such, it is an extremely

pertinent topic to study (CDC: Parasites – Toxoplasmosis 2017). *Toxoplasma gondii* infection is typically diagnosed by blood analysis, or in severe or hard to characterize cases, through tissue biopsy (Jones 2001; Hill and Dubey 2002; Montoya 2002; CDC: Dubey 2016; Parasites – Toxoplasmosis 2017). An otherwise healthy individual infected with *T. gondii* may experience mild flu-like symptoms and general malaise, whereas immunocompromised individuals (those with an autoimmune disorder, chronic stress or malnutrition) may experience severe consequences such as eye disease, encephalitis or epilepsy (Hofman et al. 1993; Montoya et al. 1997; Dubey and Jones 2008; Dubey 2016). Along with serious complications for immunocompromised people, *T. gondii* infection becomes particularly dangerous when a mother contracts the parasite while pregnant; in this case, *T. gondii* infection can lead to severe disease in the fetus, such as decreased cognitive development, microcephaly (disproportionately small head size), hydrocephalous (excess fluid in the brain) and even preterm abortion (Desmonts and Couvreur 1974; Wong and Remington 1994; Kravetz and Federman 2005). These two populations, immunocompromised individuals and expecting mothers, are targets for early action and prevention.

It is well established that humans can become infected with *T. gondii* in several ways including ingesting contaminated water or soil, eating undercooked or raw meat, receiving an organ transplant or blood infusion from an infected individual, or by trans-placental transmission from a mother to her fetus (Frenkel and Ruiz 1980; Bowie et al. 1997; Jones et al. 2001; Hill and Dubey 2002; Tenter et al. 2002; Dubey and Jones 2008; Dubey 2016). However, it is not well understood which one of these transmission routes is the most relevant, i.e., which transmission route contributes most greatly to *T. gondii* infection in the United States. More information is needed on *T. gondii* presence in an individual based on their common habits and behavioral

decisions. Our study was created to fill in this gap in the literature and to advance a novel method of diagnosing *T. gondii* infection. If we know the major risk factor for *T. gondii* infection in the United States and also have a non-invasive way of diagnosing exposure, we could move towards better prevention methods. Specifically, our study focused on examining: 1) whether human saliva examination could be used as a valid method for determining *T. gondii* antibody presence; 2) the prevalence of *T. gondii* infection within the tested population; and 3) if any behavioral factors such as water source, dietary preferences and cat affinity (as reported by surveys) could be used to predict *T. gondii* infection exposure.

To complete these three objectives, subjects at the University of Colorado Boulder volunteered to be tested for *T. gondii* presence and fill out a behavioral survey. Each volunteer gave a saliva sample that was tested for specific antibodies to determine *T. gondii* presence. In addition, each saliva sample was correlated with the participant's survey results, allowing us to correlate infection presence with certain behavioral choices. This information, along with the validation of a salivary testing method and prevalence statistics for our specific population, can be used on a broader public health scale to show what factors put an individual at higher risk for *T. gondii* infection.

Background

Toxoplasma gondii, a highly successful protozoan parasite, has the ability to infect more than 100 endothermic hosts (Tenter et al. 2000; Dubey 2016). *Toxoplasma gondii* has been found all over the world, with serological prevalence (percent of infected individuals in a population) ranging from 4% (n= 899) in Korea to 92% (n= 32,512) in Brazil (Dubey 2016). Although rates of *T. gondii* human infection tend to be higher in equatorial and developing countries (Dubey 2016), it is also relevant within the United States as mentioned above. In 2016,

the CDC estimated that 11% of the US population six years or older (over 35 million people) have been infected with *T. gondii* (CDC: Parasites – Toxoplasmosis 2017). Adaptations such as non-specificity in selection of an intermediate host and the fact that a host remains infected for life, make *T. gondii* one of the most successful parasitic species (Dubey 2016). Any endothermic animal, from a field mouse to a dog to marine mammals, can theoretically serve as an intermediate host for this parasite.

To better understand the role of this parasite's hosts and why it is so successful, it is essential to look into the complex lifecycle of *T. gondii*. *Toxoplasma gondii* has a heteroxenous life cycle, meaning that it requires two different hosts, an intermediate and a definitive host, to complete a full life cycle (Hill and Dubey 2002; Tenter et al. 2000; Dubey 2016). In general, an intermediate host supports asexual reproduction of the parasite and is used by the immature, or larval parasite, whereas the definitive host is used for sexual reproduction and maturation of the parasite (Smyth and Wakelin 1994). Specifically, the definitive host of *T. gondii* can be any member of the cat family (Felidae) which includes domestic and wild cats (Dubey 1998; Tenter et al. 2000; Hill and Dubey 2002; Dubey 2016). An intermediate host (such as a human) supports two asexual phases of *T. gondii* reproduction (Dubey 1998; Tenter et al. 2000; Dubey 2016).

Toxoplasma gondii belongs to Coccidia, a subclass of spore-forming obligate parasites with similar life cycles (Sleigh 1991; Dubey 2016). First, tachyzoites, (a non-infective stage in the development of the tissue stage of coccidial infections like *T. gondii*) multiply rapidly by endodyogeny, or the formation of two daughter cells within the mother cell (Hu et al. 2002, Tenter et al. 2002). The final generation of tachyzoites induces a second, slower form of maturation which results in the formation of tissue cysts. Within these infective tissue cysts, bradyzoites multiply by endodyogeny (Dubey 1998; Tenter et al. 2000; Dubey 2016). These

tissue cysts have a higher affinity for neural and muscular tissue within their hosts (Dubey 2016). If the tissue cyst is ingested by a definitive host (feline), the bradyzoite will initiate another phase of asexual multiplication in the new host's small intestine (Tenter et al. 2000). The last few steps of this phase initiate the sexual phase of the life cycle. Gamogony (formation of reproductive cells) and oocyst (egg cell) formation occur in the epithelial, or outer layer of, tissue in the small intestine (Dubey 1998; Tenter et al. 2000; Hill and Dubey 2002;). Oocysts can survive outside of the definitive host's body, unlike the other stages of the life cycle. Unsporulated (meaning these cells do not have infectious spores) oocysts are released into the lumen of the small intestine where they are passed into the environment through fecal matter (Tenter et al. 2000; Hill and Dubey 2002; Dubey 2016). Sporogony, formation of parasitic spores, occurs outside of the definitive host and leads to development of infectious oocysts. In summary, two of the three infectious stages in the life cycle, tachyzoites and bradyzoites within the tissue cysts, are not host specific and thus, the parasite can use any warm-blooded animal as an intermediate host (Tenter et al. 2000; Dubey 2016).

Humans could possibly have daily interactions with other intermediate hosts through consuming meat in their diet which poses a risk of infection through tissue cyst ingestion (Dubey and Jones 2008; Dubey 2016). For instance, livestock such as cattle, goats, sheep, pigs and poultry have all been documented as intermediate hosts for *T. gondii* (Dubey and Jones 2008; Herrero et al. 2016; Herrero et al. 2017; Tenter et al. 2000). Although instances of *T. gondii* exposure have been found in all of these animals, significant outbreaks of *T. gondii* have occurred only after consuming undercooked pork or lamb (Dubey and Jones 2008; Dubey 2010; Herrero et al. 2016; Herrero et al. 2017). Out of these two types of meat, pork is extremely common in the diets of Americans and it should be noted that this may present a significant

pathway for *T. gondii* infection in our study population. For example, Herrero et al. (2016) studied the seroprevalence of *T. gondii* antibodies in 1200 pigs in northern Spain. They found that 25% of pigs tested positive for *T. gondii* antibodies and that seropositive pigs were found at 97% of the farms studied (Herrero et al. 2016). In the United States, Dubey et al. (2005) surveyed commercially available meat (beef, chicken and pork) for the presence of *T. gondii*. They determined that both beef and chicken were free from *T. gondii* infection, but seven pork samples were positive for *T. gondii* presence (Dubey et al. 2005). These studies demonstrate that consuming undercooked meat presents a pertinent risk factor for human *T. gondii* infection.

In addition to pork, common wild game such as white-tailed deer, reindeer and bears, have all been found to have epidemiologically significant amounts of *T. gondii* infection (Dubey 2016; Dubey et al. 2014; Vanek et al. 1996). For example, Vanek et al. (1996) tested sera from over 1300 white-tailed deer from four hunting seasons. They determined that the prevalence of *T. gondii* was 13% in fawns, 26% in yearlings and 41% in adults in their sample population (Vanek et al. 1996). Studies such as this demonstrate that hunting and eating wild-caught meat might also represent a pertinent risk factor to human *T. gondii* infection.

Another possible infection route is ingestion of oocysts from the environment due to contaminated soil or water (Bowie et al. 1997; Bahia-Oliveira et al. 2003; Jones et al. 2001; Palanisamy et al. 2006). The *T. gondii* life cycle indicates that infectious oocysts are deposited in the environment by contaminated members of the family Felidae (Dubey 2016; Hill and Dubey 2002; Tenter et al. 2000), creating the potential for soil and water sources to be contaminated also. For example, Bowie et al. (1997) studied a significant *T. gondii* outbreak that occurred in Canada in 1995 where they determined that all of the infection cases were consistent with a waterborne source of infection and that unfiltered municipal drinking water was the likely source

of the outbreak. Waterborne outbreaks have also been reported in other countries such as Brazil and India (Bahia-Oliveira et al. 2003; Palanisamy et al. 2006). Jones et al. (2001) found that the risk for *T. gondii* infection increased for those who worked in soil-related occupations such as farming. Furthermore, hygienic measures such as washing hands and food properly have been linked to lower *T. gondii* infection risk (Frenkel and Dubey 1972; Hughes et al. 2000). These studies demonstrate that environmental exposure routes are important to consider when determining the likely risk factors of *T. gondii* infection.

Oocyst infection and resulting *T. gondii* infection has also been linked to cat interactions (Frenkel 1980; Dubey 1998; Kapperud et al. 1996; Hill and Dubey 2002). For instance, Frenkel et al. (1980) investigated the antibody prevalence of *T. gondii* in 883 Costa Ricans and found that participants who reported contact with cats had significantly higher *T. gondii* antibody prevalence than those without contact ($P = 0.02$). Their study suggested that cat contact did play a significant role in whether or not an individual was infected with *T. gondii*. Furthermore, popular media sources such as Live Science (https://www.livescience.com/56529-strange-facts-about-toxoplasma-gondii-parasite.html), the Atlantic (https://www.theatlantic.com/magazine/archive/2012/03/how-your-cat-is-making-you-crazy/308873/) and Medical Daily (http://www.medicaldaily.com/crazy-cat-lady-toxoplasmosis-parasitic-infection-brain-and-behavior-390436), among others, refer to *T. gondii* by its connection to cats. As cats are the only definitive host of *T. gondii*, it is important to look at an individual's cat affinity to see if that has any effect on their *T. gondii* infection status; however, understanding the complexity of the life cycle demonstrates that cat connections should not be the only human transmission route studied.

Humans potentially infected with *T. gondii* are diagnosed serologically (blood) or by biopsy (tissue) and subsequent microscopic examination of the tissue for parasites (Hofman et al. 1993; CDC: Parasites – Toxoplasmosis 2017). In general, serological methods are greatly preferred due to their less-invasive nature and accuracy (CDC: Parasites – Toxoplasmosis 2017). Although most serological tests require serum (blood without clotting factors) as the testing medium, it remains unclear if other bodily fluids, such as saliva, which also contains antibodies after infection, could also be used. To date, numerous serological tests have been developed to detect *T. gondii* antibodies in humans, including Sabin-Feldman dye tests, the indirect fluorescent antibody assay (IFA), the latex agglutination test (LAT), the immunosorbent agglutination assay test (IAAT) and the enzyme-linked immunosorbent assay (ELISA; Hill and Dubey, 2002; Montoya 2002). ELISA tests have been used most often and with the great success in identifying presence of *T. gondii* (Voller et al. 1978; Montoya 2002; Sampaio et al. 2014) and, therefore, we selected an ELISA method to determine prevalence in our study population.

It is important to note that ELISA also allows for detection of multiple antibodies such as Immunoglobulin G (IgG) and Immunoglobulin M (IgM) (Hill and Dubey 2002; Montoya 2002; Dubey 2016). IgG is the smallest and most common antibody in humans, making up about 75-80% of total antibodies possessed by an individual, while IgM is found mainly in blood and lymphatic fluid (Fahey 1965). IgM appears sooner after infection and can be used to identify the exact time of infection (acute stages showing symptoms), while IgG antibodies will persist for life and provide a way to test lifetime exposure (Partanen et al. 1984; Hill and Dubey 2002; Montoya 2002). IgG antibodies typically appear one to two weeks after an individual is exposed to *T. gondii*, peak at around one to two months after exposure, and will remain present for the life of the intermediate host, in this case a human (Montoya 2002). For testing whether or not an

individual has ever been exposed to *T. gondii*, IgG is the more effective choice since it remains present for life and we are not interested in studying acute infections presently. In addition, IgG can be detected from saliva since it is present in all bodily fluids, whereas IgM is present only in blood and lymphatic fluids. IgM has been detected in saliva, but the odds of it being detected in our sample size were very low (Sampaio et al. 2014). Furthermore, asking volunteers for an easy-to-obtain saliva sample instead of a blood sample increases the number of participants.

Our laboratory developed an in-house ELISA method to detect anti-*T. gondii* antibodies in human saliva. Heterogeneous (a class of assay tests that use a solid phase) ELISA systems can be described in three general steps: 1) a reactant (e.g., the antigen) is attached to a solid phase (e.g., a plastic microtiter plate); 2) the bound reagent and free reagents (added in additional steps to the substance attached to the solid phase) are separated by a simple washing step; and 3) development of color indicates results. ELISA was chosen because it is a relatively inexpensive method of detecting antibodies in saliva. In addition, although blood is typically used for *T. gondii* infection diagnosis, saliva presents a promising alternative that has not been well tested (Sampaio et al. 2014). Our study aims to further test, optimizine and validate this method.

Although toxoplasmosis is relatively uncommon compared with the prevalence of latent infection, it presents real and dangerous complications to a significant subset of the population. Clinical disease is normally restricted to fetuses and immunocompromised individuals (Tenter et al. 2002), while most healthy individuals exposed to *T. gondii* develop latent infections, meaning that the parasite is living dormant in their cells and not causing active disease (Kravetz and Federman 2005). Once infected with *T. gondii*, an intermediate host will typically stay infected for life, although most of the time the infection will stay asymptomatic. However, the proportion of immunocompromised and pregnant individuals should not be overlooked - they represent a

significant subset of the population. The term "immunocompromised" encompasses any individual whose immune system is impaired or weakened, making them unable to fight off infection. Individuals who are immunocompromised include those who have human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), are undergoing cancer treatment, are recovering from an organ transplant or serious illness, those who are malnourished, and those who experience severe or chronic stress. In these individuals, a latent *T. gondii* infection can become reactivated and lead to severe complications (Dubey and Jones 2008; Toxoplasmosis - CDC 2017). In particular, HIV-positive individuals who become infected with *T. gondii* may experience encephalitis (swelling of the brain), eye disease, or epilepsy (Hofman et al. 1993; Toxoplasmosis – CDC 2017). Similar to immunocompromised individuals, pregnant women and their fetuses are also at a higher risk for deadly toxoplasmosis complications (Desmonts and Couvreur, 1974; Wong and Remington 1994). If a woman becomes infected with *T. gondii* while she is pregnant, the parasite can be passed trans-placentally to her fetus, which could lead to preterm abortion or severe illness in the baby including epilepsy, hydrocephalus, microcephaly and cognitive developmental issues (Desmonts and Couvreur 1974; Wong and Remington 1994; Kravetz and Federman 2005; Dubey and Jones 2008; CDC: Parasites – Toxoplasmosis; Toxoplasmosis – CDC 2017). Since many individuals in the United States and around the world fall into the categories of pregnant or immunocompromised, *T. gondii* represents a pertinent public health concern and warrants more research. Also, from a global public health standpoint, it is extremely important to gain more knowledge on risk factors of *T. gondii* infection because morbidity (ill-health or disability) due to the parasite has the potential to impose severe economic consequences (Hill and Dubey 2002).

In addition to various complications that can occur with active toxoplasmosis, ongoing research studies the possible behavioral effects linked to latent infection, including increased rates of car accidents, higher grade-point-averages, and a general increase in risk-taking behaviors (Berdoy et al. 2000; Flegr et al. 2002; Webster et al. 2007; Yolken et al., 2009; Prandovsky et al. 2011; Torrey et al. 2012; Stock et al. 2017). These "risky behaviors" have been mostly studied in rodents and indicate that *T. gondii* infection correlates with modified neurotransmitter signaling, which can in turn influence a host's behavior. For example, Prandovsky et al. (2011) showed that *T. gondii* causes a significant increase in dopamine in neural cells in mice, which can lead to distinct behavioral changes that increase the parasite's probability of transmission to an uninfected host. Increases in risky behavior can also be seen in results of a study by Berdoy et al. (2000) on rats and their natural aversion to any sign of cat presence. These authors showed that infection with *T. gondii* alters the rat's perception of predation risk and can even go as far as to turn it into lethal attraction. In this case, the rats' natural aversion to the smell of cat urine was reversed due to infection with *T. gondii* (Berdoy et al. 2000). Evolutionarily, these behavioral effects are based on the manipulation hypothesis (Berdoy et al, 2000; Webster, 2007), which states that parasites specifically affect the behavior of their host to increase the probability that they are transmitted to an uninfected host. In the rat example above, *T. gondii* changes the behavior of its intermediate host to increase the likelihood of transmission and proliferation since felines are the only definitive host.

Studies involving humans infected with latent *T. gondii* infection have documented interesting outcomes as well. For example, Stock et al. (2017) demonstrated that dopamine caused a *T. gondii* positive individual to show superior performance in cognitive control situations, but decreased motivation for rewards when compared to negative subjects. This

finding suggests that individuals infected with latent *T. gondii* may have higher GPAs on average, although further research is needed. In addition to increased cognitive control, *T. gondii* infection has also been associated with increased risk of traffic accidents (Flegr et al. 2002). It is suggested that individuals with latent *T. gondii* infection have a 2.65 times higher risk of car accidents (Flegr et al. 2002). Furthermore, latent *T. gondii* has been linked to severe mental illness, such as schizophrenia, since individuals with schizophrenia had an increased prevalence of *T. gondii* specific antibodies when compared to healthy individuals (Yolken et al., 2009; Torrey et al. 2012). Although latent *T. gondii* infections do not cause active toxoplasmosis, they are correlated with behavioral changes that could be detrimental to human hosts.

Using the *T. gondii* life cycle as described above, there are three major routes through which an intermediate host, such as a human, may acquire *T. gondii* parasites. First, an individual can acquire the parasite through oral ingestion of infectious oocysts from the environment (horizontal transmission). Next, the parasite can be acquired horizontally through oral ingestion of tissue cysts contained in raw or undercooked meat. Finally, *T. gondii* can be transmitted from mother to her fetus through the placenta (vertical transmission). In the latter case, tachyzoites are passed to the fetus. For the two horizontal routes of transmission, specific risk factors such as cat ownership, water source and dietary preferences, can be accessed.

Knowledge of the multiple infection routes in humans suggests there are potentially common characteristics that could lead to increased risk of having been exposed to *T. gondii*. Here, through the use of a saliva sample and an associated survey, we tested potential risk factors that may increase exposure to *T. gondii* in undergraduate students at the University of Colorado Boulder. The survey was developed in the Leeds School of Business at the University of Colorado Boulder to assess behavioral and personality factors potentially affected by *T. gondii*

infection and how these factors might influence entrepreneurship-related activities. The survey asks for subject information including general demographics and habits (e.g., age, sex, race, zip code, drinking habits, etc.), participation in risky behaviors (e.g., drinking heavily at a social function or riding a motorcycle without a helmet), personality questions and hygiene questions. To best examine our hypotheses that cat ownership, environmental factors (such as drinking water source, camping habits and traveling exposure) and dietary preferences impact the exposure rate to *T. gondii*, we added new questions to the survey for 2017. Specifically, our questions addressed whether, 1) recreational activity (such as camping, hunting, or traveling); 2) nutritional habits (such as water source, vegetarianism and consumption of wild caught meat); and/or 3) life history (such as demographic information and affinity for cats) predict exposure to *T. gondii*. Although the population used in this study did not self-identify as malnourished or immunocompromised, their infection data is still valid and can give us an idea of the major risk factors associated with acquiring a *T. gondii* infection. These data can then be used to develop preventative measures to protect people in other locations against infection.

Methods

I. Questionnaire and saliva collection methods

We created a questionnaire to investigate potential links between students' demographic or behavioral traits and their *T. gondii* antibody status (present or absent). Demographic questions included age, sex, major field of study, and participants' childhood zip code, while behavioral questions were split into three different categories: risk taking behaviors, personality traits, and possible exposure routes (transmission). The survey was created in collaboration with researchers from the Leeds School of Business at the University of Colorado Boulder. We included questions that pertained specifically to the *T. gondii* life cycle to explore potential

exposure pathways, including cat affinity, environmental exposure and dietary decisions. To assess cat affinity, we included questions such as the number of outdoor cats owned in participants' lifetimes, how allergic they are to cats on a scale of 1 to 7 and how likely they were to pet a cat. For environmental exposure questions, we included questions trying to discern water source, contact with farm animals and general hygiene. For dietary choice exposure, we asked about the amount of time participants have spent as a vegetarian, the number of times they eat meat per week and how likely they are to eat wild-caught meat. Each transmission question was developed from historical research described in the "Background Research" section and thus, the questions capture the three major risk factors of infection.

On January 31, 2017, we sampled 404 students from two sections of General Biology II (EBIO 1220) who voluntarily tested for the salivary presence of *T. gondii* antibodies. We instructed volunteers not to eat, drink or chew gum upon entering the classroom to assist in preventing contamination of the saliva. We provided each volunteer student a uniquely labelled salivation collection vial (SARSTEDT Salivette™). In addition, we provided each student with a survey and in which we asked them to inscribe their unique vial code at the top of their survey. This allowed us to correlate answers on the survey with the *T. gondii* prevalence results. We instructed subjects to chew on the cotton swab located within the Salivette™ for 120 seconds and to complete the survey. Survey numbers and vial number were later linked and any duplicate numbers were discarded. For example, if two different participants filled out two different surveys but both indicated that they had vial H221, we discarded both surveys from the sample. Collected saliva samples were kept on ice until they were centrifuged (Fisher Healthcare Horizon Model 642E Centrifuge) at 2500 rpm for 15 minutes. After centrifugation, we discarded the

sponge leaving the participant's saliva in the base of the vial which was then stored in a standard freezer at -20°C until further analysis.

II. Antibody detection

We created an indirect competition enzyme-linked immunosorbent assay (ELISA) test in-house following a modified version of the protocols described in Voller et al. (1978). We performed the ELISA on two plates at a time over a period of two days, thus all the calculations of reagents below are made for two 96-well plates. On day one of the two-day ELISA process, we created a *T. gondii* antigen mixture using 800 µL of *T. gondii* antigen (Merdian Life Science EV8131) and 39.2 mL of carbonate buffer solution (Fisher Scientific PI-28382). We used this neutral phosphate - buffered saline (PBS) because it does not contain proteins that would compete with the antigen for attachment to the plastic plate (the solid phase). We added 200 µL of the antigen mixture to each well of a sterile 96-well microplate (Fisher 07-200-642) and then put the plates in an incubator (ThermoScientific HeraTherm Incubator) at 4°C overnight to allow the proteins in the antigen to passively attach to the plastic.

Twenty-four hours later, we removed saliva samples from the freezer and placed them on the rocker (Fisher Scientific 05-450-213) at 24 rpm to bring them to room temperature. Next, we removed excess antigen by washing each well three times with PBS Tween (PBS-T; Fisher: 50-674-79). Washing is important because it separates reacted (bound) substances from unreacted (free) substances in the ELISA. Specifically, we added 200 µL of PBS-T to each well using a multi-pipette, placed the plate on the rocker at 24 rpm for three minutes, and carefully inverted the plate to expel the solution into a sink. We repeated this process three times for each washing step. Once the plates were washed, we blocked the wells using a solution of 2% bovine serum albumin (BSA) (2g BSA/100 mL PBS-T) and placed them into the incubator at 37°C for one

hour. Using a blocking buffer prevents attachment of nonspecific proteins to the solid phase, and thereby achieves specific binding of salivary proteins in the next step.

After incubation, we washed the plate using the procedure described above and added 100 μL of undiluted saliva to each well using a sterile pipette tip for each subject. We added saliva from a specific individual to two wells to achieve repetition. In addition, each plate also contained five wells of positive controls (Accurun 135; 1:100), five negative saliva controls, and five blank wells. Of the five positive controls, three were saliva from individuals who had been sera-verified as *T. gondii* antibody positive, while the other two were verified-positive serum samples from a manufacturer (Meridian Life Sciences). Positive and negative saliva controls were obtained from individuals whose infection status was also confirmed by sera through a quantitative indirect competitive immunoenzymatic assay completed in-house (Immuno-Biological Laboratory; Hamburg, Germany) as well as a reference laboratory (Quest Diagnostics) using a sandwich competitive ELISA. Blank wells were used to baseline-correct the optical density on each different plate.

We incubated and washed plates and then added 200 μL of horseradish peroxidase-labelled anti-human IgG (Jackson ImmunoResearch Laboratories, INC 109-035-098) to each well (80 μL anti-human to 39 mL and 920 μL of PBS-T). Since we used an indirect ELISA method, the antigen is attached to the solid phase and is then targeted by added antibodies. These added antibodies are targeted by enzyme-linked antibodies produced against immunoglobulins. Specifically, we wanted to detect whether antibodies for *T. gondii* were produced in human saliva using the enzyme-linked antibodies "anti-human IgG." After incubation at 37°C for one hour and washing, we added 200 μL of tetramethyl-benzidine (TMB) membrane peroxidase substrate (Rockland TMBM- 100) to each well. We stored plates at room temperature for 15

minutes on the counter in the dark. This stage in our reaction allows a color change to develop through enzymatic catalysis. TMB was specifically chosen for its suitability to enzymes linked to the anti-human IgG and because it was used in our model study (Voller et al. 1978). We stopped the reaction by altering the pH and adding 50 µL per well of 1 M sulfuric acid (VWR: BDH7500-1). Within five minutes of stopping the reaction, we used a spectrophotometer (Biotech Synergy HT) to measure optical density (OD) of each sample at 450 nm.

To determine whether a sample was positive or negative for *T. gondii*, we used a two-standard deviation method, similar to the methods described in Johnson et al. (2018). If the optical density of a sample was at least two standard deviations greater than the average value of the negative controls on the corresponding plate, we classified the sample as positive. If not, we classified the sample as negative. For example, if the average OD of the negative samples on a plate was found to be 0.1714 and two standard deviations was calculated to be 0.126, any OD value above 0.300 was classified as positive. It is important to note that each saliva sample was run in duplicate (i.e., two wells were filled with the same individual's saliva on the same plate). For instances in which the two samples generated conflicting results, we classified the subject as 'ambiguous' (i.e., one sample positive and one sample negative) and re-tested two additional samples on another plate. The validity of this two-standard deviation method was evaluated by using it to classify the known positive and negative samples included on each plate. When running a binary classification test, sensitivity and specificity are both measures of the accuracy of the test, with sensitivity measuring the proportion of positives that are identified correctly and specificity measuring the proportion of negatives that are identified correctly (Altman and Bland 1994).

III. Statistical analysis

Prior to conducting analyses, we cleaned up the data set to exclude outliers, missing answers, and questions that were not relevant to *T. gondii* transmission. To determine the final data set, we made histograms (using R) of all the variables of interest to catch outliers visually. If any answer was biologically implausible, we excluded the response from the data set. For example, it is biologically impossible for an individual to eat 360 servings of meat during one week so that response was considered an outlier and excluded from the dataset. On the other hand, we kept an individual who reported ownership of 45 cats throughout their lifetime in the data, as this is possible. Next, we decided which variables to transform using log10 +1 by viewing the histograms of all the data. Each variable was log transformed and the two histograms (the log transformed histogram and the untransformed histogram) were compared. If the log transformed histogram was less skewed and better approximated a normal distribution, we decided to use the log transformed variable in later analyses.

In addition to cleaning up the data, we also ran correlation analyses of predictor variables to check for collinearity using R (R Core team 2013). Eliminating collinear variables reduces standard error within the models. Collinearity was determined to be any correlation coefficient (r) greater than 0.500. Variables such as the number of times an individual went camping in a *year* versus how many times they had been camping in their *life* were determined to be collinear (r = 0.580). Hygiene variables such as "I wash my hands before meals" and "I wash my hands after using public transportation" were also found to be collinear (r = 0.615). In the first case, we chose to use yearly number of camping days as opposed to lifetime number of camping days as we thought that would be a more accurate number. In the second case, we chose "I wash my hands before meals" because this variable could show a direct line of contact from contaminated

water to ingestion, while the other variable does not. Even though not collinear, we also chose to exclude the "how many years have you been a vegetarian" variable in favor of "how many times a week do you eat meat" because they are mutually exclusive and we felt that the amount of times an individual eats meat per week more accurately determines the effect of eating meat on *T. gondii* presence, and there was a high response rate in the surveys for eating meat.

We investigated associations between students' antibody status and their indicated field of study (i.e., their stated major at CU) and their geographic origin as reported by the zip code they spent their childhood in on the questionnaire. Each of the 38 reported majors were classified as "Natural Sciences", "Social Sciences" or "Arts and Humanities" based on the University of Colorado's divisions (https://www.colorado.edu/artsandsciences/student-resources/fields-study/natural-sciences). They were also more broadly classified as "Natural Science" and "Not Natural Science" during a follow-up analysis. For the major analysis, we used 320 samples, as these were the volunteers that had reported their major. We used chi-squared analyses to determine whether there was a significant association between major and *T. gondii* exposure status. In this case, our null hypothesis was that major and *T. gondii* presence were independent. If the test statistic (as determined by using the chisq.test function in R) yielded a P-value of 0.05 or less, it would indicate that the two variables (major and *T. gondii* presence) are significantly associated with each other. For the first test, we investigated majors split into six categories: the three majors with the highest number of representatives (Ecology and Evolutionary Biology, n= 39; Integrated Physiology, n = 80; Psychology, n = 105), along with other natural science majors (n = 62), social science majors (n = 15) and arts and humanities majors (n = 19). For the second, we used just two divisions: natural science majors (n = 286) and other (n =34). To evaluate associations between infection and geographic origin, we used the Federal Government Zip Code

Database (http://federalgovernmentzipcodes.us/) to convert each student's reported zip code into the appropriate city and state. We divided states into one of five geographic regions based on their location. These regions included Central-Mountain (n = 156; CO, MT), Midwest (n = 27; KS, MO, NE, IL, IN, MI, MN, OH, WI), Northeast (n = 39; NJ, NY, MD, PA, VA, CT, MA, NH, RI, VT), South (n = 32; AR, NM, OK, TX, FL) and West (n = 41; AK, ID, OR, WA, AZ, CA). Similar to major, we used Chi-squared (chisq.test command in R) to analyze the relationship between geographic region and *T. gondii* antibody presence. In this case our null hypothesis is that geographic location and *T. gondii* presence are independent of one another.

Finally, we used generalized linear models (GLMs) in R to analyze the effects of cat affinity, environmental factors, and dietary decisions on individual infection status. These specific models were chosen to include variables that captured the major exposure pathways of *T. gondii* as described in the 'Background Research' section of this thesis. For each GLM we included 245 samples as the other 155 samples had missing values for one or more of the included variables. We included sex as a predictor variable in all the models based on previous evidence that infection status can vary between males and females (Walker et al. 1997; Roberts et al. 1995). It is also important to note that these models used a binomial distribution for the response (positive or negative) and a logit-link function. In the cat exposure model, predictor variables included the number of outdoor cats an individual has owned in his/her lifetime, how allergic they are to cats on a scale of 1 (not at all allergic) to 7, and how likely (as indicated by a percentage from 0-100) they are to pet a cat when encountered. Both the outdoor cat and allergic variables were log10- transformed. For the environmental exposure model, variables included: 1) the number of times camping in the year, 2) the frequency on a scale of 1 (almost never) to 7 (almost always) an individual: washes fruits and vegetables before consumption, washes their

hands after using the restroom, washes their hands before meals, washes their hands after touching animals, washes their hands after petting stray animals, and 3) if an individual had frequent contact with farm animals while growing up as measured by a yes or no question. In this model, the only variable that was log10 transformed was yearly camping days. Finally, we created the dietary choice model using predictor variables including how many times in an individual's life they have been hunting and how many times a week that an individual eats meat. Both variables were log transformed. We ran each of these models (cat exposure, environmental exposure, and dietary choice models) with the trimmed dataset (245 samples). In addition, we created an intercept only model (with no predictor variables) as well as a combined, global model (including all the predictor variables) to look at the combined predictive power of all of the variables of interest. We made comparisons among the three models using AIC values (AIC package, R Development Core Team 2013).

Results

To evaluate our salivary testing method, we assessed the method's efficacy in classifying the status (presence or absence of *T. gondii* antibodies) of known positive or negative samples (i.e., control samples). Using the two-standard deviation method described above, our salivary ELISA classified 66 out of 70 known positive samples correctly (94.2% sensitivity) and 70 out of 70 known negatives correctly (100% specificity). All four of the known positive samples that were not classified correctly were sera-confirmed saliva samples as opposed to manufactured controls.

Of the 400 saliva samples collected, 22 were duplicates (students had written incorrect vial numbers on their surveys) and 26 were considered 'ambiguous' even after retesting. Consequently, these 48 samples were removed from the analysis. Among the remaining 352

samples, the prevalence of testing positive for the presence of *T. gondii* antibodies within our study population was 27.6%. Within the data set used, 28.4% of males (33 out of 116) were positive for *T. gondii* infection, while 27.9% of females (64 out of 229) were determined to be positive and seven did not report their gender (Figure 1). One hundred and sixty-nine students (42%) reported Colorado as the state in which they spent the majority of their childhood while the remaining 58% grew up in other states.

Based on an analysis of the relationship between field of study (divided into six categories) and *T. gondii* antibody presence, we detected a significant association between these two variables (X^2 = 11.2, df = 5, P = 0.048; Figure 2). The prevalence of exposure was highest among Ecology and Evolutionary Biology (EBIO) majors (41%), other natural science majors (37%), and Integrated Physiology (IPHY) (28%) majors (Table 1). We saw lower prevalence in arts and humanities (26%) and social science majors (0.0%). Dividing field of study into two categories (natural science majors versus other), there was a marginal association between major and *T. gondii* antibody presence (X^2 = 3.20, df = 1, P = 0.0739; Figure 3). Natural science majors had *T. gondii* antibody prevalence of 31.1%, while other majors showed a prevalence of 14.7%.

We found no significant association between geographic region and *T. gondii* antibody presence (X^2 = 1.02, df = 4, P = 0.908; Figure 4). Overall, the prevalence of exposure was broadly similar among the five regions evaluated. The Midwest region had the highest rate of *T. gondii* antibody presence at 33%, with the South close behind at 31%. The West followed with a *T. gondii* antibody prevalence of 27%, while the Central-Mountain and Northeast regions both had a slightly lower antibody prevalence of 26% (Table 2).

In evaluating the three potential models of *T. gondii* exposure, the dietary choice model had the lowest AIC value (291) while the environmental exposure and cat exposure models had

higher AIC values (both at 295). However, none of the individual predictor variables were statistically significant (Table 3), and the intercept-only model (null model) had an AIC value of 289 (Table 4), suggesting it was comparable to the dietary choice model (models within 2 delta AIC units are often considered equivalent; Symonds and Moussalli 2011; Burnham and Anderson 2002). Including sex as a predictor variable did not significantly change the predictive power of these models. Restricting the geographic region of origin (as identified by participants) to focus only on Colorado also had no appreciable effect on the models.

Discussion

I. Salivary ELISA

Our in-house ELISA method produced accurate results when classifying known positive and known negative samples. Our results for sensitivity and specificity are comparable to a previous study conducted by Johnson et al. (2018) which tested 201 known positive samples and 186 known negative samples to provide an accurate measure of the salivary ELISA method. Johnson et al. (2018) used the same methods as described by the present thesis and found 197 out of 201 known positive samples to be classified correctly using the salivary ELISA method, and 186 out of 186 known negatives to be classified correctly (Johnson et al. 2018). Based on these standard methods, their ELISA method had a 97% sensitivity and a 100% specificity (Johnson et al. 2018). This is similar to the results from our study which showed 94% sensitivity and 100% specificity. The results from our 2017 salivary analysis coupled with the results from Johnson et al. (2018) show that the in-house ELISA method is an accurate way to test for *T. gondii* antibody presence in a non-invasive manner.

Our sensitivity and specificity results confirm that ELISA tests performed on saliva represent an accurate method of diagnosing *T. gondii* antibody presence. This has broader

implications in the public health and health care spheres as it presents a non-invasive way of determining infection. Another benefit of this method is the ease of testing. Instead of getting their blood drawn or a tissue sample taken, patients can be tested for infection just by submitting a simple saliva sample, which makes it easier to test children. In addition, saliva analysis presents less potential for infection or hematoma. In some cases, venipuncture can lead to severe infections including infection by *Staphylococcus aureus*, *Klebsiella, Serratia* and *Psuedomanas aeruginosa* (Nentwich 1990). Our non-invasive technique of submitting a sample by chewing on a sponge, greatly reduced the risk of contracting a potentially life-threatening infection. Furthermore, our method cuts down the cost of testing large numbers of individuals for *T. gondii* presence as it does not require hiring phlebotomists or other trained professionals.

II. Prevalence and risk factors in sample population

Our results indicate that the prevalence of *T. gondii* infection is much higher in University of Colorado Boulder students compared with the national average as reported by the CDC (27.3% versus 11.0%). Dubey and Jones (2008) also analyzed *T. gondii* antibody prevalence in the United States from 1999-2004, specifically on women aged 12-49 years, and Dubey reported a seroprevalence of 10.8% (Dubey and Jones 2008; Dubey 2016). One explanation for the discrepancies between our prevalence data and the national prevalence could be the restricted age range of our sample. When the CDC reported their prevalence statistic, they took into account all individuals ages six and older, while Dubey and Jones considered individuals ages 12-49. Our sample population age ranged from 18-22. It is possible that young children, who have not had many years to become exposed to the parasite, account for a large proportion of the uninfected population. Since the age range in our sample only encompassed young adults, we could be missing the part of the population that is traditionally uninfected, thus

skewing our antibody prevalence percentage and making it seem disproportionally higher than the national average. Another explanation for the fact that CU Boulder students had such a high percentage of antibody-positive individuals could be that, in general, people in Boulder spend more time outdoors, which could lead to higher instances of environmental exposure to *T. gondii*. With access to wilderness and national parks less than an hour's drive away, it is easy to imagine that most CU Boulder students take advantage of this and spend large amounts of time outdoors. Although our environmental exposure model did not have any significant predictor variables, this could be due to the fact that we did not ask the correct questions, or we did not have enough specificity in our questions to accurately gauge the routes of environmental exposure.

In addition to a higher antibody prevalence in CU Boulder students, our results indicate that certain fields of study such as natural science or EBIO, are significantly associated with *T. gondii* antibody presence. Again, this could be due to natural science majors having an increased appreciation for the natural world, and thus spending more time outdoors than their arts and humanities or social science classmates. Here, it seems possible that environmental exposure might play the largest role in prevalence. To support this hypothesis with evidence, the survey questions would need to be revised to see which specific aspects of exposure cause this trend. Our survey questions might have just missed the exact variable that is making prevalence so high in these particular instances.

In terms of the prevalence found by geographic regions, instead of the extreme variation we see between field of study prevalence rates (i.e., 41% in EBIO majors versus 0.0% in social science majors), the region prevalence rates do not vary greatly which could explain why the chi-square analysis fails to reject the null hypothesis that region and *T. gondii* antibody presence are

independent. The sample size in some of the regions was quite small which could be skewing the overall result of the region. For example, the Midwest region had the highest antibody-positive percentage, but the smallest size. If we consider that one of the major draws of Boulder as a college town might be the fact that access to wilderness is extremely easy, we might only be getting 'outdoorsy' people from the Midwest coming here. If that is the case, it could mean that our geographic prevalence percentages are not indicative of the actual prevalence in those regions. For comparison, Jones et al. (2001) did an analysis of *T. gondii* seroprevalence throughout the United States using data from 17,658 individuals. They found prevalence in the Northeast to be the highest (29.2%), with the Midwest (20.5%) and West (17.5%) trailing behind (Jones et al. 2001). This is almost opposite of our findings which show the Northeast with the lowest prevalence (26.0%) and Midwest with the highest prevalence (33,0%) and the West region falling in the middle (27.0%). To get a more accurate representation of the regional variation in *T. gondii* antibody presence in our study, a larger sample from each region would be needed.

Although none of the variables we tested in any of the three models were significantly associated with *T. gondii* antibody presence, the dietary choice exposure model offered the highest predictive powers of the three. This result is interesting because it means that we need to look at dietary choices rather than cat ownership as the major driving factor behind *T. gondii* infection. As mentioned in the "Background" section, much of the popular media portrays the *T. gondii* infection as mostly cat related, often times failing to even mention other possible routes. In the broader context of preventing *T. gondii* prevention, especially in pregnant women and immunocompromised individuals, our results indicate that dietary choices put an individual at the highest risk for infection. While other studies do indicate eating undercooked meat as an

important exposure route, they have not classified it as the most important route, which is what our findings suggest. Moving forward, this information can be used by health care and public health professionals to counsel at risk individuals on how best to avoid infection. Looking at the literature in combination with our study's results, it can be concluded that undercooked meat is an important exposure route of *T. gondii* infection.

It is also important to note, however, that our results could be a reflection of the way in which questions were asked on the survey. It is possible that the main driving variable for *T. gondii* antibody presence in CU Boulder students was missed on the questionnaire even though it contained questions about all of the literature-supported routes of infection. Another possible limitation of our research is the restricted study population. Although our study population (as part of a public university), has student representatives from many states in the United States, it is not the most accurate representation of the United States population as a whole. Therefore, while our analysis of risk factors may be extremely pertinent to students at the University of Colorado Boulder, it may be hard to generalize these results to the greater United States population as a whole.

Moving forward, our results indicate that it is important to further study dietary choices and their impact on *T. gondii* infection. Specifically, preventing human exposure by preventing livestock exposure could potentially help to lower infection rates. In addition, it is important for public health and health care professionals to educate the population on the importance of properly cooking all meat. Our research has presented a novel way for easily testing for *T. gondii* antibody presence which can be used on larger scales to gather prevalence data all around the world. With the prevalence data, researchers can begin to look more deeply into exactly which variables or behaviors are most closely associated with *T. gondii* antibody presence to determine

how best to prevent infection. Rather than focusing on just cats, our research shows that future research should look into dietary choices and how those specifically impact the likelihood an individual is infected with *T. gondii*.

Tables

Table 1: *Toxoplasma gondii* antibody prevalence by field of study (major). Ecology and Evolutionary Biology majors had the highest antibody prevalence with 41% of the sample testing positive, while social science majors had an antibody prevalence of 0.0%.

Major	**Sample size**	***T. gondii* antibody prevalence**
Ecology and Evolutionary Biology	39	0.41
Other Natural Science	62	0.37
Integrated Physiology	80	0.28
Psychology	105	0.27
Arts/Humanities	19	0.26
Social Sciences	15	0

Table 2: *Toxoplasma gondii* antibody prevalence by geographic region. The Midwest had the highest percent of *T. gondii* antibody-positive individuals and the smallest sample size. The Northeast and Central-Mountain regions were tied for the lowest antibody prevalence.

Region	**Sample Size**	***T. gondii* antibody prevalence**
Midwest	27	0.33
South	32	0.31
West	41	0.27
Central-Mountain	156	0.26
Northeast	39	0.26

Table 3: Results from the exposure model GLMs. None of the predictor variables have significant association with *T. gondii* antibody presence.

Model	Estimate (β)	Standard error	Z-value	P-value
Dietary choice exposure				
> log10(# of times eat meat per week +1)	0.549	0.459	1.19	0.232
> log10(# of times gone hunting +1)	0.402	0.469	0.855	0.393
Environmental exposure				
> log10(# of times camping per year +1)	-0.435	0.353	-1.23	0.218
> frequent contact with farm animals (Y/N)	-0.202	0.405	-0.498	0.618
> frequency wash fruits/vegetables before eating	0.128	0.099	1.29	0.196
> frequency wash hands after restroom	-0.057	0.155	-0.368	0.713
> frequency wash hands before meals	0.059	0.109	0.545	0.586
> frequency wash hands after touching animals	-0.062	0.089	-0.689	0.491
> frequency pet stray animals	-0.115	0.085	-1.36	0.173
Cat exposure				
> log10(# outdoor cats in life +1)	-0.435	0.532	-0.818	0.413
> log10(how allergic to cats +1)	-0.256	0.696	-0.368	0.713
> how likely to pet unknown cat	-0.005	0.004	-1.22	0.221

Table 4: Comparison of AIC values between the different models. There is a two AIC value difference between the null (intercept-only) model and the dietary choice exposure model which indicates the models are comparable. The dietary choice exposure model is four AIC values lower than both the environmental exposure and cat exposure models. The global (combined) model had the highest AIC.

Model	**df**	**AIC**	**Δ AIC**
Null	1	289	
Dietary choice exposure	4	291	2
Environmental exposure	9	295	6
Cat exposure	5	295	6
Global	15	303	14

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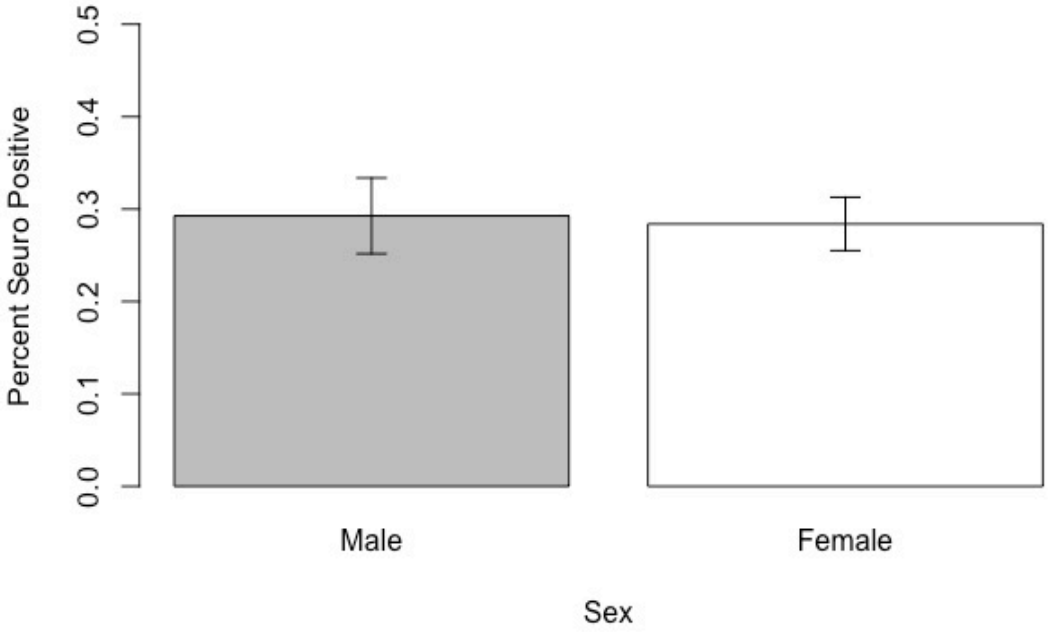

Figure 1: The antibody prevalence of *T. gondii* by sex with bars indicating standard error. Antibody prevalence in males was 28.4% (33 out of 116 individuals were antibody-positive), while in females it was 27.9% (64 out of 229). There was no significant difference in the percentage of antibody-positive individuals between the sexes.

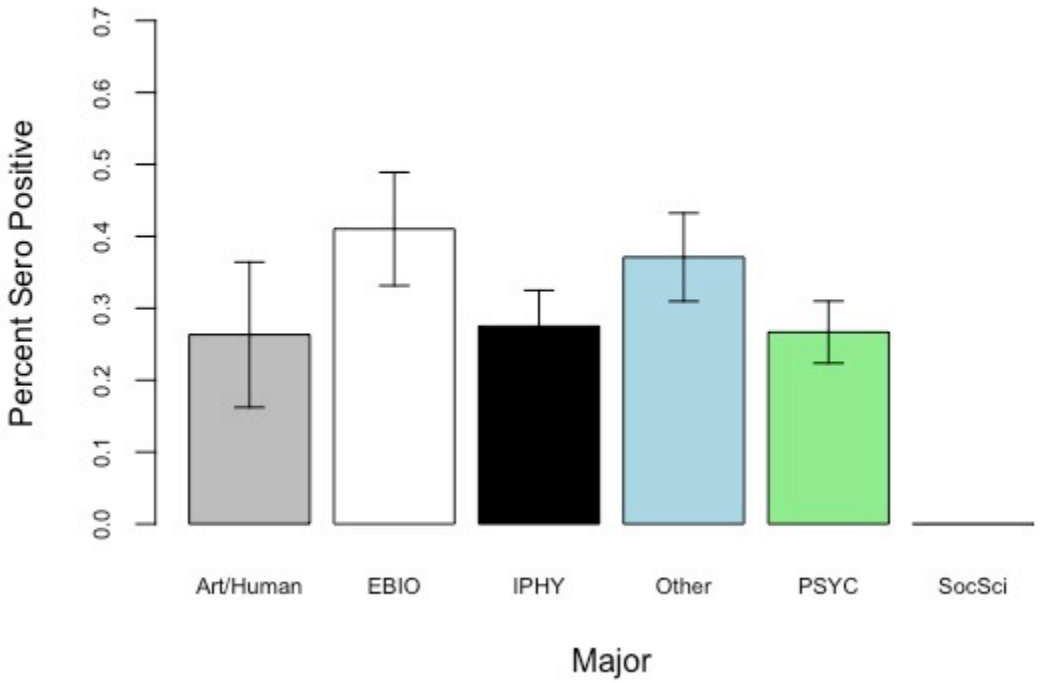

Figure 2: The antibody prevalence of *T. gondii* among different fields of study with bars indicating standard error. Ecology and Evolutionary Biology (EBIO) had the highest percentage of antibody-positive individuals at 41% while social science majors had the lowest at 0.0%. Chi-squared analysis showed a significant association between field of study and *T. gondii* antibody presence (X^2 = 11.2, df = 5, P = 0.0482).

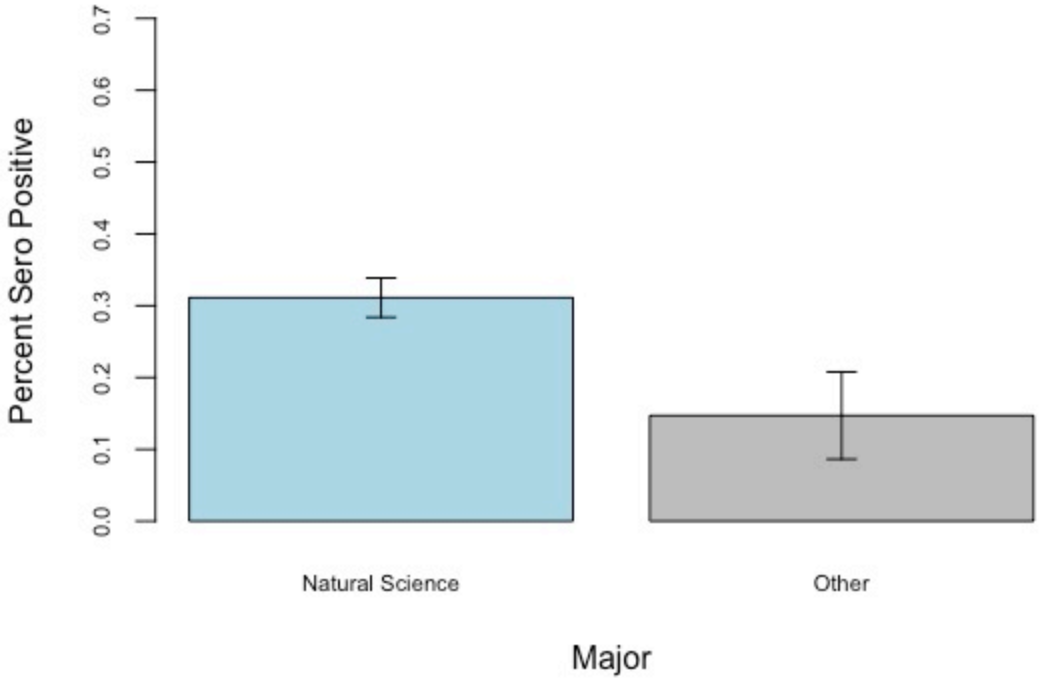

Figure 3: The antibody prevalence of *T. gondii* among natural science majors and other fields of study with bars indicating standard error. Natural science majors had *T. gondii* antibody prevalence of 31%, while other majors showed a prevalence of 15%. Chi-squared analysis showed a marginally significant association between field of study (natural science versus other) and *T. gondii* antibody presence ($X^2 = 3.20$, df = 1, P = 0.0739).

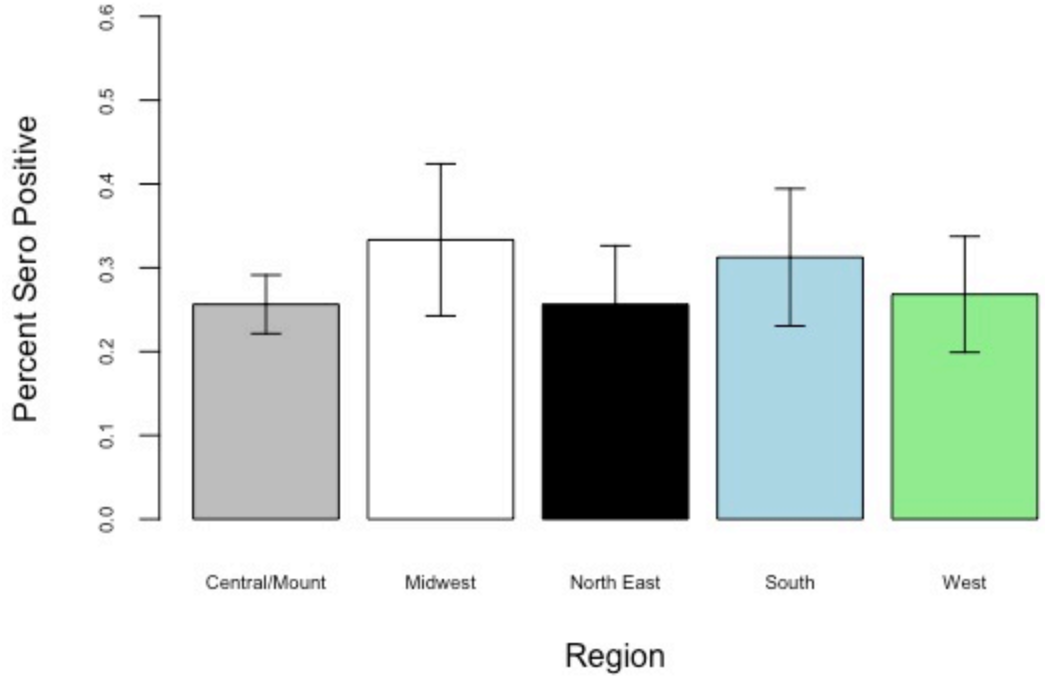

Figure 4: The antibody prevalence of *T. gondii* among the five regions of the United States. Bars indicate standard error. The Midwest had the highest percentage of antibody-positive individuals at 33%, while the Central Mountain and Northeast regions were tied with the lowest percentage (26%). Chi-squared analysis indicated that geographic region and *T. gondii* antibody presence were independent of one another (X^2 = 1.02, df = 4, P = 0.906).

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