HABITAT – HOST – VECTOR INTERACTIONS OF *ORNITHODOROS TURICATA* DUGÈS (IXODIDA: ARGASIDAE) IN TEXAS, USA.

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2017

Major Subject: Entomology

ABSTRACT

Ticks (Class Arachnida: Order Ixodida) are obligatory ectoparasites with diverse vertebrate host groups and vectors of more than 35 human and zoonotic pathogens, which causes untold economic damages. For the past several decades, applications of ecological principles based on tick-host-pathogen interactions have led to the development of effective and efficient tick surveillance and control. However, the progression in tick ecological studies has been largely in Ixodid and not in Argasid ticks. *Ornithodoros turicata* Dugès (Ixodida: Argasidae) is well established as a vector and reservoir of *Borrelia turicatae,* the causative agent of Tick-Borne Relapsing Fever. Furthermore, *O. turicata* is capable of transmitting African swine fever virus, an acute hemorrhagic disease of swine with global implications. Nevertheless, the ecology of *O. turicata* is poorly understood. The studies conducted in this dissertation determined the *O. turicata* habitat-host-vector interactions in TX via field observations at the cave environments, immunoassays of *O. turicata* challenge host sera, and two types of bloodmeal analysis techniques.

The *O. turicata* habitat studies were conducted in 2015-2016 at the caves of Government Canyon State Natural State Area, TX. The study revealed that *O. turicata* are active year around with peak activities in the months of June and August. Moreover, the relative humidity and temperature profiles among four *O. turicata*-active caves in GCSNA remain similar in their values and pattern changes throughout the year. Also, activities of 20 vertebrate animals species, of which only seven were the previously known hosts of *O.*

turicata, were noted. There were no correlations among the *O. turicata* phenology and relative humidity nor animal activities. However, there were significant correlations between *O. turicata* phenology and temperatures throughout the year.

The enzyme-linked immunosorbent assays of *O. turicata* challenged domestic pig sera against *O. turicata* salivary gland extract (SGE) showed a significant increase in the production of immunoglobulin G (IgG) production against SGE as early as three weeks post-challenge. Up to 8,000 fold increase in IgG production in some of the *O. turicata* challenged pigs were observed. Western blot showed post challenge pig sera began reacting with SGE protein(s), size 25kDa, starting three weeks post-challenge.

The bloodmeal analyses of *O. turicata* fed on chicken, goat, and swine blood using a qPCR method showed that *O. turicata* could retain cytochrome b (*cyt*b) genes of hosts beyond 330 days through multiple molting. Also, the qPCR-based bloodmeal analyses could discern ctyb genes of multiple hosts if *O. turicata* had taken bloodmeals from multiple hosts. The bloodmeal analyses based on stable isotopic ratios of Carbon ($\delta^{13}C$) and Nitrogen ($\delta^{15}N$) in *O. turicata* generated unique $\delta^{13}C$ and $\delta^{15}N$ signatures based on the host blood consumed. However, the stable isotope analyses were not able to discern *O. turicata* which acquired multiple host bloodmeals.

DEDICATION

To my God, what an amazing world you have created!

How little do I know of your infinite brilliance...

To my wife and children, you are my motivations and strengths for all that I do…

To Dr. Jimmy Karl Olson, February 18, 1942 – July 2, 2015, Thank You…

ACKNOWLEDGEMENTS

I would like to thank my Lord and Savior Christ Jesus whom all things are possible. I would like to thank my committee members for their supports. Dr. Teel, thank you for the mentorship and guidance over the years. Who would have thought that my phone call as a 14-year-old kid who was just looking for some advice on his high school science fair project would lead us to the path of Ph.D. in Entomology, 23 years later? Dr. Gabe Hamer, I always appreciated your calm presence. Thank you for helping me to have a new perspective and embark on "re-searches" when I encountered challenges. Dr. Sarah Hamer, you are a superwoman! I am so impressed with your dedication and commitment to excel as a professional and a mother (of three children so far…WOW!). Both Gabe and Sarah, you guys are a great team and shining example for many young professional couples to follow. Dr. Lopez, thank you for your generosity in sharing your knowledge and resources. I have learned so much from you. Dr. Tomberlin, I do not know how you manage to be "one of the first" to arrive and the "one of the last" to leave work, exercise daily, be there for your family, and reply e-mails within minutes (even if it was sent to you at 2 am in the morning). I personally think you do not sleep at all! You are a great model to follow for those of us dealing with conflicts between work and family. Thank you for leading by example.

I would also like to thank my team at Dr. Teel's Tick Laboratory and those who have generously offered their assistance during this study. Mr. Otto Strey, thank you for showing me how to raise these blood-sucking creatures. I appreciated your meticulous and

patient teaching methods. Taylor, Samantha, and Brian thank you for the conversations, laughs, and anger. I am going to miss you guys. Dr. Clay Ashely and Ms. Destiny Taylor at the Texas A&M University Veterinary Medical Park, thank you for your support on the pigs during our study. Ms. Lisa Auckland and the team at Drs. Hamer's Laboratory, thank you guys for your patience during our qPCR experiments. I have learned so much from you guys. Brittany, Amy, and Hannah at Dr. Lopez laboratory, I appreciate your patience and sympathy when I was totally lost and confused by the experiment protocols. Dr. Longnecker, I truly appreciate your guidance during our studies. You were the "beacon of hope" when I was lost in the "sea of data".

To current and former members at GCSNA at San Antonio, TX, I thank you for your willingness to help me during our field work. Ms. Nikki Lake, I admired your love for nature, and I hope we can work together again someday. Ms. Kelsey Woodward, your commitment to your job was quite motivating to watch. I wish you the very best in the future. Mr. Kyle McDaniel, you are the "unsung hero" of my work at the GCSNA. I could not have done it without you.

To my friends and families, thank you for your support and endurance during my time at Texas A&M University. My fellow graduate students, thank you for sharing your daily struggles and reminding me that we are all in this together (plus, that we need to get out of here as soon as possible). Liz and Chris, thanks for letting me crash at your place every time I went out to San Antonio to do my fieldwork. I miss you. To my brothers-and-sisters-in-Arms, thank you for "holding down the fort" while I was given the privilege to come back to earn Ph.D. God willing, I will put my utmost efforts to utilize the skill set

learned in the past three years to serve you and your loved ones. To my mentor LTC Anthony (Tony) Schuster, thank you for listening to all my complaints and hear my cries. I know you supported me on many levels (including some “behind the scene stuff”). I pray that I can adequately carry forward your wisdom and kindness to the next generations of Army entomologist. To my lovely wife Mirka, no words can express my gratitude towards your dedications and supports throughout our marriage and my career. I love you more every day. To my brood Aila and Eelis, Appa loves you so much! I thank God every day for gifting mom and dad with the greatest joy in our lives. I pray that one day you can read this and be proud of what Appa has accomplished.

CONTRIBUTORS AND FUNDING SOURCES

Part 1, faculty committee recognition

The research and experiments reported in this dissertation were supervised by a committee consisting of the chair of committee, Dr. Pete D. Teel, Department of Entomology, the members of committee, Dr. Gabriel L. Hamer, Department of Entomology, Dr. Jeffery K. Tomberlin, Department of Entomology, Dr. Sarah A. Hamer, Department of Veterinary Integrative Biosciences, and Dr. Job E. Lopez of Pediatrics-Tropical Medicine, Baylor College of Medicine Houston, TX.

Part 2, student/collaborator contributions

All work for the dissertation was completed by the student, under the advisement of the committee members listed above. Also, statistical data analyses in Chapters 2 and 3 were conducted by the student under the advisement of Dr. Michael Longnecker of the Department of The Statistics at Texas A&M University. The Stable Isotope Analysis in Chapter 3 was conducted by Dr. Brendan Roark and Dr. Christopher Maupin of the Department of The Geography at Texas A&M University. All other work carried out for the dissertation was completed by the student independently.

Funding Source

This project was funded in part by the United States Department of Agriculture, Agricultural Research Service, (USDS-ARS) through Cooperative Agreement Number

59-3094-5-001, by the Texas A&M AgriLife Research, and by the U.S. Army Medical Department (AMEDD) Long Term Health Education and Training for FY 2015-2017. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDS-ARS, Texas A&M AgriLife Research, and AMEDD.

NOMENCLATURE

*cty*b	Cytochrome b gene
CG	Fed on Chicken and Goat
CS	Fed on Chicken and Swine
EC	Exclusively fed on Chicken
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Exclusively fed on Swine
GCSNA	Government Canyon State Natural Area
qPCR	quantitative Polymerase Chain Reaction
SI	Stable Isotope

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Ticks (Class Arachnida: Order Ixodida) are obligatory ectoparasites with diverse vertebrate host groups and vectors of more than 35 human and zoonotic pathogens (Guglielmone et al. 2003, Nicholson et al. 2009). Nearly 900 tick species have been described (Barker and Murrell 2004). They are divided into three families: Ixodidae, Nuttallielidae, and Argasidae (Nava et al. 2009). Morphological features of the gnathosoma and idiosoma can differentiate these families. The family Ixodidae, or hard ticks, have the idiosoma consisting of a heavily sclerotized scutum, or shield, that is often ornate and easily discernable from the integument (i.e., alloscutum). The scutum of male hard ticks covers their entire dorsal plane whereas the scutum of female hard ticks only covers the anterior portion to allow greater expansions of the alloscutum during feeding (Walker 2003). The gnathosoma of all ticks consist of the basis capituli, chelicerae, palps, and hypostome. The gnathosoma of Ixodid ticks is readily visible from both the ventral and dorsal planes in all life stages (Walker 2003, Sonenshine and Roe 2014). The fourth segment of the hard tick palp is reduced and arises from a ventral pit of the third segment. The family Argasidae, or soft ticks, is characterized by the absence of the scutum, and leathery integument covers their entire idiosoma. The anteroventral gnathosoma is not visible dorsally except in the larval stage. Unlike Ixodid ticks, the fourth palpal segments of soft ticks are not reduced and are apically oriented (Walker 2003). The family Nuttallielidae has morphological features resembling both Ixodid and Argasid ticks.

Nuttallielidae possesses a weakly sclerotized scutum in all life stages, which is not readily discernable from the alloscutum. The gnathosoma of Nuttallielidae is visible from both ventral and dorsal planes during all stages and the fourth palpal segment is not reduced and is apically oriented (El Shoura 1990, Mans et al. 2011).

Ixodidae is represented by 13 genera and approximately 650 species (Sonenshine and Roe 2014). Nuttallielidae is represented by a single genus and species *Nuttalliella namaqua* Bedford (Ixodida: Nuttallielidae) (Guglielmone et al. 2010, Mans et al. 2011). In contrast, Argasidae is represented by an estimated 190 species, but the total number of genera is debatable. Over the past five decades, several taxonomic organizations of Argasid genera have been suggested including the Soviet, American, French, and Cladistic systems. Each taxonomic system proposed a different scheme with varying numbers of genera ranging from four to eleven. Both the Soviet and American systems suggested five genera schemes but listed different genus names consisting of *Argas*, *Alveonasus*, *Antricola, Ornithodoros*, *Otobius* (Filippova 1966, Pospelova-Shtrom 1969, Burger et al. 2014) and *Argas*, *Antricola, Nothoaspis, Ornithodoros*, *Otobius*, (Clifford et al. 1964, Hoogstraal 1985, Burger et al. 2014), respectively. The French system proposed the scheme with eleven genera including *Alectorbius*, *Alveonasus*, *Antricola*, *Argas*, *Carios*, *Microargas*, *Nothoaspis*, *Ogadenus*, *Ornithodoros*, *Otobius*, and *Parantricola* (Camicas and Morel 1977, Camicas et al. 1998, Burger et al. 2014). The Cladistics system proposed the scheme with four genera including *Argas*, *Carios*, *Ornithodoros*, and *Otobius* (Klompen and Oliver 1993). Genus *Argas*, *Otobius*, and *Ornithodoros* are the only genera consistently represented in all taxonomic systems.

The habitat-host-vector interactions and their relationships with the environment are vital components of vector ecology. These interactions have been studied extensively in hard ticks. Ixodid ticks exhibit three distinctive "types" of life cycles based on their host interactions and result from deep historical and evolutionary consequences (Hoogstraal and Aeschlimann 1982). There are three common features in all hard tick life cycles types; 1.) all life cycles include four stages that consist of the egg and three obligatory hematophagous stages (e.g., three-legged larvae, four-legged nymph, and adult) (Sonenshine and Roe 2014); 2.) all hematophagous stages of Ixodid ticks require a single successful bloodmeal to molt to the next stage unless forcibly interrupted (Sonenshine and Roe 2014); and, 3.) there is a single gonotrophic cycle per an individual tick's lifespan (Sonenshine and Roe 2014).

The majority of hard ticks exhibit a three-host life cycle and this is thought to be the most primitive (Hoogstraal and Aeschlimann 1982, Oliver 1989, Sonenshine and Roe 2014). Generally, oviposition takes place off the host at the soil/vegetation interface and newly hatched larvae typically aggregate in clusters on vegetation while awaiting their first host. Engorged larvae then drop off from the first host and complete ecdysis in the environment to become nymphs (Oliver 1989, Sonenshine and Roe 2014). Nymphs then attach to the second host to acquire their bloodmeal and drop off once feeding to repletion. Engorged nymphs complete ecdysis off their host to become adults, which then seek their third host. Generally, feeding to repletion and mating occurs on the third host, and fertilized females drop off from the host and lay their eggs in the environment (Oliver 1989, Sonenshine and Roe 2014). However, in some Prostriata tick species, copulation

can take place off the host (Kiszewski et al. 2001, Sonenshine and Roe 2014). In the three-host tick life cycle, generally small sized hosts (i.e., rodent and leporids) are utilized in the larvae and nymph stages whereas larger hosts (i.e., ungulates) are utilized in the adult stage (Sonenshine and Roe 2014). However, host specificity in three-host ticks varies by species. For example, *Amblyomma americanum* L. (Ixodida: Ixodidae) and *Amblyomma cajennense* Fabricius have wide and overlapping host ranges across the three blood-feeding stages. Finding all three stages of these tick species blood-feeding on the same large ungulate hosts is not uncommon, in addition to using small to medium sized hosts for larvae and nymphs (Lopes et al. 1998, Childs and Paddock 2003). The generation time of the three-host tick may be greatly regulated by temperature fluctuations between seasons and can vary from one to three years (Oliver 1989).

The life cycle of two-host Ixodid such as *Hyalomma marginatum* Koch (Ixodida: Ixodidae), differs from that of three-host tick life cycle in the post-feeding behavior of larvae. Host-seeking larvae will attach, feed, and molt to the nymphal stage on the first host and then drop off into the environment once engorged (Sonenshine and Roe 2014). Subsequent host-seeking adult ticks then attach to the second host (Sonenshine and Roe 2014). Mating occurs on the second host and only the fertilized female drops off into the environment for oviposition as adult males remain on the host until death (Oliver 1989). The generation time of two-host ticks is typically shorter than that of three-host ticks. Two-host ticks can complete their life cycle in less than a year in some cases (Oliver 1989).

The one-host tick life cycle is thought to be the most recently evolved (Hoogstraal and Aeschlimann 1982, Oliver 1989). In the life cycle of one-host ticks such as

Dermacentor albipictus Packard (Ixodida: Ixodidae), and all six *Rhipicephalus (Boophilus)* Say (Ixodida: Ixodidae) species of all hematophagous stages utilize a single large ungulate host and only fertilized females drop off to lay their eggs in the environment (Oliver 1989). Compared to three-, and two-host ticks, the generation time in the one-host tick life cycle can be short and multiple generations can be produced in a single year (Oliver 1989). The number of hosts utilized in the lifecycles of two-host and one-host ticks can also vary. *Hyalomma excavatum* Koch can complete its life cycle as either three-host or two-host ticks, while in a more extreme example, such as *Hyalomma dormedarii* Koch, can utilize one to three hosts depending on the host availability and climatic conditions (Sonenshine and Roe 2014).

Both biotic and abiotic factors drive population changes in Ixodid ticks regardless of types (i.e., one-, two-, or three-host life cycle) of the life cycle. A primary example of a biotic factor is bloodmeal acquisition, which is driven by host diversity and host density. On the other hand, abiotic factors include environmental constraints such as temperature and humidity, which can lead to desiccation and eventual increase in off-host death rate (Sutherst et al. 1978, Spickett 1994). In addition, host-seeking activities are influenced by the length of diapause/aestivation and the suitable host availability at each life stage. Diapause in ticks can be behavioral or morphogenetic (physiological). The behavioral diapause denotes the suppression of host-seeking activity (sometimes referred to as "quiescence") in unfed ticks or delay of engorgement in ticks already attached on the host (Oliver 1989). The morphogenetic diapause, on the other hand, represents a delay during embryogenesis, in ecdysis, and/or in oviposition.

Proportionalities of influences of biotic or abiotic factors on a tick population are different based on its life cycle. For example, the population of three-tick host tick such as *A. americanum* is more influenced by abiotic than biotic factors since it can spend 97% or more of its lifespan in the off-host phase that is exposed to the environmental constraints (Needham and Teel 1991). On the other hand, the population of a one-host tick such as lifespan *Rhipicephalus (Boophilus) microplus* Canestrini heavily depend on the availability of a suitable host. This rationale is based on the fact that one-host ticks spend most of their lifespan on a single host, which provides nutrients to all stages of the tick, as well as providing protection from environmental constraints. This speculation can be indirectly observed by the popular control strategies employed for *R.(B). microplus* management. Currently, the vast majority of *R.(B). microplus* control strategies rely on treatment of tick infested hosts (Graf et al. 2004). This approach is due to the fact there are few effective or efficient control strategies targeting off-host fertilized females, eggs or larvae.

The life cycle of Argasid ticks can vary significantly between species; however, five common biological features can be observed; 1.) four distinctive stages include egg, larva, nymph (with multiple instars) and adult. Multiple-instar nymphs typically increase the total number of hematophagous events compared to those in Ixodid ticks (Sonenshine and Roe 2014); 2.) ticks may feed multiple times (intermittent feedings) in each hematophagous stage unlike the single feeding to repletion behavior exhibited in all stages of Ixodid ticks (Sonenshine and Roe 2014); 3.) the feeding duration (minutes) is relatively shorter than that of Ixodid ticks (days) and varies among species and developmental stages

(Oliver 1989); 4.) There are multiple gonotrophic cycles during the lifespan with relatively small batches of eggs laid compared to a single gonotrophic cycle in Ixodid ticks with large egg masses (Sonenshine and Roe 2014); and 5.) Argasid ticks are generally associated with nidicolous habitats (Oliver 1989, Sonenshine and Roe 2014). However, there are different variations of life cycles observed in Argasid ticks, and the following sections highlight different life cycle types, biology, and tick-host-habitat relationships using examples from the three common Argasid genera *Otobius, Argas,* and *Ornithodoros*.

Argasid ticks in the genus *Otobius* are thought to be more recently evolved compared to those in genus *Ornithodoros* and *Argas* (Hoogstraal and Aeschlimann 1982). Furthermore, *Otobius* ticks exhibit significantly different developmental biology and tick-host-habitat relationships. Genus *Otobius* are represented by two species, *Otobius megnini* Dugès and *Otobius lagophilous* Cooley & Kohls (Cooley and Kohls 1944). *Otobius* tick biology is known from *O. megnini*. The life cycle consists of egg, larvae, two nymphal instars, and adult stages (Sonenshine and Roe 2014). Only larvae and nymphs of *O. megnini* are hematophagous. Adults have vestigial mouthparts and thus do not seek or feed on hosts, but mate and oviposit on the ground (Cooley and Kohls 1944, Walker 2003, Sonenshine and Roe 2014). Unlike other Argasid ticks, *O. megnini* is considered a "modified" one-host tick and is known to utilize domestic animals such as cattle, sheep, goats, horses, donkeys, dogs, cats as well as humans as hosts (Cooley and Kohls 1944, Walker 2003). In addition, hematophagous stages of *O. megnini* are often found in the host ear cavities. (Cooley and Kohls 1944, Walker 2003) Finally, an *O. megnini* is capable of autogenous oviposition and produce a relatively large number of eggs during its lifespan

(~1,500 eggs) compared to a typically small egg production (<500 per lifespan) by other Argasid ticks (Sonenshine and Roe 2014).

The life-history of Argasid ticks in the type genus *Argas* have four stages: egg, larvae, nymph (up to four instars) and adult. (Walker 2003). Variation in the number of instars is driven by quantity and frequency of bloodmeals acquired during nymphal stage. (Sonenshine and Roe 2014). *Argas* ticks often utilize bird nests and prefer to feed on various bird species (Cooley and Kohls 1944, Walker 2003). Consequently, wider (global) distributions of *Argas* spp. can be observed. *Argas persicus* Oken, for example, is found in Europe, Asia, Africa, Americas, and Australia (Cooley and Kohls 1944). Finally, multiple gonotrophic cycles in *Argas* ticks require blood-feeding at adult stages and autogenous oviposition has not been observed.

Argasid ticks in the genus *Ornithodoros* have the same four stages previously described with up to eight nymphal instars (Sonenshine and Roe 2014). Variation in a total number of nymphal instars found is not only species specific but also affected by various biotic and abiotic factors (Sonenshine and Roe 2014). For example, *Ornithodoros turicata* Dugès typically have six nymphal instars (Beck et al. 1986); however, the number of nymphal instars can be reduced to five or less. The reduced nymphal instars in *O. turicata* is typical when ticks are allowed to feed to complete repletion from larvae to 4th instar nymph (laboratory observation). This ability results in the *O. turicata* to skip late nymphal instar(s) and molt directly to adult (Sonenshine and Roe 2014). In addition, some *Ornithodoros* spp. larvae such as *Ornithodoros moubata* Murray and O*rnithodoros capensis* Neumann, do not feed and molt directly to the first instar nymph (Walker 2003,

Sonenshine and Roe 2014), whereas other *Ornithodoros* ticks, such as *Ornithodoros talaje* Dunn, larvae feed for days (Dunn 1927). Generally, *Ornithodoros* ticks are parasites of diverse vertebrate hosts including mammals, birds, and reptiles (Davis 1941). However, host specificity for bats has been shown by *Ornithodoros kelleyi* Cooley & Kohls (Sonenshine and Anastos 1960). *Ornithodoros* ticks are generally associated with various nidicolous habitats that include caves, nests, and burrows (Sonenshine and Roe 2014). Finally, gonotrophic cycles in *Ornithodoros* ticks are not always initiated by bloodmeal acquisitions in the adult stage. Autogenous oviposition has been observed in *Ornithodoros lahorensis* Neumann and *Ornithodoros papillipes* Birula soon after molting to adults without a bloodmeal (Sonenshine and Roe 2014).

Ornithodoros turicata is one of five species of *Ornithodoros* found in the U.S. (Felsenfeld 1973, Dworkin et al. 2002). Although first described in 1876 from specimens collected from Guanajuato, Mexico (Dugès 1876, Cooley and Kohls 1944), *O. turicata* has been collected from western to central states including California, Arizona, New Mexico, Utah, Colorado, Kansas, Oklahoma, and Texas (Davis 1940, Cooley and Kohls 1944, Butler and Gibbs 1984). Interestingly, *O. turicata* is not readily collected in the southeastern U.S. except in Florida, which has led to further speculation that the *O. turicata* population found in Florida should be considered an allopatric subspecies, *Ornithodoros turicata americanus* Marx (Beck et al. 1986). As mentioned earlier, the life cycle of *O. turicata* includes an egg, larvae, up to six nymphal instars, and the adult stage (Beck et al. 1986). *Ornithodoros turicata* exhibits remarkable longevity and endurance even as immatures, surviving over 10 years when fed regularly and up to five years when

starved in a laboratory setting (Francis 1938, Cooley and Kohls 1944). A mark-and-recapture study also revealed that *O. turicata* nymphs survived for over a year without feeding (Adeyeye and Butler 1989). In addition, adults feed and copulate multiple times and produce relatively small, multiple batches of eggs, totaling less than 400 per individual female lifespan (Francis 1938, Davis 1941).

Ornithodoros turicata is well established as a vector and reservoir of *Borrelia turicatae* Steinhaus (Spirochaetales: Spirochaetaceae), one of the spirochetes that can cause Tick-Borne Relapsing Fever (TBRF) (Davis 1936, Thompson et al. 1969, Fihn and Larson 1980, Goubau 1984, Rawlings 1995, Trevejo et al. 1998, Dworkin et al. 2002, Cutler 2010). Clinical TBRF is defined by recurring symptoms of fever and nonspecific symptoms such as a headache, myalgia, arthralgia, shaking chills, and abdominal discomforts (Dworkin et al. 2002). TBRF is caused by multiple *Borrelia* spp. that are transmitted by several species of *Ornithodoros* ticks (Dworkin et al. 2002). Typically, TBRF B*orrelia* spp. is named after their *Ornithodoros* vector tick species. For example, *B. turicatae*, *Borrelia parkeri* Steinhaus, and *Borrelia hermsii* Steinhaus are transmitted by *O. turicata, Ornithodoros. parkeri* Cooley, and *Ornithodoros hermsii* Wheeler, Herms & Mayer, respectively (Dworkin et al. 2002, Dworkin et al. 2008). In the U.S., TBRF cases have been reported from Arizona, California, Idaho, Kansas, Montana, Nevada, New Mexico, New York, Utah, Oklahoma, Oregon, Oklahoma, Texas, and Washington (Palmer and Crawford 1933, Davis 1936, 1940, Southern Jr and Sanford 1969, Dworkin et al. 2008).

Ornithodoros turicata may also pose a potential threat to livestock, specifically in swine populations, in the U.S. Studies conducted by Butler and Gibbs (1984) and Hess et al. (1987) which examined vector competencies of four North American *Ornithodoros* ticks for African swine fever virus (ASFV) in the laboratory settings reported that *O. turicata* was capable of transmitting ASFV along with *Ornithodoros coriaceus* Koch and *Ornithodoros (Alectorobius) puertoricensis* Fox. African swine fever (ASF) is an acute hemorrhagic disease of swine with global implications due to its near 100 percent mortality and lack of effective vaccine and treatments. African swine fever is readily reported from most Sub-Saharan African countries with reports extending back since the 1920's (Costard et al. 2009). In 1957, the first case of ASF outside of Africa was reported in Portugal (Costard et al. 2009), and subsequent ASF outbreaks were reported in European countries from the 1960's into the 1980's (Plowright et al. 1994). In the Americas, ASF was reported in Cuba in 1971 and 1980, Brazil in 1978, the Dominican Republic and Haiti in 1979 (Ordas-Alvarez and Marcotegui 1987, Simeón-Negrín and Frías-Lepoureau 2002). The depopulation of swine was used to eradicate each of these Western Hemisphere outbreaks successfully. The potential vector capacity of *O. turicata* coupled with its extreme longevity and capability of enduring harsh environmental conditions poses a unique dilemma where a vector survives longer than most of its hosts and serves as a pathogen reservoir.

The general habitat, geographic distribution, and host preference of *O. turicata* in the U.S. are relatively unknown. This limitation is despite its early description in 1876 and subsequent studies linking its vector potential to TBRF as early as the 1930's. This

knowledge gap may be a result of biological and behavioral attributes of *O. turicata* that pose challenges to conducting basic surveillance in its native environment. *Ornithodoros turicata* is generally considered a nocturnal organism with an affinity toward micro-habitats found in caves, burrows, nests and cavities with host activity, and seldom found in relatively accessible open environments (Cooley and Kohls 1944, Beck et al. 1986, Milstrey 1987, Rawlings 1995). Their ability to feed to repletion in minutes (Davis 1941) means these ticks are infrequently found attached to their hosts. Therefore, current survey methods for *O. turicata* and Argasid ticks, in general, are limited to labor intensive and time consuming techniques such as the CO_2 baited debris-filtering method (Niebuhr et al. 2013) and animal burrow vacuuming techniques (Butler et al. 1985).

Ecological studies of *O. turicata* are met with challenges due to the lack of *O. turicata*-specific techniques. This deficiency resulted in the utilization of non-specific and indirect surveillance techniques, such as CO_2 baited traps or excavation of suspected habitats, which are labor intensive and inefficient in acquiring meaningful data for vector and disease ecology studies. Therefore, innovative methods for examining unique vector-host-habitat interactions for *O. turicata* is needed. The studies conducted in this dissertation aim to build the foundations for habitat-host-vector interactions that can be used to examine vector/disease ecology and develop future systematic surveillance methods for *O. turicata* using habitat description, host immune responses, comparative bloodmeal analyses.

There were three main objectives in this dissertation. The first objective was to conduct field studies at known *O. turicata* cave habitat in order to establish correlations

between *O. turicata* phenology and quantifiable biotic and abiotic factors. The second objective was to determine the effects of the repeated *O. turicata* challenges on the immune responses of model hosts. The third objective to assess the applicability of bloodmeal analyses using DNA-based and Stable Isotope-based methods on *O. turicata* cohorts.

CHAPTER II

INTERACTIONS BETWEEN *ORNITHODOROS TURICATA* DUGÈS (IXODIDA: ARGASIDAE) AND BIOTIC/ABIOTIC FACTORS FOUND IN CAVE ENVIRONMENTS AT GOVERNMENT CANYON STATE NATURAL AREA, TEXAS

Introduction

Ticks and tick-borne diseases have a tremendous impact on global economy and health of both animal and human. It is estimated ten percent of all known tick species are vectors of one or more pathogens (Jongejan and Uilenberg 2004). Subsequent cost of tick-borne disease control may cause untold economic global burden. For example, a study by Kivaria (2006) estimated that $364 million annual loss in Tanzania due to the direct economic cost associated with Ixodid tick-borne disease in cattle alone. Moreover, the "foothill abortion" (epizootic bovine abortion), a bacterial disease transmitted by *O. coriaceus* in cattle, causes estimated death of 45,000 to 90,000 calves in the U.S. annually (Bailey 2015). In human, Mae et al. (1998) estimated that the national expenditure over five years for therapeutic intervention for Lyme diseases in the U.S. could be $2.5 billion (1996 UDS).

Ecological studies of ticks can reduce both economic and physical burdens of ticks and tick-borne diseases. Indeed, for the past several decades, applications of ecological principles based on tick-host-pathogen interactions have led to the development of effective and efficient surveillance techniques and control tactics (Sutherst et al. 1978,

Spickett 1994, Jongejan and Uilenberg 2004). However, the progress in tick ecology studies has been unevenly matched between Ixodid ticks and Argasid ticks.

The popularity of ecological studies in Ixodid ticks may stem from the fact that a majority of human tick-borne diseases are transmitted by the Ixodid ticks (Parola and Raoult 2001, Jongejan and Uilenberg 2004), relative ease of surveillance techniques, and their ability to thrive readily in laboratory colonies. The richness in Ixodid tick ecological studies has enabled development of several successful epidemiological risk assessments and management strategies about Ixodid tick-associated damages and diseases throughout the world (Sutherst et al. 1979, Needham and Teel 1991, Nari 1995, de Castro 1997, Randolph 2004, Lindgren and Jaenson 2006, Piesman and Eisen 2008).

On the other hand, ecological studies of Argasid ticks are relatively rare in their numbers and tend to have narrow foci which address specific Argasid tick-pathogen relationships and events. For example, Duffy (1983) conducted an ecology study of *Ornithodoros (Alectorobius) amblus* Chamberlin, to determine nest abandonment among Peruvian seabirds due to Argasid tick parasitism. Furthermore, the ecology of *O. moubata* has been extensively studied due to its ability to transmit ASFV, which has devastating ecological and economic consequences on the affected area (Walton 1957, Peirce 1974, Butler et al. 1984a, Vial 2009, Bernard et al. 2016).

Ecology of *O. turicata* is poorly understood because the ecological studies of *O. turicata* has been limited to laboratory observations (Beck et al. 1986, Adeyeye and Phillips 1996, Phillips and Adeyeye 1996) or conducted in the context of its interactions with specific pathogens (Davis 1936, Francis 1938, Rawlings 1995, Dworkin et al. 2002,

Dworkin et al. 2008). There are limited studies which examined interactions between *O. turicata* and its habitat. Cooley and Kohl (1944) briefly summarized *O. turicata* distributions, morphological features, and host range. A dissertation by Milstrey (1987) examined the ecology of *O. turicata* within the gopher tortoise, *Gopherus polyphemus* Daudin (Testudines: Testudinidae), burrows in Florida. Recently, Donaldson et al. (2016) employed an ecological niche modeling approach in assessing geographic distribution based on environmental variables, host range, and *O. turicata* transmitted disease cases in the U.S. In all these studies, the distribution of *O. turicata* is reported as mainly in the southern U.S. from California to Texas with allopatric populations in Florida, which received a subspecies designation, *O.t. americanum* (Beck et al. 1986). However, current ecological understanding of *O. turicata* is inadequate in rationalizing the gap in their distribution in the U.S. and their dispersal strategies. Therefore, ecological studies in a field setting that examines interactions between *O. turicata* with abiotic (e.g., temperature and relative humidity), and biotic factors (e.g., animal activities) may serve to fill the knowledge gaps in *O. turicata* ecology in the U.S.

There were two objectives in this study. The first objective was to quantify biotic and abiotic factors in four *O. turicata*-active caves in Government Canyon State Natural Area (GCSNA), TX. The second objective was to assess correlations among *O. turicata* phenology with biotic and abiotic factors across four *O. turicata*-active caves in GCSNA.

Materials and Methods

***Study sites*.** All cave habitats investigated in this study were found within GCSNA, TX. GCSNA is located in the northwest of San Antonio in Bexar County, TX. It encompasses 47.04 km^2 of designated karst preserve that is part of the critical recharge zone of karstic Edwards (Balcones Fault Zone) Aquifer (Veni 2013). GCSNA is part of "Dry Climate Karst" which extends across entire central Texas where carbonate rock formations are commonly found at or near the land surface (Weary and Doctor 2014). As of 2013, 37 open caves were discovered in GCSNA. The typical features of caves found in GCSNA are relatively small and shallow (Miller 2012). The habitats found in GCSNA harbor diverse epigean and hypogean fauna that include mammals, herpetofauna, and invertebrates. The cave ecosystem found in GCSNA is unique in the sense that it is biologically rich with more than 65 identified vertebrate and invertebrate species (Miller and Reddell 2011). There were no prior collection records of *O. turicata* or Argasid ticks in general from GCSNA other than the anecdotal encounters by the GCSNA park rangers. Therefore, preliminary site surveys were conducted in ten caves selected based on accessibility using the method described later in the chapter to determine following four *O. turicata*-active caves located at GCSNA.

Ornithodoros turicata-active cave determination. Ten caves along Joe Johnston Route in GCSNA were surveyed during four separate visits between June to September 2015 based on their accessibility. Each cave was surveyed for 60 minutes using a tick survey method which will be described later in the chapter. Surveyed caves were deemed

as *O. turicata*-active cave if *O. turicata* was found within a 60-minute survey session. Four *O. turicata*-active caves and their brief descriptions are as follow.

Ornithodoros turicata-active cave: The Little Crevice Cave. The Little Crevice Cave (LCC) has the length and depth of 8.31 m and 3.78 m, respectively with the cave entrances facing the North. (Figure 1). There are two main entrances at LCC that forms a short (<1 m) bedding place squeeze which opens to a 3 m tall cliff face. From the cliff face inside of the LCC, the crevice floor slopes up steeply for about 3 m in length where it becomes an extremely narrow passage to the left. To the right, the crevice ends after about a meter at a small hole (Figure 1). This cave is developed in the cavernous hydrostratigraphic member of the Glen Rose Limestone (Miller 2017).

Ornithodoros turicata-active cave: The Mad Crow Cave. The Mad Crow Cave (MCC) has the length and depth of 8.56 m and 2.00 m, respectively with the cave entrance facing the East (Figure 2). This cave is located high on a cliff facing out to the GCSNA. This cave has the entrance measuring at 2 m which quickly narrows to less than 30 cm tall within few steps (<1.5 m). Beyond 5.5 m into the cave, it becomes impassable to humans (Figure 2). The floor of the cave is composed of brittle and dusty sediments (Miller 2017).

Ornithodoros turicata-active cave: The Log Cave. The Log Cave (LOG) has the length and depth of 17.5 m and 4.8 m, respectively with the cave entrance facing the East (Figure 3). The cave entrance is located 1.5 m high on a cliff face. The entrance is measuring at 2.8 m high by 7 m wide. However, the fractured floor rises within 2 m inside of cave making the distance to the ceiling of the cave to less than 40 cm. The cave extends

about 11 m before becoming impassible to humans (Figure 3). The cave floor is mostly covered with dry, dusty silt (Miller 2017).

Ornithodoros turicata-active cave: The Wash Out Cave. The Wash Out Cave (WOC) has the length and depth of 21 m (69 ft) and 6 m (20 ft), respectively with the cave entrance facing the East (Figure 4). This cave is the largest found in GCSNA to date (Miller 2017). This cave is composed of a large collapsed entrance that opens to the nearby creek bed. The entrance of cave measures at 6 m high by 10 m wide. Within the 8 m deep into the cave, the cave height becomes less than a meter tall, where the floor sloped up to the height of 50 cm or less. The cave passage forks to the south at about 15.5 m then becomes impassable after about 4 m (Figure 4). The cave floor is composed of soft dirt and scattered rocks with several speleothems (cave formations) along the back ceiling. The cave is formed in the cavernous hydrostratigraphic member of the upper member of the Glen Rose Limestone (Miller 2017).

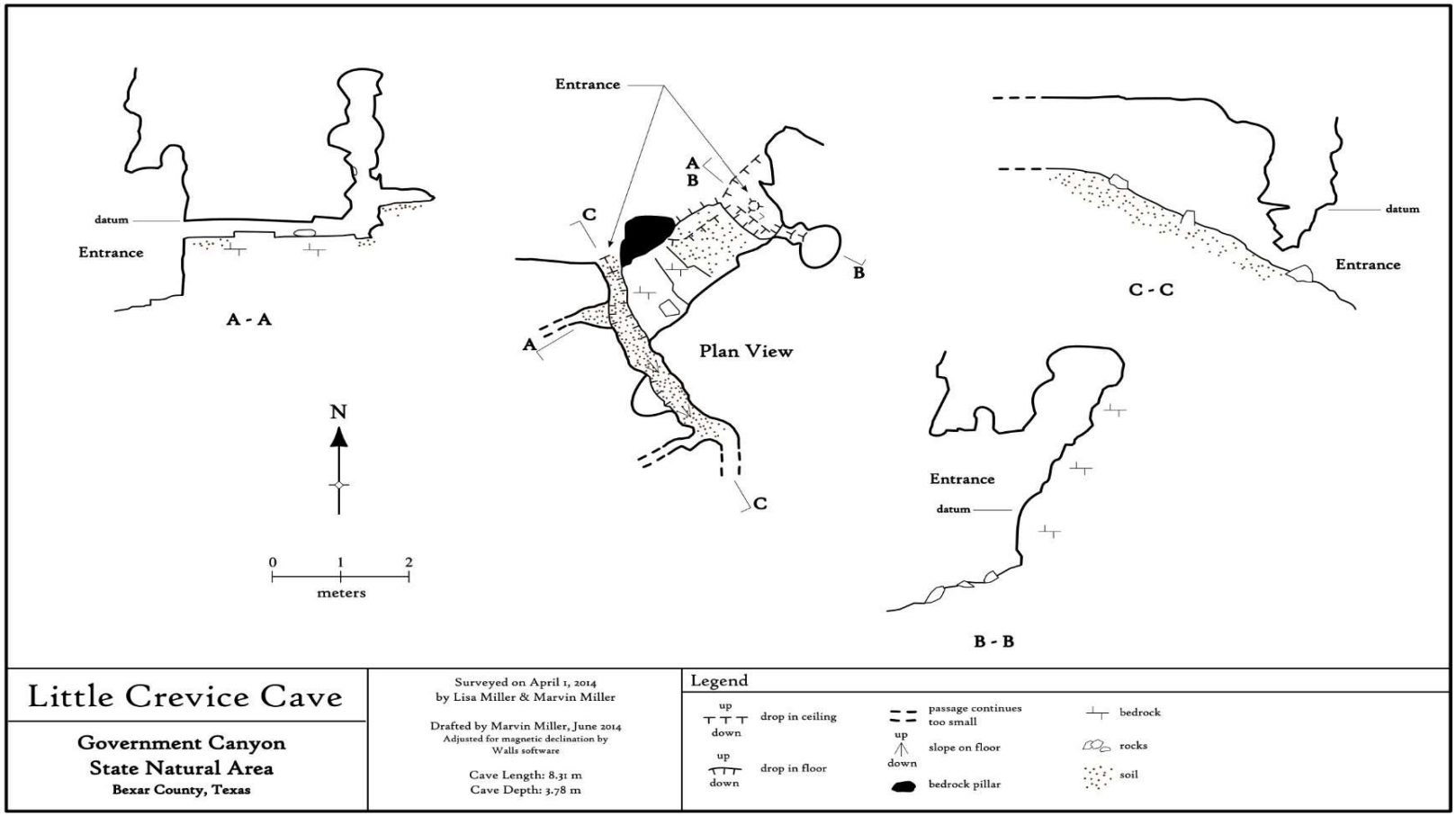

Figure 1. The Little Crevice Cave at the GCSNA, TX. Reproduced from Miller (2017).

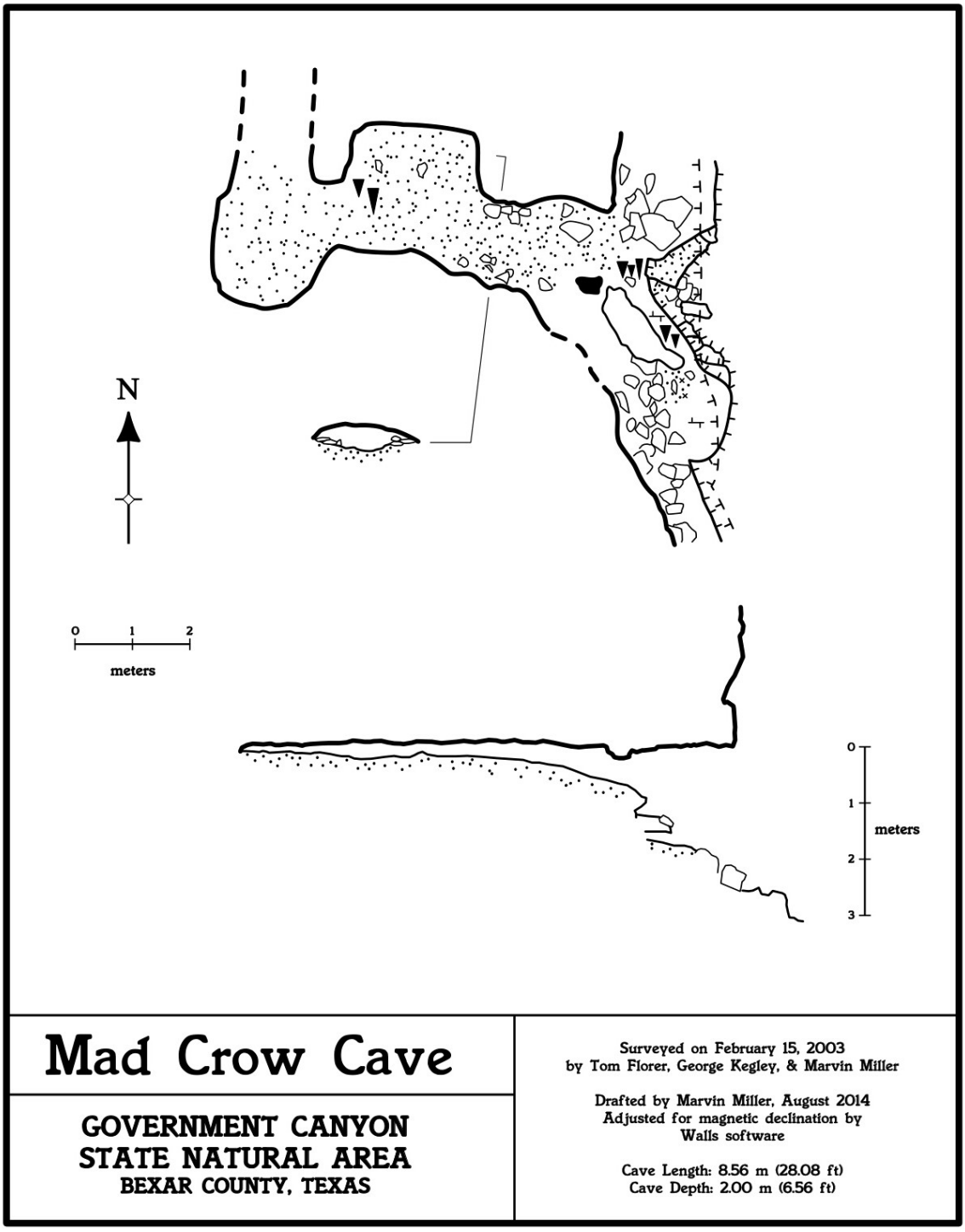

Figure 2. The Mad Crow Cave at the GCSNA, TX. Reproduced from Miller (2017).

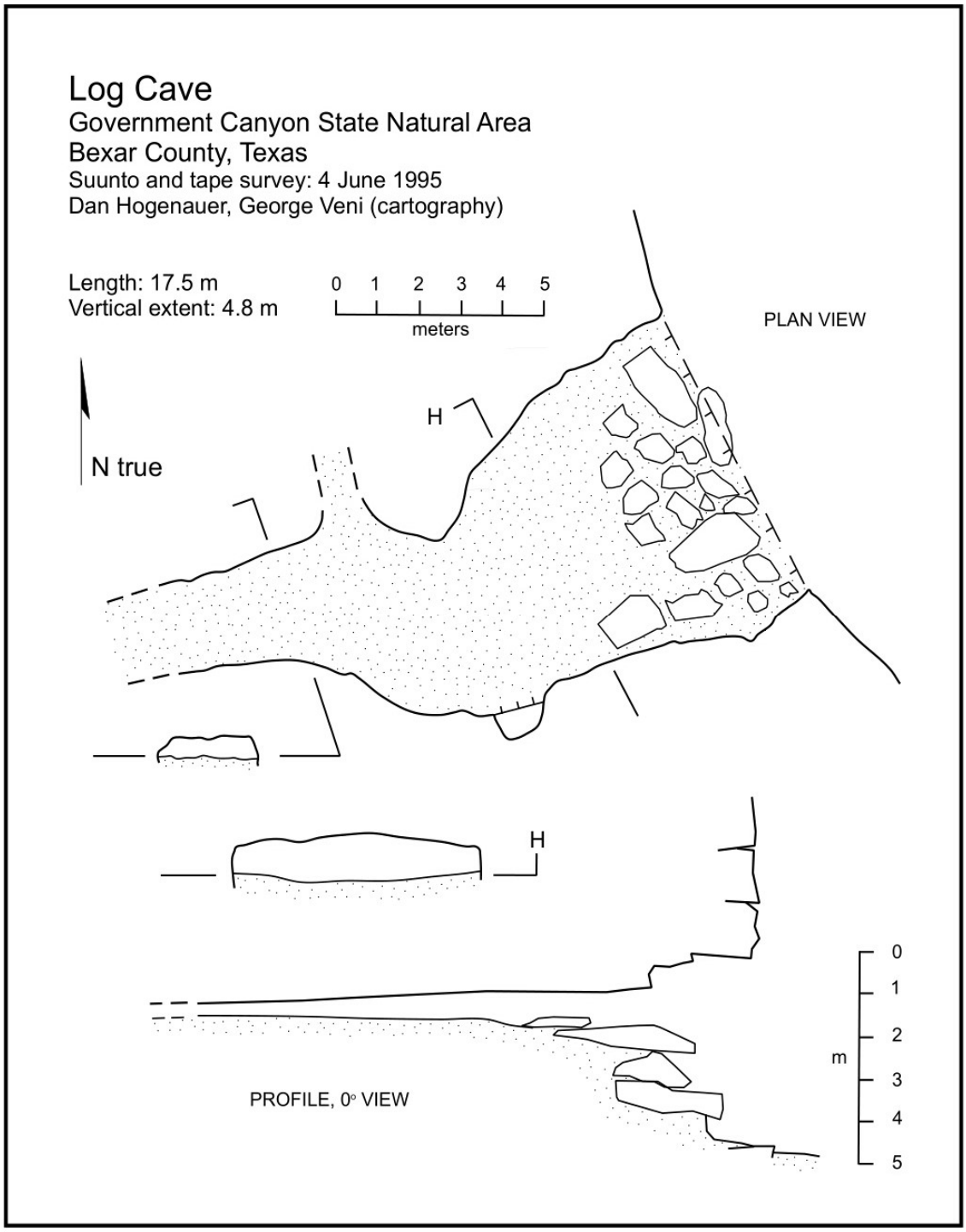

Figure 3. The Log Cave at the GCSNA, TX. Reproduced from Miller (2017).

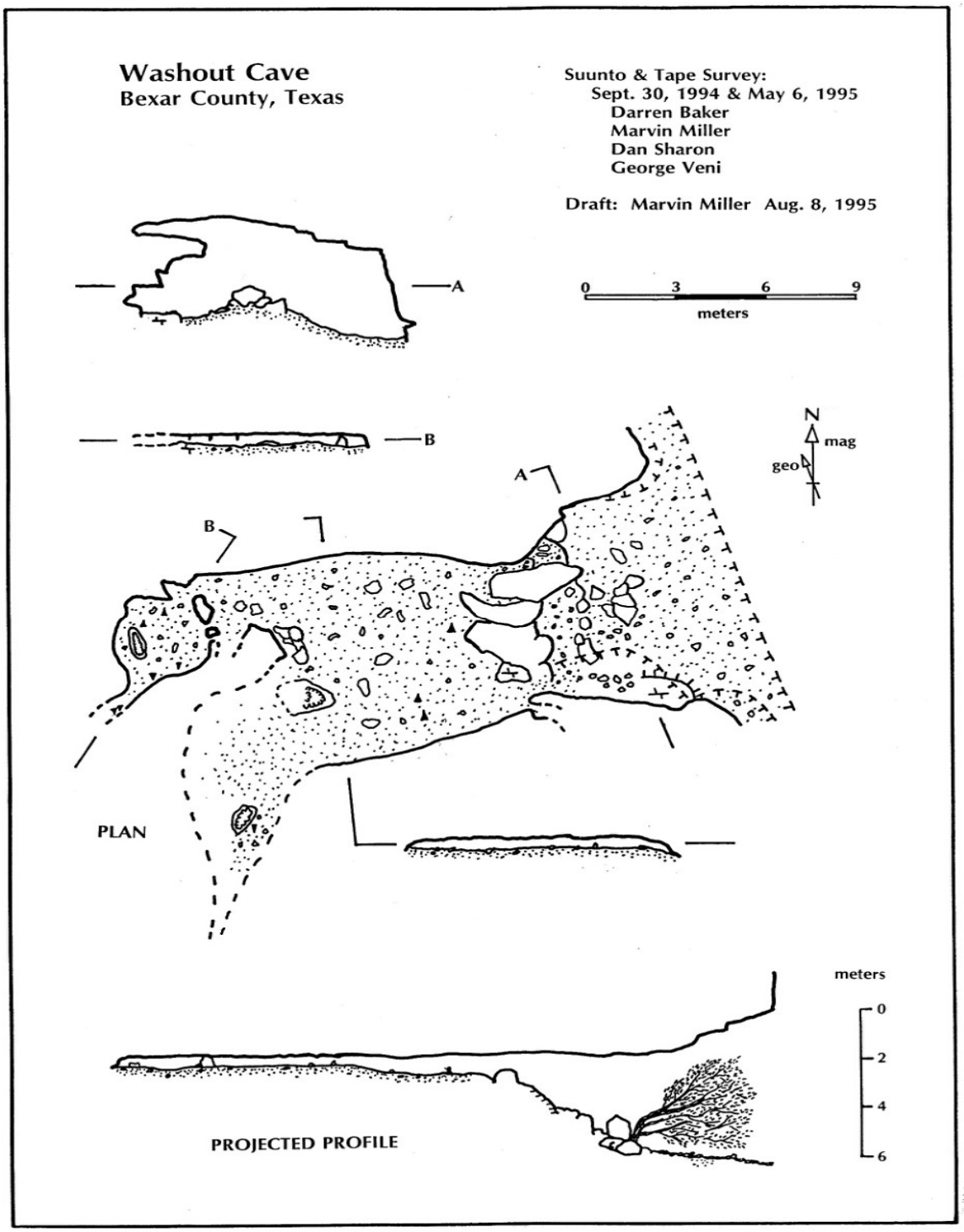

Figure 4. The Wash Out Cave at the GCSNA, TX. Reproduced from Miller (2017).

Tick survey. Tick surveys at each *O. turicata*-active cave were conducted during each visit to GCSNA. A total of six tick surveys were conducted at approximately 60-day intervals. The *O. turicata* survey method described by Adeyeye and Butler (1991) was used with the following modification: A flannel cloth measuring 50 cm x 50 cm was placed on the cave floor then 113 g (~4 oz) of dry ice was placed at the center of the flannel cloth. A smaller flannel cloth measuring 20 cm x 20 cm was then placed on top of the dry ice to limit direct contact between *O. turicata* and the dry ice during the survey (Figure 5). Each tick survey was conducted for 60 minutes. The captured ticks were released back into the cave once their numbers were recorded.

Kissing bug collection. Kissing bugs, *Triatoma* spp. Laporte (Hemiptera: Reduviidae) that were present during the tick survey were collected and submitted to Dr. Sarah Hamer's laboratory through the citizen science survey (http://kissingbug.tamu.edu) at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences to screening for *Trypanosoma cruzi* Chagas (Kinetoplastida: Trypanosomatida). Samples were tested for *T. cruzi* DNA using a probe-based qPCR method described by Duffy et al. (2013).

Figure 5. An *Ornithodoros turicata* survey setup at the Little Crevice Cave at the GCSNA, TX. A flannel cloth cut to 50 cm x 50 cm (A.) was used with 113 g (~4 oz) of dry ice as the attractant. A smaller flannel cloth cut to 20 cm by 20 cm was used to cover the dry ice to limit direct contact between *O. turicata* and dry ice.

Abiotic data collection. The environmental data from each *O. turicata*-active cave was collected from September 2015 to October 2016. Several abiotic environmental factors that may influence *O. turicata* activities were measured. These abiotic factors include light intensity, relative humidity, and temperature. Onset® HOBO® Data Loggers (Onset Computer Corporation, Bourne, MA) were used to measure all abiotic data. Light intensity was measured using HOBO® Data Logger Model: UA-002-08 in lux. The measurements were taken at 30-minute intervals for the entire study period. Light intensity was selected to validate the photoperiod responses of *O. turicata* reported in previous studies. For example, the study by Adeyeye and Butler (1991) indicated that there was no correlation between the number of *O. turicata* captured and the time of the day when tick survey was performed in the field. Furthermore, Adeyeye and Philips (1996) reports that there were no correlations between *O. turicata* development time and changes in photoperiods. Nevertheless, *O. turicata* is described as a nocturnally active tick that tends to feed in the dark (Cooley and Kohls 1944, Beck et al. 1986). The relative humidity and temperature were collected using HOBO® Data Logger Model: U23-001 Temp/RH in °C and %, respectively. The measurements were taken at 5-minute intervals for the entire study period. Both temperature and humidity were selected as relevant factors since ticks are vulnerable to desiccation and extreme temperature (Sutherst et al. 1978, Spickett 1994). All probes were placed within the area covered by the tick collection sheet (50 cm x 50 cm) used during the tick survey. Onset HOBOware® software version 3.7.10 was used to extract and combine data from the data loggers.

Biotic data collection. The animal activity data from each *O. turicata*-active cave were collected concurrently with environmental data from September 2015 to October 2016. Motion sensor triggered Trail Camera, Stealth CAM® Model# STC-G42NG (Stealth CAM, LCC, Grand Prairie, TX), was used to capture both still and video images of animals that visit the tick survey site at each *O. turicata*-active cave. Trail cameras were positioned 1 to 1.5 meters from the tick survey site within the cave to ensure captured images will have full tick survey site in view as well as animals that may have triggered the trail camera sensors (Figure 6). Moreover, cameras were positioned to capture different viewpoints of tick survey area within the cave without causing mutual flash interference when the terrains within the caves were conducive. Images from each camera were retrieved in approximately 60-day intervals. Next, one of the two cameras were set to capture three burst images in 8-megapixel resolution with 30 second reset time when triggered, and another camera was configured to capture a 10-second video clip at 1280 x 720 pixel resolution with 30 second reset time. Each image and video clip was time stamped which made assembly of data and information processing possible. Three sets of data were extracted from captured images and video clips. The first data set was the animal species that visited each cave. The identification and verification of each species performed by the GCSNA park rangers and resource managers. The second data set was the number of visits by species per week. The third data set was the average duration of visits by each species per week. Finally, animal species visited *O. turicata*-active caves in this study were compared to previously known *O. turicata* hosts reported by Cooley and Kohls (1944) and Donaldson et al. (2016).

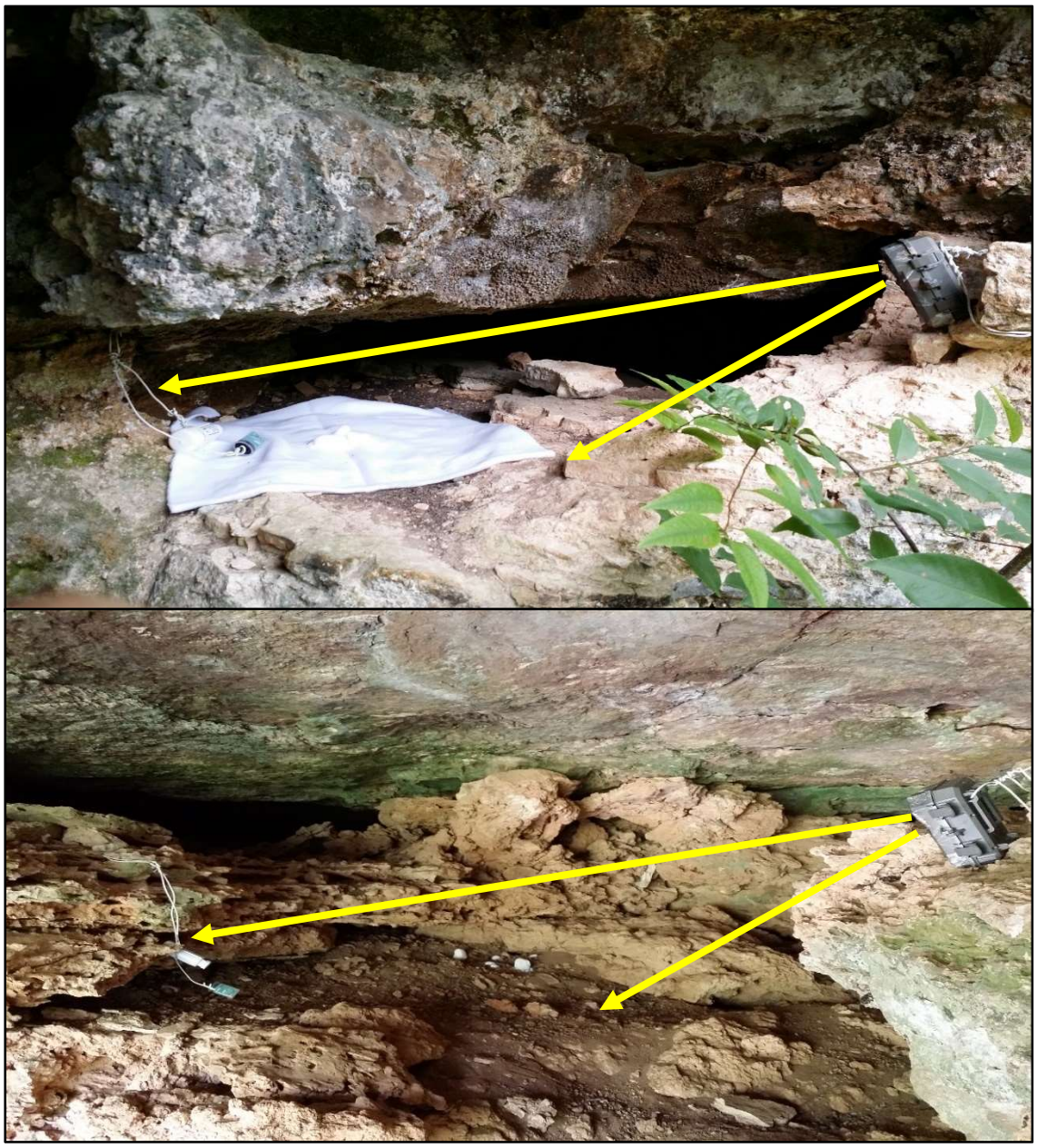

Figure 6. The animal activity collection setup using motion sensor triggered trail camera at the GCSNA, TX. Two trail cameras, Stealth CAM® Model# STC-G42NG (Stealth CAM, LCC, Grand Prairie, TX), were installed at each *Ornithodoros turicata*-active cave from September 2015 to October 2016. Trail cameras were positioned 1 to 1.5 meters from the tick survey site within the cave to ensure captured images would have full tick survey site in view (indicated by the yellow arrows) as well as animals that may have triggered the trail camera sensors. Top and bottom: Trail camera set up at Little Crevice Cave and Wash Out Cave, respectively.

Statistical data analysis. JMP® Pro 12 statistical software program (SAS Co., Cary, NC) was used for all statistical analyses. Transfer function modeling was used to assess correlations among caves based on light intensity, relative humidity, and temperature. Transfer function models is a time series analysis often employed in settings where correlative or causal relationships exist between temporally or spatially related variables (Montgomery and Weatherby 1980). Essentially, transfer function model estimates one set of time series data (the output series) using the second set of time series data (the input series) (Montgomery and Weatherby 1980). Popular applications for transfer function include predicting a company's sales during "period A" (the output series) using advertising expenditures during "period B" (the input series) or predicting the daily maximum temperature during a month (the output series) to the daily cloud coverage (the input series) (Montgomery and Weatherby 1980). The transfer function model used to assess correlations between caves using measured values of Cave A as the output series and the values of Cave B as the input is represented as follow:

$$\text{Cave } A_t = \beta_o + \beta_1 \text{ x Cave } B_t + e_t$$

Where Cave A_t = output series (values of Cave A observed during period t), β_o = intercept, β_1= input coefficient of measured values of Cave B, Cave B_t = input series (values of Cave B observed during period t), and e_t = noise series. Statistical significance of β_1, R^2, and the Mean Absolute Percent Error (MAPE) was then used to establish the trend similarities among the measures values of caves. Tick captured data from six survey visits to caves were normalized as a proportion of tick captured per visit out of total ticks captured during the study period per each cave. Linear regression analyses using

normalized tick captured data as the function of the weekly average of light intensity, relative humidity, and temperature data from the week of, one-, two-, and three-weeks prior to the tick survey visits were assessed.

Results

Ornithodoros turicata phenology. A total 448 *O. turicata* were captured from four *O. turicata*-active caves during six survey visits at GCSNA. However, the number of ticks collected from each cave varied widely. For example, a relatively higher number of ticks were collected from LCC and MCC consistently whereas a relatively low number of ticks were collected from LOG and WOC (Table 1). These difference in tick collection resulted in wide range of standard errors that were greater than the average of tick collected during several survey visits (Table 1). When the raw tick collection data were compared, there was no significant difference (df = 5, F = 1.20, $P = 0.35$) in the numbers of ticks collected from survey visits (Figure 7). On the contrary, when tick collection data were normalized by depicting ticks collected during each survey as the proportion of the entire ticks collected from the corresponding cave, a discernable pattern emerged, that significant differences (df = 5, F = 11.87, $P < 0.001$) between the ticks collected from survey visits (Figure 8). The post-hoc tests (Tukey's HSD, $P < 0.05$) showed significant differences ($P < 0.001$ each) among months of June and August 2016 compared to months of November 2015, January 2016, and March 2016 (Figure 8).

Table 1. *Ornithodoros turicata* captured during six survey visits at the GCSNA, TX in 2015-2016. Total ticks and normalized captured records (proportion of tick collected during each visit) are presented.

		Ornithodoros turicata captured					
Survey Visits	Month-Year	LCC	MCC	LOG	WOC	Average	SD
1	November 2015	30	14	7	2	13.25	12.20
2	January 2016	12	0	4	2	4.50	5.26
3	March 2016	22	8	2	1	8.25	9.67
4	June 2016	88	24	13	5	32.50	37.81
5	August 2016	90	33	8	12	35.75	37.79
6	October 2016	33	24	9	5	17.75	13.05
	Total per cave	275	103	43	27		
	Combined total			448			
		Proportion of *Ornithodoros turicata* captured					
Survey Visits	Month-Year	LCC	MCC	LOG	WOC		
1	November 2015	0.11	0.14	0.16	0.07	Average	SD
2	January 2016	0.04	0.00	0.09	0.07	0.12	0.04
3	March 2016	0.08	0.08	0.05	0.04	0.05	0.04
4	June 2016	0.33	0.23	0.19	0.19	0.06	0.02
5	August 2016	0.32	0.32	0.30	0.44	0.30	0.11
6	October 2016	0.12	0.23	0.21	0.19	0.28	0.07

LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

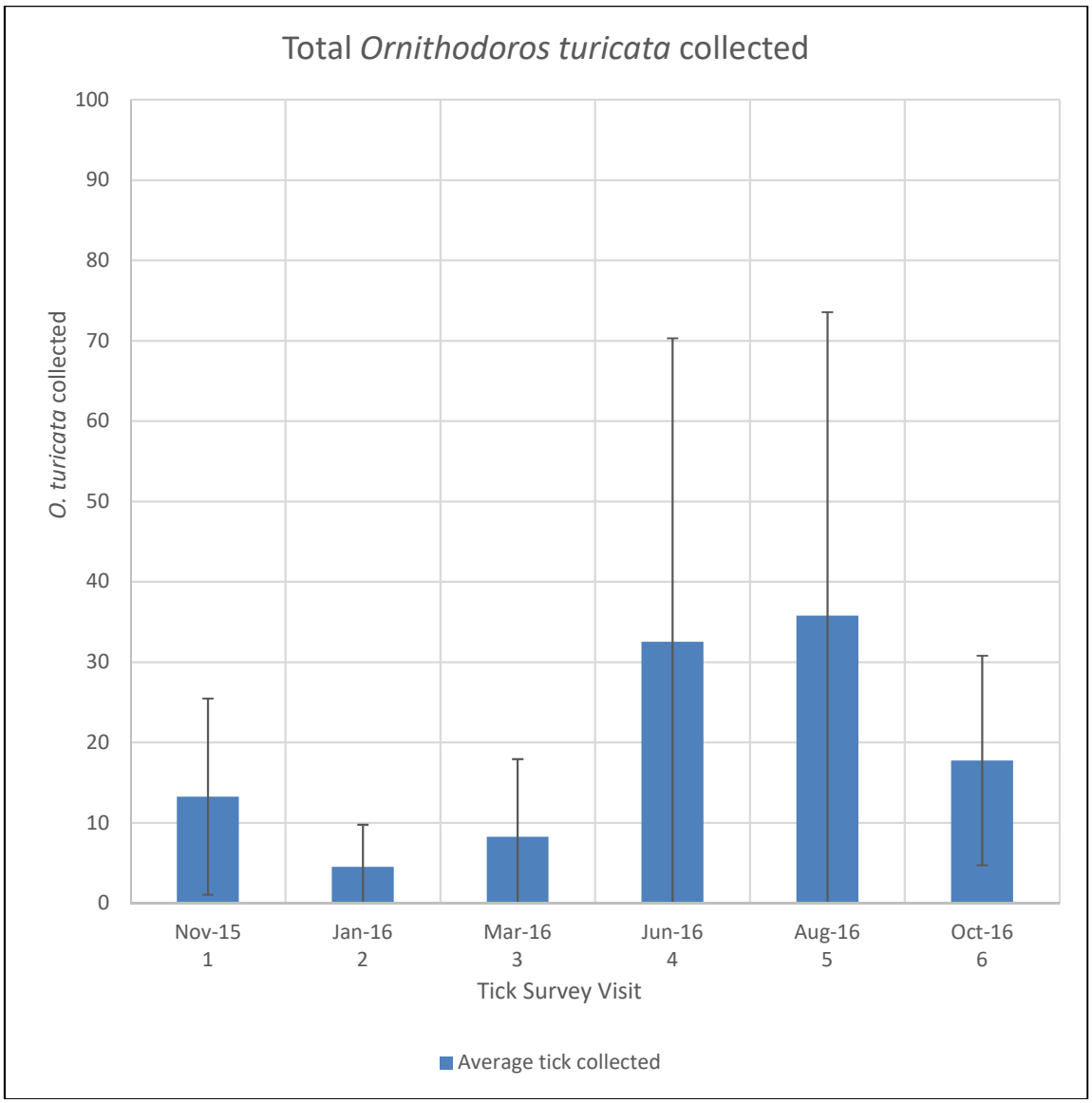

Figure 7. Total *Ornithodoros turicata* captured from four caves located at the GCSNA, TX during six survey visits from November 2015 to October 2016. The numbers of ticks collected from each cave varied widely, resulting in no significant difference in the numbers of ticks collected from survey visits (df = 5, F = 1.20, $P = 0.35$).

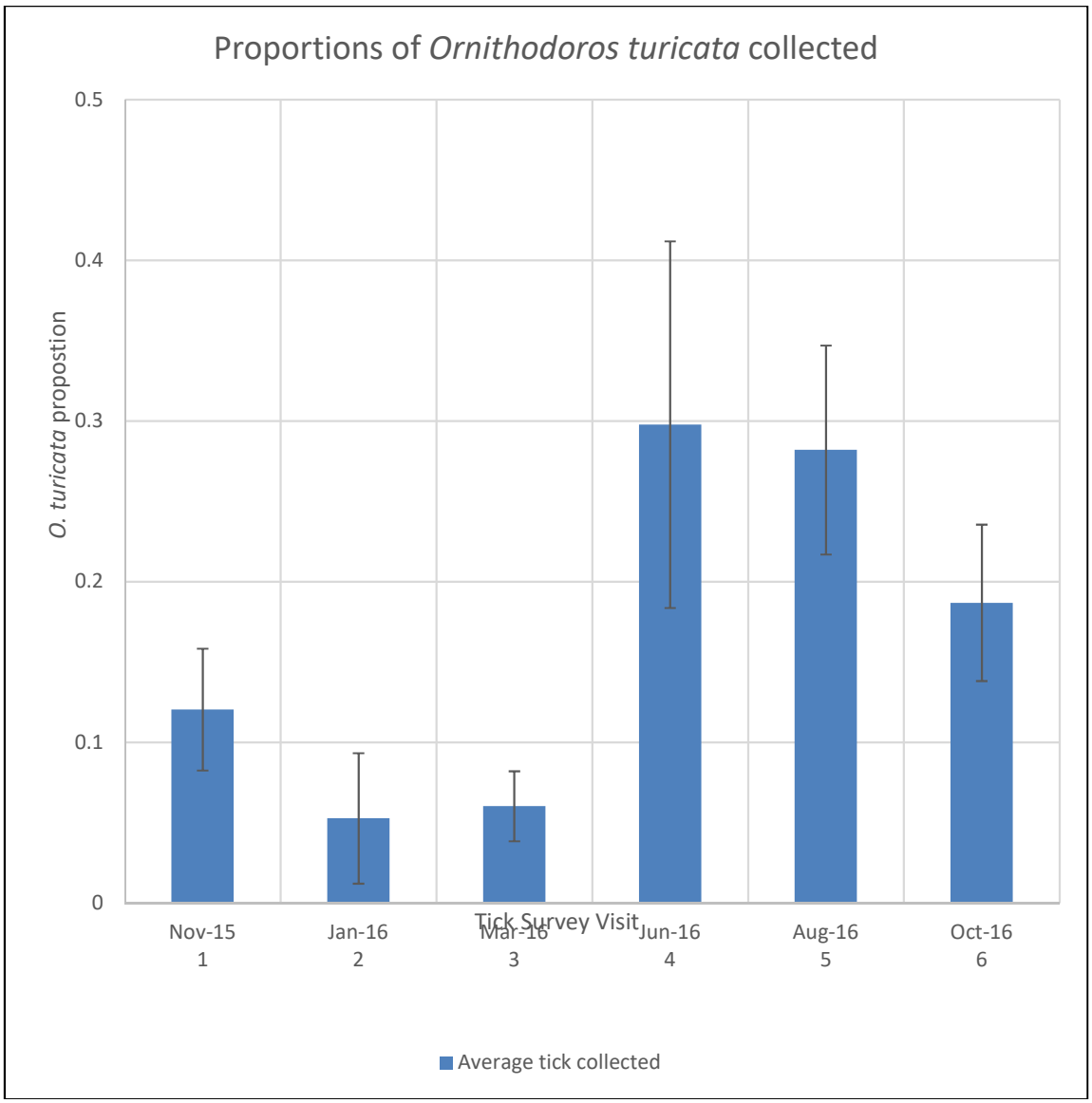

Figure 8. Normalized *Ornithodoros turicata* captured data from four caves located at the GCSNA, TX during six survey visits from November 2015 to October 2016. The tick captured data was normalized by depicting ticks collected during each survey as the proportion of the entire ticks collected from the corresponding cave. The proportion of ticks collected were significantly different (df = 5, F = 11.87, $P < 0.001$) where tick collected from the months of June and August 2016 were significantly higher than those from the months of November 2015, January 2016, and March 2016 (Tukey's HSD, $\alpha = 0.05$, $P < 0.001$ each).

Abiotic factor comparison among Ornithodoros turicata-active caves. The weekly average light intensities of four *O. turicata*-active caves showed no discernable change in patterns which encompasses all four caves. However, a similar change in patterns between LCC - WOC and MCC - LOG is detected (Figure 9). Transfer function analyses using light intensity values showed no significant correlations (R^2 range = 0.0016 to 0.16, β_1 *P*-value range = 0.40 to 0.94, MAPE range = 31.93 to 87.85) among caves except between LCC - WOC and MCC - LOG. The LCC - WOC and MCC - LOG showed transfer function R^2 values of, 0.29 and 0.30, respectively with β_1 *P* values of 0.01 each. However, MAPE range among these caves were 27.85 to 71.89 which denotes a significant error in predicting light intensity values of each other (Table 2).

The weekly average relative humidity of four *O. turicata*-active caves showed discernable changes in a pattern which encompasses all four caves (Figure 10). Transfer function analyses using relative humidity values showed significant correlations (R^2 range = 0.72 to 0.97, β_1 $P < 0.01$ each, MPAE range = 1.73 to 6.54) among all caves (Table 3). Similarly, the weekly average temperature of four *O. turicata*-active caves showed discernable changes in a pattern which encompass all four caves (Figure 11). Transfer function analyses using temperature values showed significant correlations (R^2 range = 0.98 to 0.99, β_1 $P < 0.01$ each, MPAE range = 1.71 to 3.87) among all caves (Table 4).

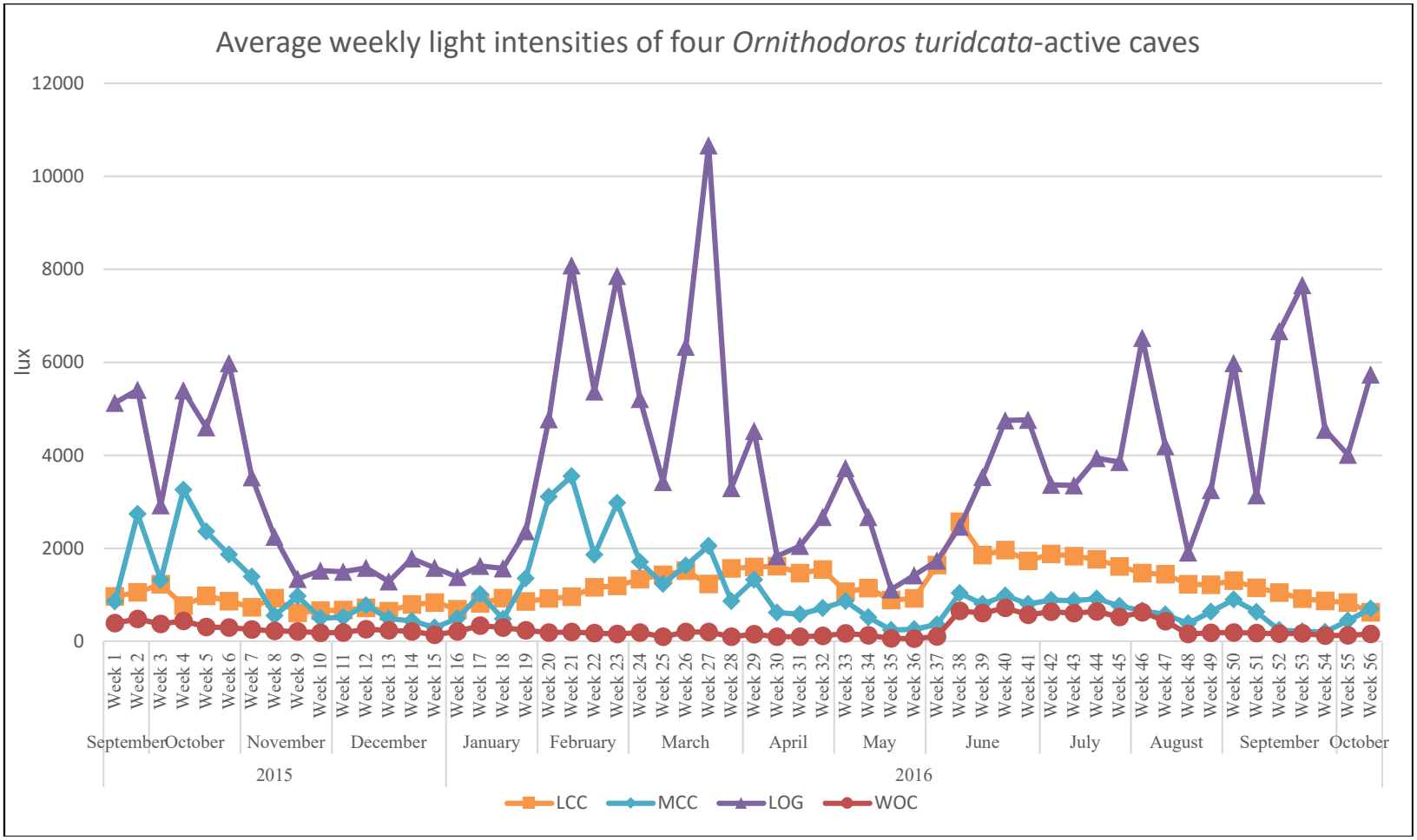

Figure 9. Weekly averages light intensity of four *Ornithodoros turicata*-active caves at the GCSNA, TX from September 2015 to October 2016. LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

Table 2. A summary of transfer function analyses of *Ornithodoros turicata*-active caves at GCSNA, TX using weekly average light intensity values from September 2015 to October 2016.

Transfer function model: Cave $A_t = \beta_o + \beta_1$ x Cave $B_t + e_t$	DF	*P*, $\beta_o = 0$	*P*, $\beta_1 = 0$	R^2	MAPE (%)
LIGHT LCC_t = 1210.57 + -0.021*LIGHT $MCC_t + e_t$	54	0.01	0.78	0.0016	31.93
LIGHT LCC_t = 1105.96 + 0.022*LIGHT $LOG_t + e_t$	54	0.01	0.42	0.012	31.48
LIGHT LCC_t = 839.29 + 1.26*LIGHT $WOC_t + e_t$	54	0.01	0.01	0.29	27.85
LIGHT MCC_t = 1146.82+ - 0.078*LIGHT $LCC_t + e_t$	54	0.01	0.77	0.0016	87.85
LIGHT MCC_t = 233.88 + 0.22*LIGHT $LOG_t + e_t$	54	0.22	0.01	0.30	71.89
LIGHT MCC_t = 911.83 + 0.51*LIGHT WOCt + e_t	54	0.01	0.40	0.013	84.03
LIGHT LOG_t = 3174.20 + 0.53*LIGHT $LCC_t + e_t$	54	0.01	0.42	0.012	60.46
LIGHT LOG_t = 2323.15 + 1.41*LIGHT $MCC_t + e_t$	54	0.01	0.01	0.30	46.45
LIGHT LOG_t = 3405.71 + 1.45*LIGHT $WOC_t + e_t$	54	0.01	0.94	0.016	59.88
LIGHT WOC_t = 6.72 + 0.23*LIGHT $LCC_t + e_t$	54	0.91	0.01	0.29	66.78
LIGHT WOC_t = 252.05+ 0.03*LIGHT $MCC_t + e_t$	54	0.01	0.40	0.013	65.64
LIGHT WOC_t = 237.35 + 0.01*LIGHT $LOG_t + e_t$	54	0.01	0.94	0.016	66.29

LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

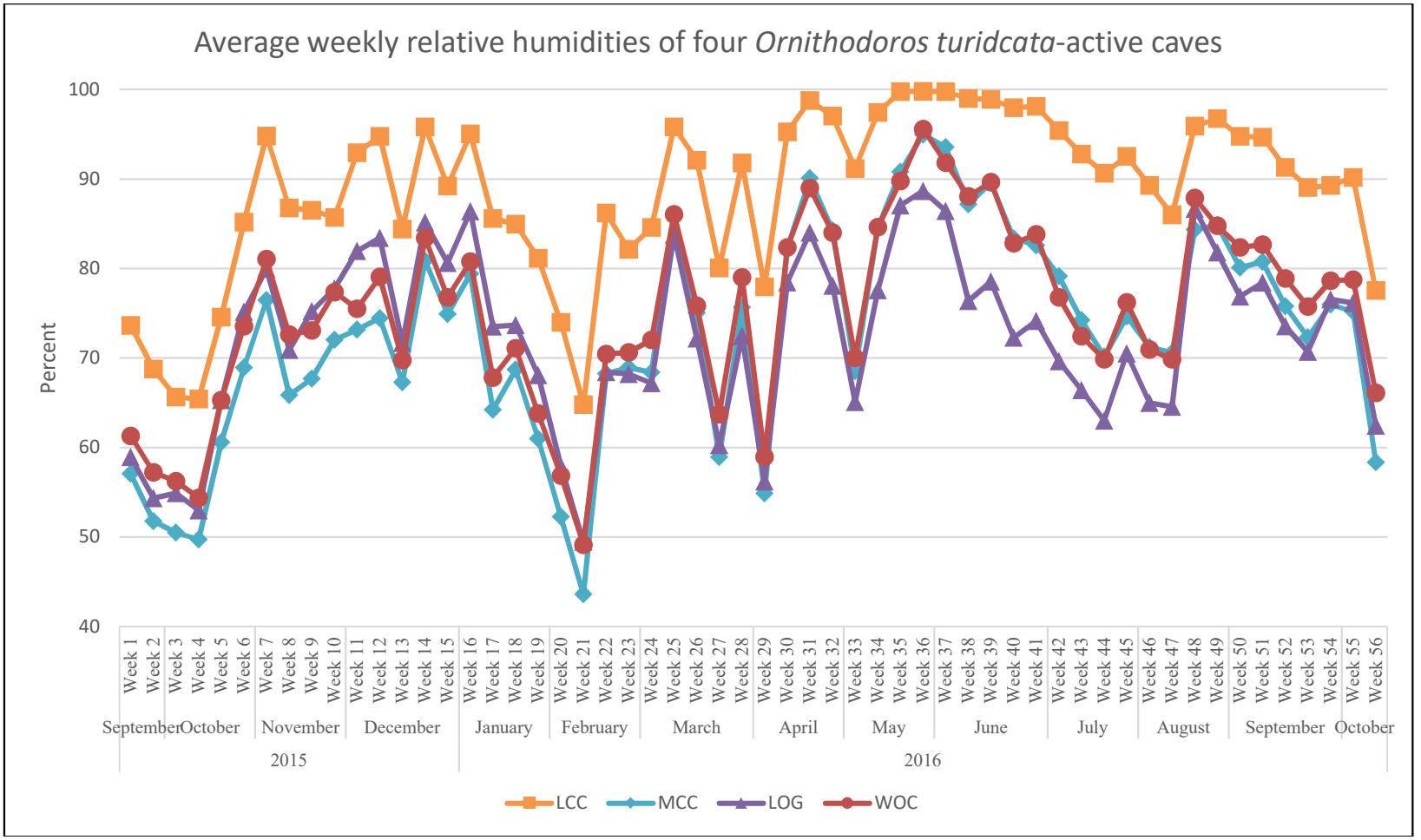

Figure 10. Weekly averages relative humidity of four *Ornithodoros turicata*-active caves at the GCSNA, TX from September 2015 to October 2016. LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

Table 3. A summary of transfer function analyses of *Ornithodoros turicata*-active caves GCSNA, TX using weekly average relative humidity values from September 2015 to October 2016.

Transfer function model: Cave $A_t = \beta_o + \beta_1$ x Cave $B_t + e_t$	DF	*P*, $\beta_o = 0$	*P*, $\beta_1 = 0$	R^2	MAPE (%)
RH LCCt = 33.65 + 0.75 x RH MCCt + et	54	0.01	0.01	0.91	2.52
RH LCCt = 28.95 + 0.82 x RH LOGt + et	54	0.01	0.01	0.72	4.73
RH LCCt = 24.46 + 0.85 x RH WOCt + et	54	0.01	0.01	0.89	2.84
RH MCCt = -34.51 + 1.21 x RH LCCt + et	54	0.01	0.01	0.91	3.93
RH MCCt = -5.27 + 1.08 x RH LOGt + et	54	0.36	0.01	0.77	6.54
RH MCCt = -12.38 + 1.13 x RH WOCt + et	54	0.01	0.01	0.97	2.03
RH LOGt = -5.22 + 0.88 x RH LCCt + et	54	0.42	0.01	0.72	5.78
RH LOGt = 20.39 + 0.71 x RH MCCt + et	54	0.01	0.01	0.77	5.14
RH LOGt = 8.26 + 0.854 x RH WOCt + et	54	0.04	0.01	0.83	4.28
RH WOCt = -16.81 + 1.04 x RH LCCt + et	54	0.01	0.01	0.89	3.76
RH WOCt = 12.61 + 0.86 x RH MCCt + et	54	0.01	0.01	0.97	1.73
RH WOCt = 4.48 + 0.98 x RH LOGt + et	54	0.3	0.01	0.83	4.38

LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

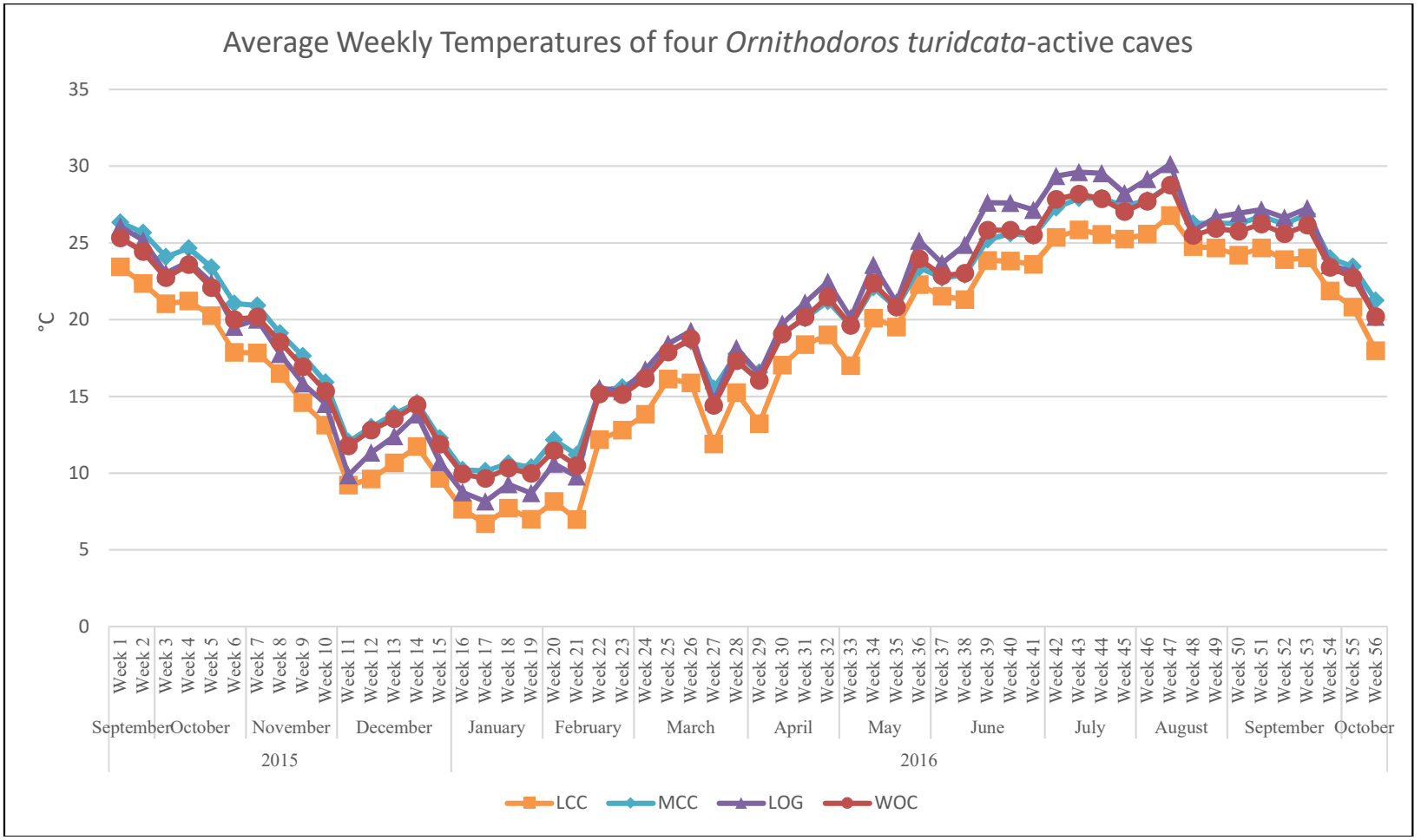

Figure 11. Weekly averages temperature of four *Ornithodoros turicata*-active caves at the GCSNA, TX from September 2015 to October 2016. LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

Table 4. A summary of transfer function analyses of *Ornithodoros turicata*-active caves GCSNA, TX using weekly average temperature values from September 2015 to October 2016.

Transfer function model: Cave $A_t = \beta_o + \beta_1$ x Cave $B_t + e_t$	DF	*P*, $\beta_o = 0$	*P*, $\beta_1 = 0$	R^2	MAPE (%)
TEMP LCCt = -3.99 + 1.07 x TEMP MCCt + et	54	0.01	0.01	0.99	3.03
TEMP LCCt = -0.87 + 0.92 x TEMP LOGt + et	54	0.01	0.01	0.99	3.56
TEMP LCCt = -3.43 + 1.06 x TEMP WOCt + et	54	0.01	0.01	0.99	2.17
TEMP MCCt = 3.89 + 0.92 x TEMP LCCt + et	54	0.01	0.01	0.99	2.30
TEMP MCCt = 3.06 + 0.85 x TEMP LOGt + et	54	0.01	0.01	0.98	3.25
TEMP MCCt = 0.64 + 0.99 x TEMP WOCt + et	54	0.01	0.01	0.99	1.85
TEMP LOGt = 1.19 + 1.07 x TEMP LCCt + et	54	0.01	0.01	0.99	3.35
TEMP LOGt = -3.13 + 1.15 x TEMP MCCt + et	54	0.01	0.01	0.98	3.87
TEMP LOGt = -2.62 + 1.15 x TEMP WOCt + et	54	0.01	0.01	0.99	2.43
TEMP WOCt = 3.31 + 0.94 x TEMP LCCt + et	54	0.01	0.01	0.99	1.71
TEMP WOCt = -0.51 + 1.01 x TEMP MCCt + et	54	0.01	0.01	0.99	1.87
TEMP WOCt = 2.40 + 0.87 x TEMP LOGt + et	54	0.01	0.01	0.99	2.08

LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

Animal activities at the Ornithodoros turicata-active caves. A total of twenty species of vertebrate animal activities were recorded from the four *O. turicata*-active caves during the study. Among the twenty-animal species, seven were previous known *O. turicata* hosts (Table 5). There were total 392 days each species of vertebrates could have visited. Canyon wrens, *Catherpes mexicanus* Swainson (Passeriformes: Troglodytidae), had the most frequent visits of 361 days (Table 5). However, their average visit duration was relatively short, with average duration of 2.70 minutes, compared to black vultures, *Coragyps atratus* Bechstein (Cathartiformes: Cathartidae) and raccoons, *Procyon lotor* L. (Carnivora: Procyonidae), which had 30 daily visit with average duration of 33.73 minutes and 106 daily visit with average duration 16.68 minutes, respectively (Table 5). Also, different numbers and types of vertebrate animal species visited each *O. turicata*-active caves. For example, both LCC and MCC had 13 species of vertebrate animals visited during the study (Tables 6 and 7), whereas LOG and WOC had eight and nine animal species, respectively (Tables 8 and 9). However, canyon wrens, raccoons, turkey vultures, *Cathartes aura* L. (Cathartiformes: Cathartidae), ring-tailed cats, *Bassariscus astutus* Lichtenstein (Carnivora: Procyonidae), and opossums visited all *O. turicata*-active caves during the entire study period (Tables 6 to 9). Furthermore, despite the fact that *O. turicata-active* caves in this study were off limits for people, there were human visits to MCC and WOC. At MCC, three visits by humans with an average duration of 14 minutes were recorded. At WOC, eight visits with an average duration of 10 minutes were recorded (Tables 7 and 9).

Table 5. A summary of all vertebrate animal visits and their durations from September 2015 to October 2016 at the *Ornithodoros turicata*-active caves at the GCSNA, TX. Maximum 392 days of visits per species were possible. Both total times spent, and the average duration of animal visits at the cave was measured in minutes. Asterisk denotes previously known *O. turicata* host.

Common name	Binomial name	Days visited	Time spent	Average duration
Black Vulture	*Coragyps atratus* Bechstei)	30	1012	33.73
Black-crested Titmouse	*Baeolophus atricristatus* Cassin	4	4	1.00
Black-tailed Jack Rabbit	*Lepus californicus* Gray	1	16	16.00
Bobcat	*Lynx rufus* Schreber	4	5	1.25
Canyon Wren	*Catherpes mexicanus* Swainson	361	973	2.70
Coyote*	*Canis latrans* Say	1	2	2.00
Eastern Fox Squirrel	*Sciurus niger* L.	22	55	2.50
Feral Swine*	*Sus scrofa* L.	1	2	2.00
Grey Fox	*Urocyon cinereoargenteus* Schreber	2	4	2.00
Human*	*Homo sapiens* L.	11	122	11.09
Nine-banded Armadillo*	*Dasypus novemcinctus* L.	2	2	1.00
Northern Cardinal	*Cardinalis cardinalis* L.	2	2	1.00
Opossum	*Didelphis virginiana* Kerr	39	299	7.67
Porcupine	*Erethizon dorsatum* L.	40	214	5.35
Raccoon	*Procyon lotor* L.	106	1768	16.68
Ring-tailed Cat*	*Bassariscus astutus* Lichtenstein	102	233	2.28
Rock Squirrel*	*Otospermophilus variegatus* Erxleben	113	417	3.69
Rattlesnake*	*Crotalus* spp. L.	3	5	1.67
Turkey Vulture	*Cathartes aura* L.	131	1503	11.47
White-footed Mouse	*Peromyscus leucopus* Rafinesque	80	179	2.23

* Denote known hosts of *O. turicata* from previous studies.

Table 6. A summary of all vertebrate animal visits and their durations from September 2015 to October 2016 at the Little Crevice Cave. There were total maximum 392 possible daily visit days per species. Both total times spent, and the average duration of animal visits at the cave was measured in minutes.

Common name	Binomial name	Days visited	Time spent	Average duration
Canyon Wren	*Catherpes mexicanus* Swainson	93	234	2.52
White-footed Mouse	*Peromyscus leucopus* Rafinesque	80	179	2.24
Raccoon	*Procyon lotor* L.	51	662	12.98
Ring-tailed Cat	*Bassariscus astutus* Lichtenstein	20	39	1.95
Black Vulture	*Coragyps atratus* Bechstein	18	911	50.61
Turkey Vulture	*Cathartes aura* L.	13	104	8.00
Porcupine	*Erethizon dorsatum* L.	11	47	4.27
Opossum	*Didelphis virginiana* Kerr	7	24	3.43
Rock Squirrel	*Otospermophilus variegatus* Erxleben	5	18	3.60
Bobcat	*Lynx rufus* Schreber	2	2	1.00
Nine-banded Armadillo	*Dasypus novemcinctus* L.	2	2	1.00
Feral Swine	*Sus scrofa* L.	1	2	2.00
Snake	*Crotalus* spp. L.	1	2	2.00

Table 7. A summary of all vertebrate animal visits and their durations from September 2015 to October 2016 at the Mad Crow Cave. There were total maximum 392 possible daily visit days per species. Both total times spent, and the average duration of animal visits at the cave was measured in minutes.

Common name	Binomial name	Days visited	Time spent	Average duration
Canyon Wren	*Catherpes mexicanus* Swainson	168	589	3.51
Turkey Vulture	*Cathartes aura* L.	95	963	10.14
Ring-tailed Cat	*Bassariscus astutus* Lichtenstein	43	95	2.21
Raccoon	*Procyon lotor* L.	33	780	23.64
Opossum	*Didelphis virginiana* Kerr	25	217	8.68
Eastern Fox Squirrel	*Sciurus niger* L.	22	55	2.50
Porcupine	*Erethizon dorsatum* L.	21	132	6.29
Black Vulture	*Coragyps atratus* Bechstein	6	41	6.83
Black-crested Titmouse	*Baeolophus atricristatus* Cassin	4	4	1.00
Human	*Homo sapiens* L.	3	42	14.00
Grey Fox	*Urocyon cinereoargenteus* Schreber	2	4	2.00
Black-tailed Jack Rabbit	*Lepus californicus* Gray	1	16	16.00
Coyote	*Canis latrans* Say	1	2	2.00

Table 8. A summary of all vertebrate animal visits and their durations from September 2015 to October 2016 at the Log Cave. There were total maximum 392 possible daily visit days per species. Both total times spent, and the average duration of animal visits at the cave was measured in minutes.

Common name	Binomial name	Days visited	Time spent	Average duration
Rock Squirrel	*Otospermophilus variegatus* Erxleben	108	399	3.69
Raccoon	*Procyon lotor* L.	11	154	14.00
Canyon Wren	*Catherpes mexicanus* Swainson	10	12	1.20
Ring-tailed Cat	*Bassariscus astutus* Lichtenstein	10	31	3.10
Turkey Vulture	*Cathartes aura* L.	9	218	24.22
Black Vulture	*Coragyps atratus* Bechstein	6	60	10.00
Bobcat	*Lynx rufus* Schreber	2	3	1.50
Opossum	*Didelphis virginiana* Kerr	2	29	14.50

Table 9. A summary of all vertebrate animal visits and their durations from September 2015 to October 2016 at the Wash Out Cave. There were total maximum 392 possible daily visit days per species. Both total times spent, and the average duration of animal visits at the cave was measured in minutes.

Common name	Binomial name	Days visited	Time spent	Average duration
Canyon Wren	*Catherpes mexicanus* Swainson	90	138	1.53
Ring-tailed Cat	*Bassariscus astutus* Lichtenstein	29	68	2.34
Turkey Vulture	*Cathartes aura* L.	14	218	15.57
Raccoon	*Procyon lotor* L.	11	172	15.64
Human	*Homo sapiens* L.	8	80	10.00
Porcupine	*Erethizon dorsatum* L.	8	35	4.38
Opossum	*Didelphis virginiana* Kerr	5	29	5.80
Northern Cardinal	*Cardinalis* L.	2	2	1.00
Rattlesnake	*Crotalus* spp. L.	2	3	1.50

Correlations between Ornithodoros turicata phenology and abiotic/biotic data from O. turicata-active caves. There were no correlations between the *O. turicata* phenology (i.e., the proportion of ticks collected during each survey) and the light intensity values from the week of, one-, two-, and three-weeks prior to the tick survey (RMSE = 0.12 each, $P = 0.79, 0.81, 0.92, 0.93$ for the week of, one-, two-, and three-weeks prior to tick survey, respectively) (Figure 12). Similarly, there were no correlations between the *O. turicata* phenology and the relative humidity values from the week of, one-, two-, and

three-weeks prior to the tick survey data (RMSE = 0.11 each, P = 0.02, 0.11, 0.19, 0.42 for the week of, one-, two-, and three-weeks prior to tick survey, respectively) (Figure 13). However, there were significant correlations between the *O. turicata* phenology and the temperatures from the week of, one-, two-, and three-weeks prior to the tick survey (P = 0.01 each, RMSE = 0.082, 0.071, 0.080, and 0.086 for the week of, one-, two-, and three-weeks prior to tick survey, respectively) (Figure 14).

There were no correlations between the *O. turicata* phenology, and a number of animal species visited the *O. turicata*-active caves in the week of, one-, two-, and three-weeks prior to the tick survey (RMSE = 0.11 each, P = 0.72, 0.40, 0.63, 0.39 for the week of, one-, two-, and three-weeks prior to tick survey, respectively) (Figure 15). Similarly, there were no correlations between the *O. turicata* phenology and the average animal visit durations at the *O. turicata*-active caves in the week of, one-, two-, and three-weeks prior to the tick survey (RMSE range 0.10 to 0.11, P = 0.04, 0.07, 0.07, 0.02 for the week of, one-, two-, and three-weeks prior to tick survey, respectively) (Figure 16). However, two *O. turicata*-active caves with the highest animal visits also had the highest total *O. turicata* collections (Table 10).

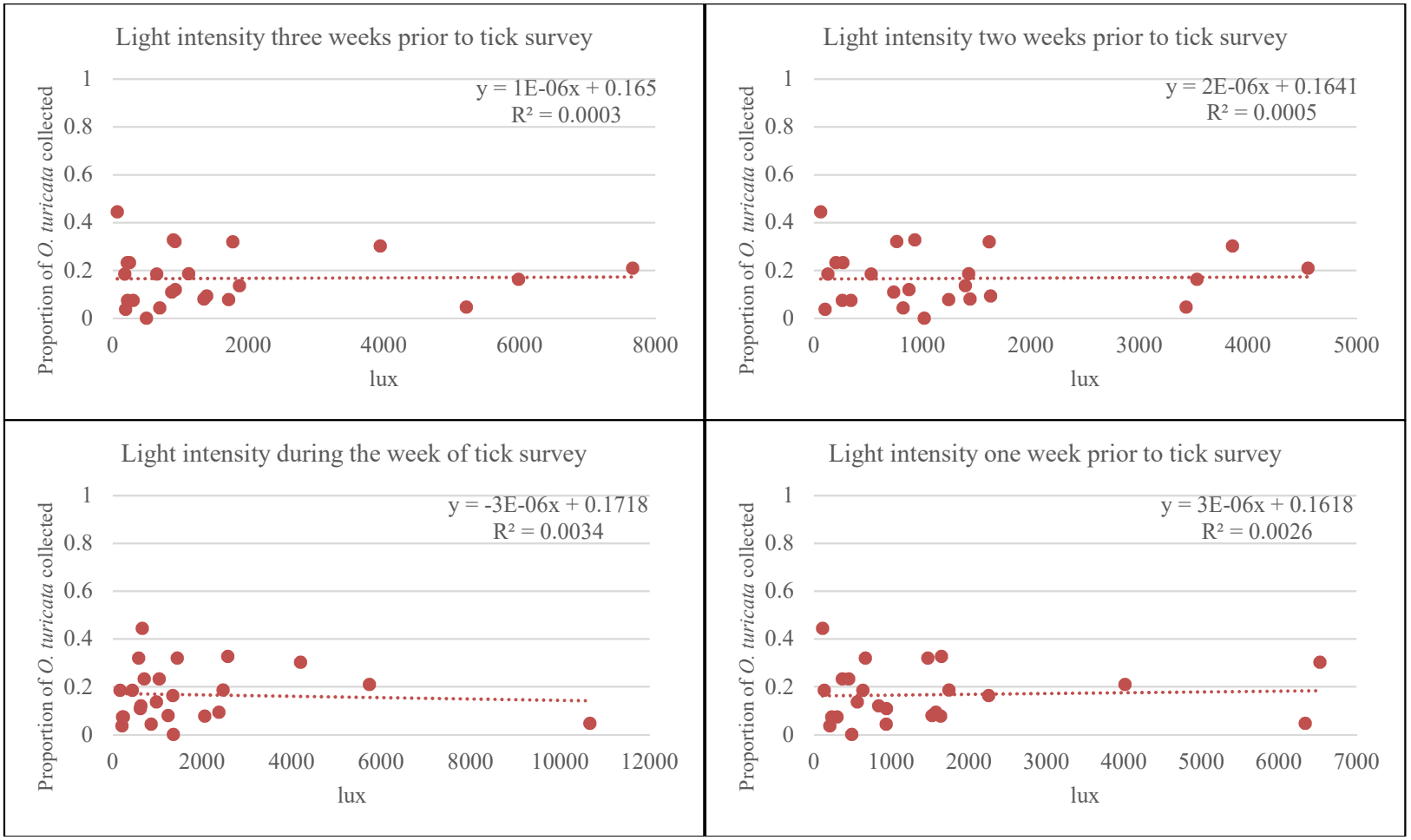

Figure 12. The proportion of *Ornithodoros turicata* captured expressed as functions of the combined average weekly light intensity values from September 2015 to October 2016 at the GCSNA, TX. The average weekly light intensity values of *O. turicata*-active caves from the week of, one-, two-, and three-weeks prior to tick survey are presented. There were no significant correlations between light intensities, and normalized tick captured data (RMSE = 0.12 each, $P = 0.79, 0.81, 0.92, 0.93$ for the week of, one-, two-, and three-weeks prior to tick survey, respectively).

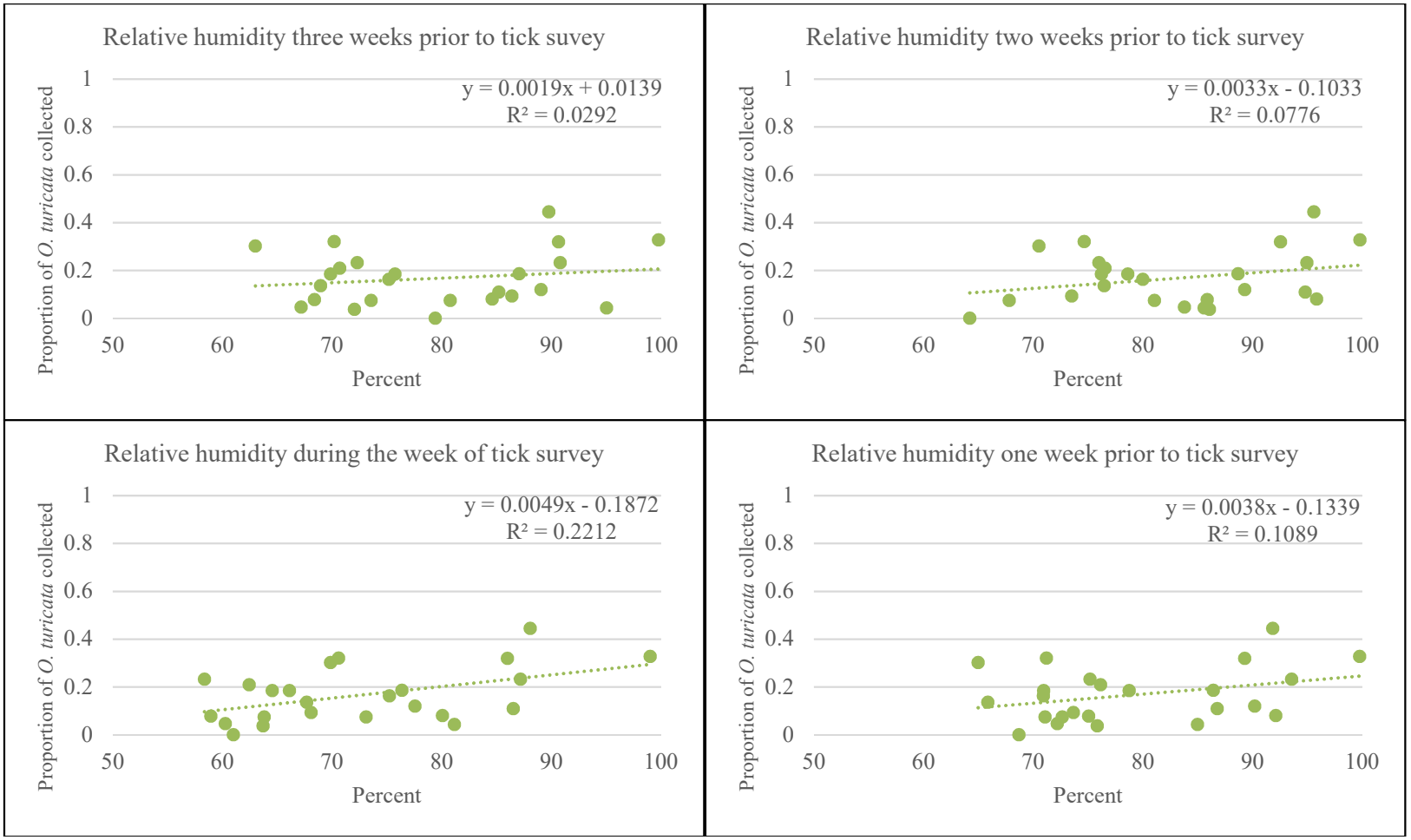

Figure 13. The proportion of *Ornithodoros turicata* captured expressed as functions of the combined average weekly relative humidity values from September 2015 to October 2016 at the GCSNA, TX. The average weekly relative humidity values of *O. turicata*-active caves from the week of, one-, two-, and three-weeks prior to tick survey are presented. There were no significant correlations between relative humidity, and normalized tick captured data (RMSE = 0.11 each, $P = 0.02, 0.11, 0.19, 0.42$ for the week of, one-, two-, and three-weeks prior to tick survey, respectively).

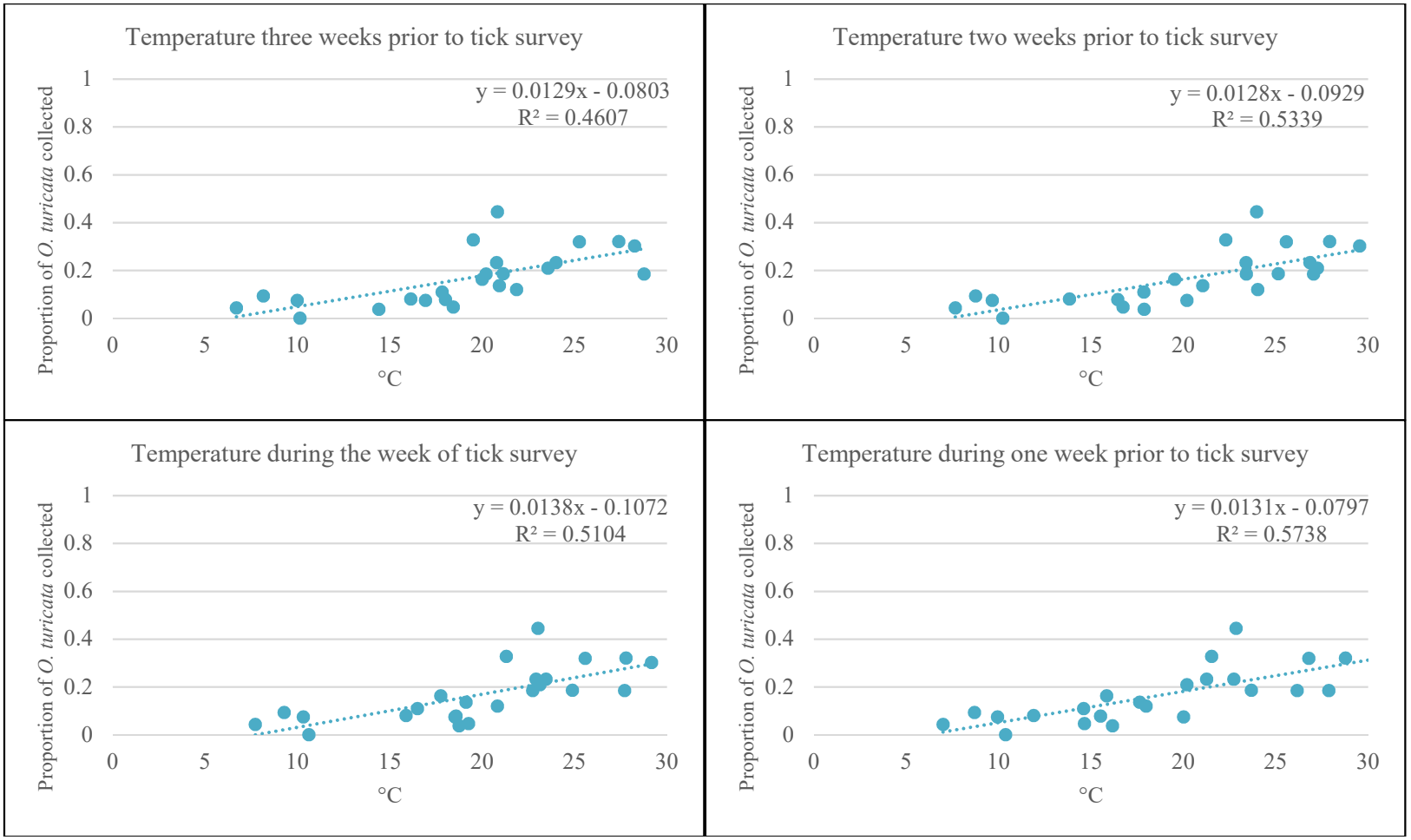

Figure 14. The proportion of *Ornithodoros turicata* captured expressed as functions of the combined average weekly temperature values from September 2015 to October 2016 at the GCSNA, TX. The average weekly temperature values of *O. turicata*-active caves from the week of, one-, two-, and three-weeks prior to tick survey are presented. There were significant correlations between temperature, and normalized tick captured data (P = 0.01 each, RMSE = 0.082, 0.071, 0.080, and 0.086 for the week of, one-, two-, and three-weeks prior to tick survey, respectively).

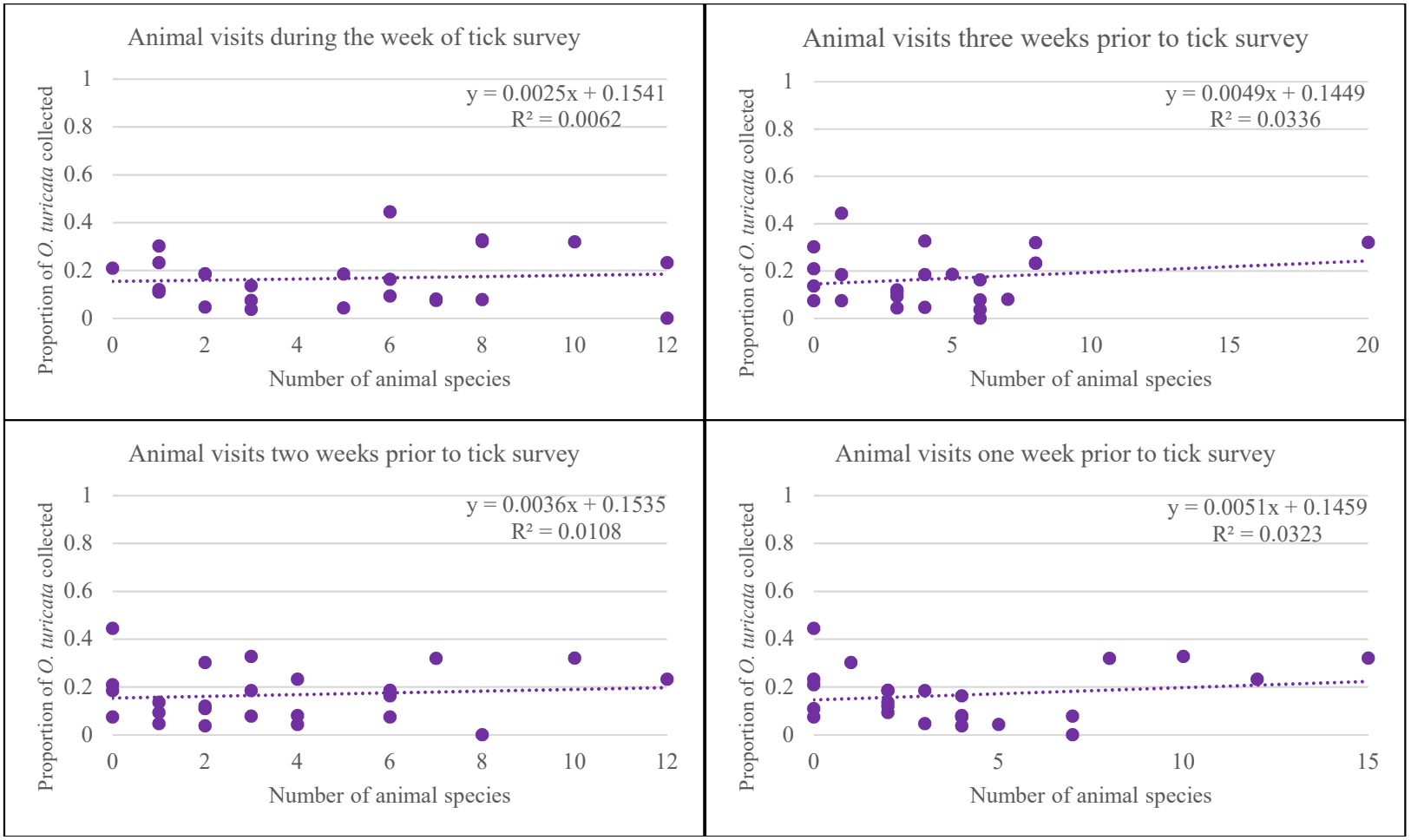

Figure 15. The proportion of *Ornithodoros turicata* captured expressed as functions of the number of animal species visits from September 2015 to October 2016 at the GCSNA, TX. The combined animal species visits at the *O. turicata*-active caves from the week of, one-, two-, and three-weeks prior to tick survey are presented. There were no significant correlations between animal visits, and normalized tick captured data (RMSE = 0.11 each, P = 0.72, 0.40, 0.63, 0.39 for the week of, one-, two-, and three-weeks prior to tick survey, respectively).

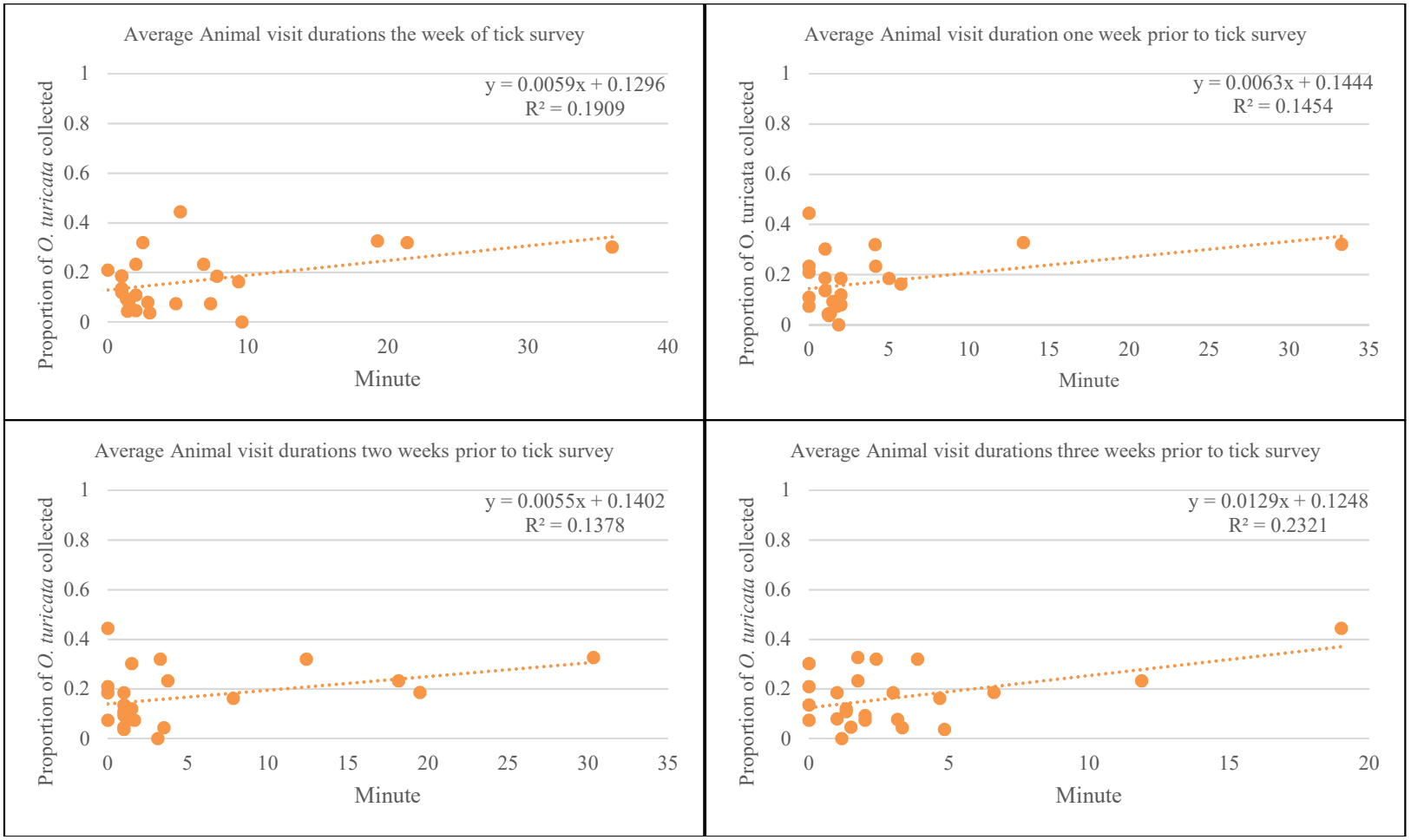

Figure 16. The proportion of *Ornithodoros turicata* captured expressed as functions of the average animal visit duration from September 2015 to October 2016 at the GCSNA, TX. The average animal visit duration at the *O. turicata*-active caves from the week of, one-, two-, and three-weeks prior to tick survey are presented. There were no significant correlations between animal visits, and normalized tick captured data (RMSE range 0.10 to 0.11, P = 0.04, 0.07, 0.07, 0.02 for the week of, one-, two-, and three-weeks prior to tick survey, respectively).

Table 10. A summary table of total *Ornithodoros turicata* captured and total vertebrate animal visits at the *O turicata*-active caves from September 2015 to October 2016 at the GCSNA, TX.

	LCC	MCC	LOG	WOC
O. turicata Captured	275	103	43	27
Animal Visits	304	424	158	169

LCC= Little Crevice Cave, MCC= Mad Crow Cave, LOG= Log Cave, WOC= Wash Out Cave.

Kissing bug capture and pathogen analysis. Nine kissing bugs (*Triatoma* spp.) were collected, and five were tested positive for *T. cruzi*.

Ornithodoros turicata at the cave. The coloration of *O. turicata* matched the substrates on the cave floor in which they were found. Ticks were difficult to locate without their movement (Figure 17) During the tick survey; ticks often appeared within minutes of placing attractant (i.e., dry ice). Furthermore, ticks often dropped from the small crevices and cavities located on the cave walls and the ceiling near the tick survey site. Finally, there were multiple stages of *O. turicata* captured during each tick survey. The composition of *O. turicata* collected included adults and all nymphal instars. However, no *O. turicata* larvae were collected.

Harvestmen, Leiobunum townsendi Weed, (Opiliones: Sclerosomatidae) aggregation at the Ornithodoros turicata-active caves. Aggregations of harvestmen were readily observed in all *O. turicata*-active caves. The harvestmen clusters were present not only during tick survey visits, but the size and the movements of these harvestmen clusters were sufficient to trigger the motion sensors of trail cameras used in this study and subsequently recorded the animal activities in and around the harvestmen cluster (Figure 18).

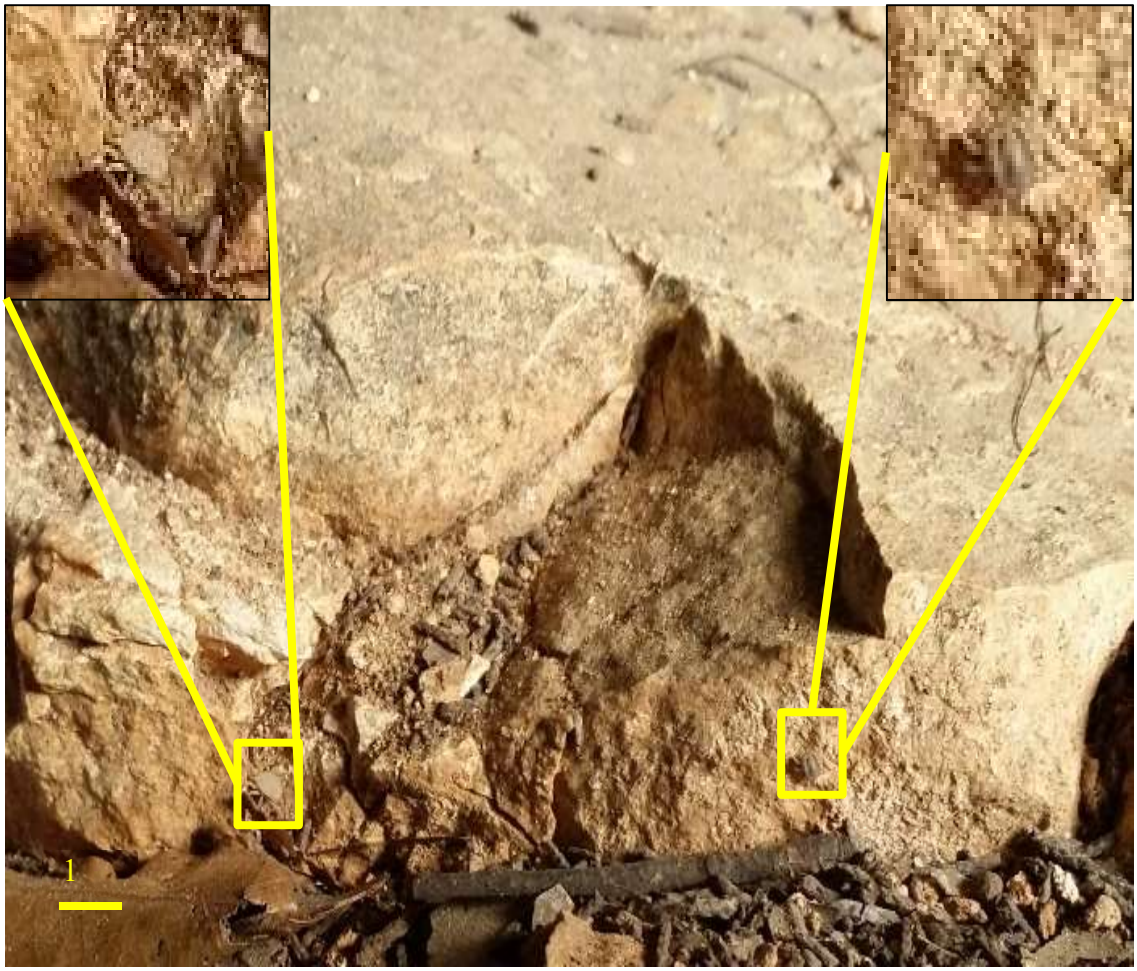

Figure 17. An image of *Ornithodoros turicata* at the GCSNA, TX. Two *O. turicata* (yellow boxes) are seen climbing over the rock toward the source of CO_2 (Dry ice block, not shown) at the Little Crevice Cave on the June 9th, 2016. Their coloration matches the substrate, and it is difficult to locate them without their movement.

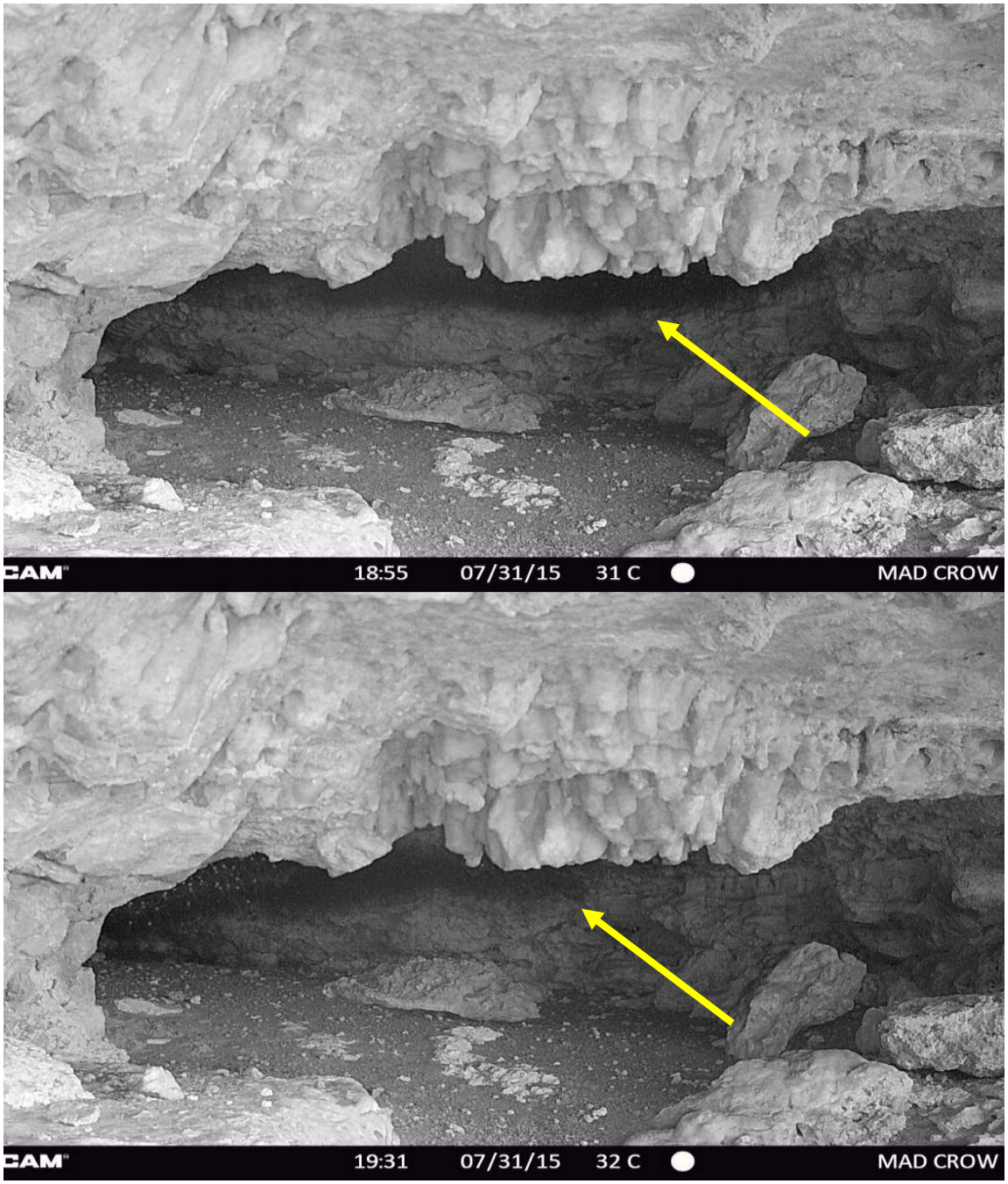

Figure 18. Images of Harvestmen, *Leiobunum townsendi*, aggregation at the GCSNA, TX. Harvestmen clusters (indicated by yellow arrows) recorded on July 31, 2015, at the Mad Crow Cave. The size and the movement of the harvestmen clusters are sufficient to trigger the motion sensors of trail cameras used in this study.

Vertebrate animal predation of harvestmen clusters at the Ornithodoros turicata-active caves. Several vertebrate species consumptions of harvestmen clusters were observed during this study (Figures 19, 22, 23, and 24). However, each animal employed different tactics to capture and consume the harvestmen cluster. For example, raccoons actively reached up into the harvestmen cluster, grasping a heap of harvestmen from the cluster to consume (Figure 20) and often consume the harvestmen cluster as a group (Figure 21). On the other hand, canyon wrens, targeted a single harvestmen among the cluster at a time (Figure 22), whereas ring-tailed cats did not actively pursue the harvestmen cluster located on the ceiling and only consumed when the cluster broke off and landed on the cave floor (Figure 23). Finally, a single incident where a rock squirrel was standing in the midst of a cluster to consume the harvestmen was observed (Figure 24).

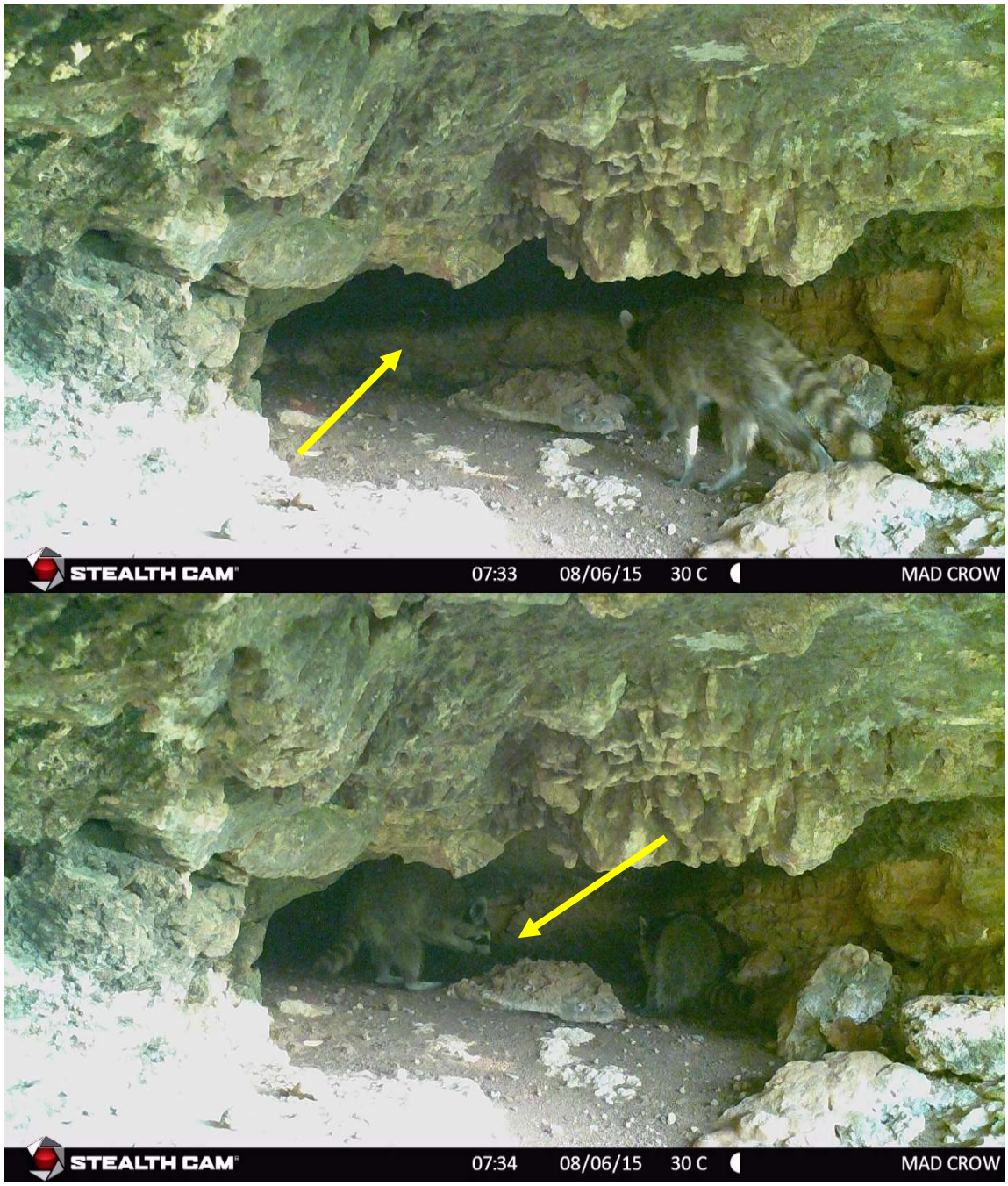

Figure 19. Images of raccoon, *Procyon lotor*, consuming Harvestmen, *Leiobunum townsendi*, at the GCSNA, TX. Above: A raccoon is approaching harvestmen aggregation cluster (indicated by yellow arrows) Below: Two raccoons consume the harvestmen clusters after knocking them down from the cave ceiling. Images captured on August 6th, 2015 at the Mad Crow Cave

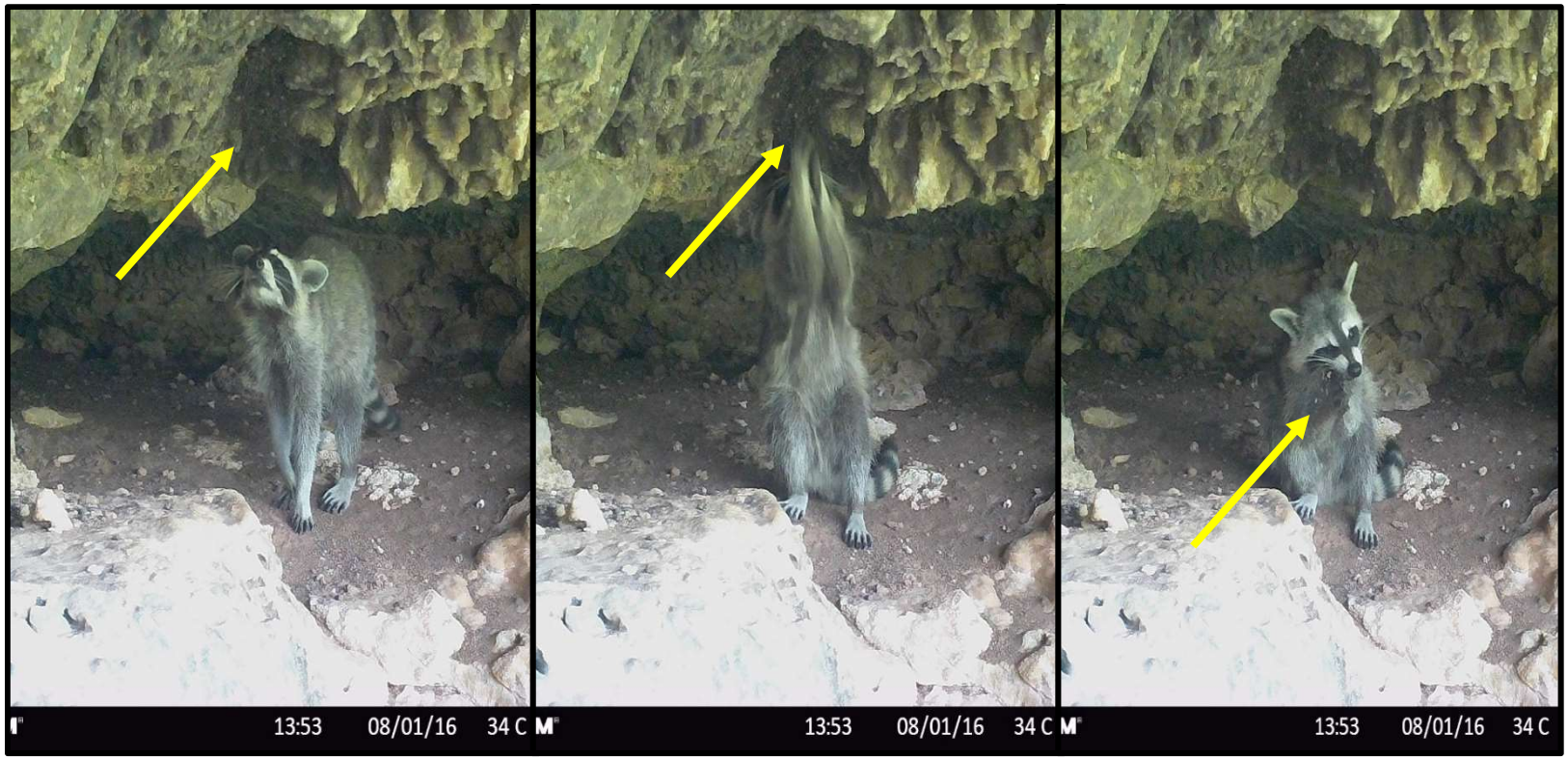

Figure 20. Sequential images of a raccoon, *Procyon lotor*, eating harvestmen, *Leiobunum townsendi*, at the GCSNA, TX. A raccoon approaches the harvestmen cluster located on the cave ceiling (indicated by yellow arrows), removes a portion of the cluster to consume. Images recorded on August 1st, 2016 at the Mad Crow Cave

Figure 21. An image of a group of raccoons, *Procyon lotor*, consuming harvestmen, *Leiobunum townsendi*, aggregation clusters at the GCSNA, TX. Several Harvestmen clusters. Image recorded on August 14th, 2016 at the Mad Crow Cave.

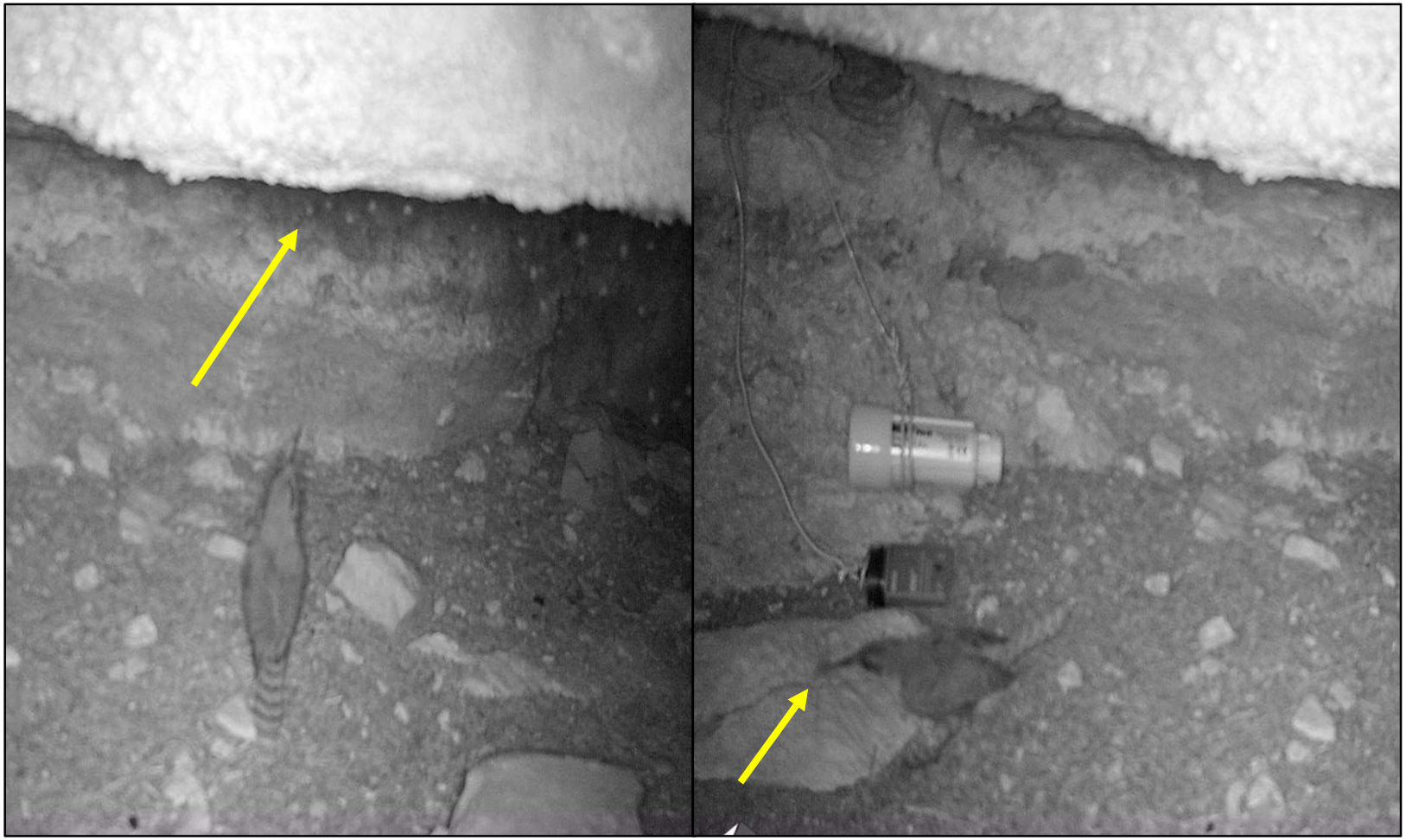

Figure 22. Sequential images of a canyon wren, *Catherpes mexicanus*, consuming a harvestman, *Leiobunum townsendi*, at the GCSNA, TX. A canyon wren approaches the harvestmen cluster (yellow arrows) located on the cave ceiling but removes only single harvestmen at a time to consume. Image recorded on May 14th, 2016.

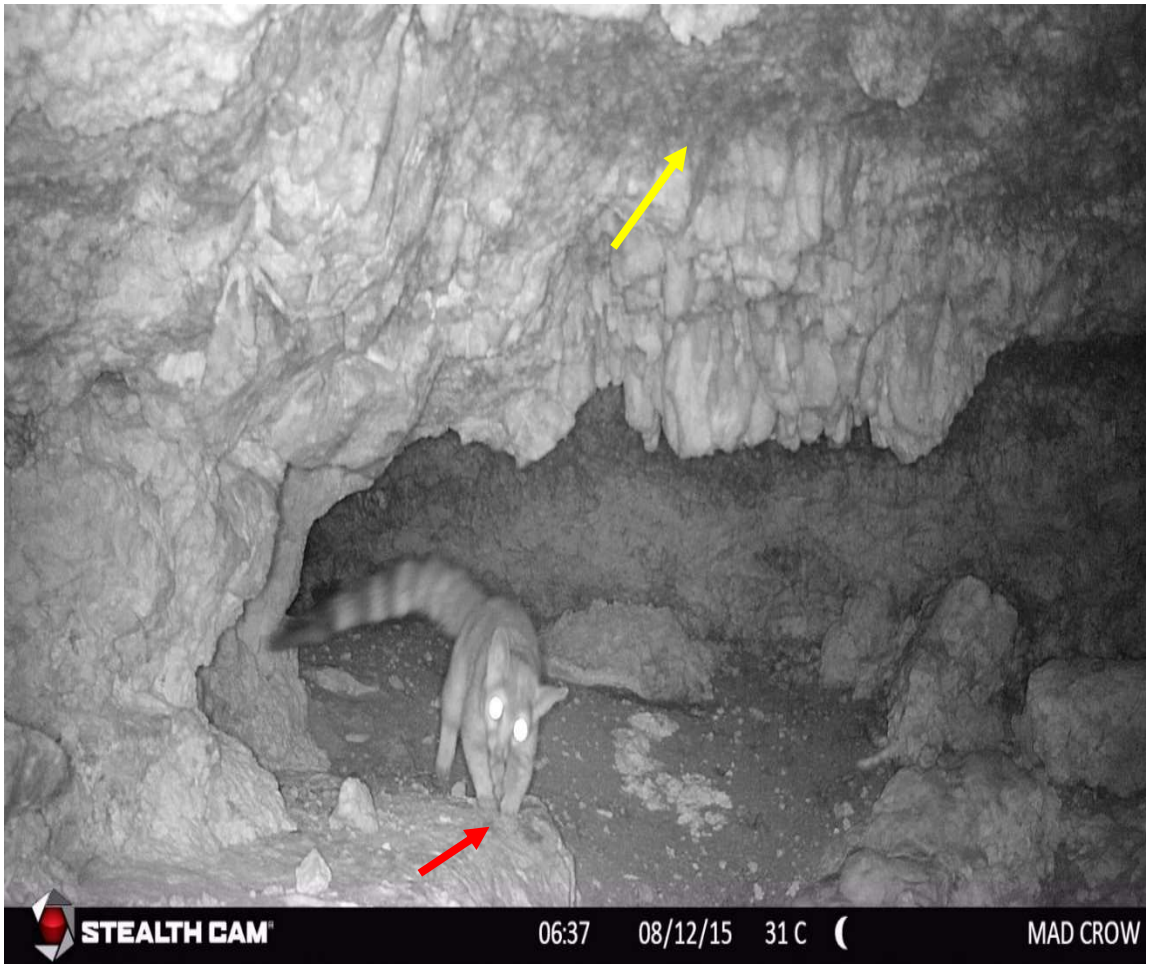

Figure 23. An image of a ring-tailed cat, *Bassariscus astutus*, consuming harvestmen, *Leiobunum townsendi*, at the GCSNA, TX. Harvestmen clusters can be seen on the ceiling of the cave (yellow arrow), once a portion of harvestmen cluster drops on the cave floor, a ring-tail subdue them using its front paws (indicated by red arrow) to consume. Image recorded on August 12th, 2015 at the Mad Crow Cave.

Figure 24. An image of a rock squirrel, *Otospermophilus variegatus*, consuming harvestmen, *Leiobunum townsendi*, at the GCSNA, TX. A rock squirrel. A rock squirrel (indicated by red arrow) is standing in the midst of harvestmen cluster (indicated by yellow arrow) to consume. Image recorded on August 27th, 2015 at the Little Crevice Cave.

Vertebrate animal grooming activities at the Ornithodoros turicata-active caves. Several animals visiting the *O. turicata*-cave conducted extensive grooming near the site where tick surveys were conducted. For example, there were several incidences where opossum groomed for 20+ minutes while sitting on top of the *O. turicata* survey site at the MCC. On the other hand, raccoons commonly conducted grooming sessions after consuming harvestmen clusters while sitting on top of the *O turicata* survey sites at the MCC. Finally, black vultures often utilized *O. turicata*-caves to take refuge from inclement weather and were recorded grooming themselves at the LCC *O. turicata* survey site (Figure 25).

Canyon wren, Catherpes mexicanus, eating Ornithodoros turicata at O. turicata-active caves. A series of images taken immediately upon tick survey completion at LCC on July 28th, 2015 revealed that canyon wren actively sought after *O. turicata* remaining at the tick survey site and proceeded to catch and consume them (Figure 26).

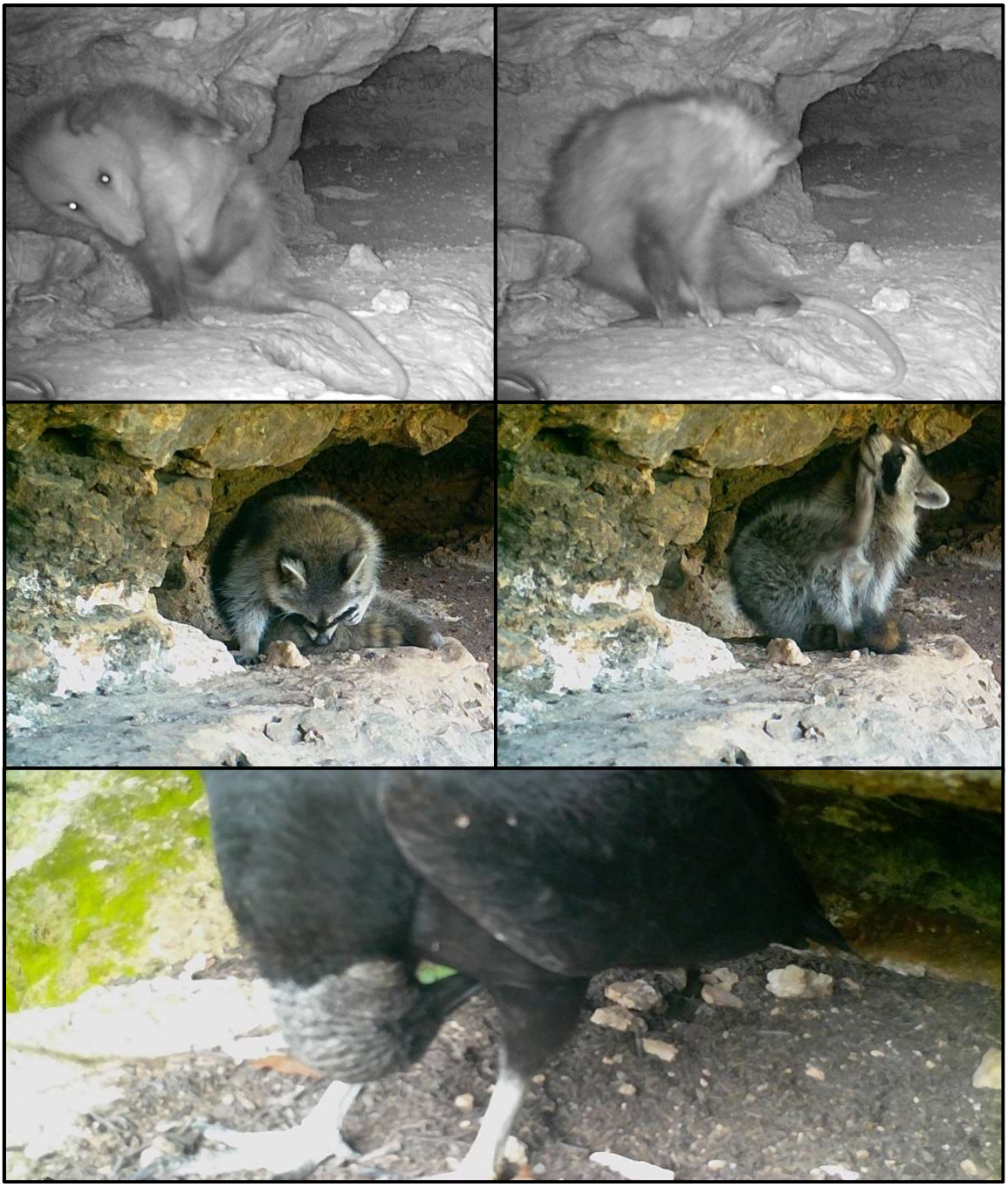

Figure 25. Images of Vertebrate animal grooming activities at the *Ornithodoros turicata*-active caves at the GCSNA, TX. Several animals visiting the *O. turicata*-cave conducted extensive grooming sessions near the site where tick surveys were conducted. Top: An opossum, *Didelphis virginiana,* groomed for 20+ minutes while sitting on top of the *O. turicata* survey site at the Mad Crow Cave. Middle: A raccoon, *Procyon lotor,* conducts grooming session after consuming harvestmen cluster for 20+ minutes while sitting on top of the *O turicata* survey sites at the Mad Crow Cave. Bottom: A black vulture, *Coragyps atratus,* takes a refuge from the rain while grooming itself at the Little Crevice Cave *O. turicata* survey site.

Figure 26. Sequential images of a canyon wren, *Catherpes mexicanus*, eating *Ornithodoros turicata* at the GCSNA, TX. This series of images were taken after tick survey where several *O. turicata* were mobile on the cave floor. A canyon wren approached and consumed an *O. turicata* (yellow arrows) located on the cave floor on top of the pebble. Images recorded on July 28th, 2015 at the Little Crevice Cave.

Discussion

This is the first field study that reports correlations between *O. turicata* phenology and the abiotic and biotic factors found in the cave environment. Interestingly, there were strong correlations between changes in patterns of relative humidity and temperature across all four *O. turicata*-active caves during the study (Tables 3 and 4), while no significant correlations were observed for light intensity (Table 2). The difference in the light intensity was expected due to the difference in the orientation of cave entrances among *O. turicata*-active caves. However, LCC was the only cave with North facing entrance while remaining cave entrances faced East, yet there were no light intensity correlations among the three East-facing *O. turicata*-active caves. Another explanation for the inconsistent light intensities observed among *O. turicata*-active caves may be the varying degrees of vegetation coverage near the cave entrance. Indeed, seasonal changes in the vegetation coverage near the cave were observed. However, this field study did not accurately measure such variation.

The light intensity may not play a vital role in assessing *O. turicata* activity in cave environments based on the fact that there were no correlations between *O. turicata* phenology and the weekly average of light intensity (Figure 12). This inference was made based on the notion that aligning host seeking behavior to circadian rhythm (i.e., only during the night) may be maladaptive since it would reduce their chance to acquire bloodmeal. The study by Adeyeye and Philips (1996), which examined the photoperiodic response in *O. turicata* in a laboratory setting made a similar conclusion where circadian rhythm may not be relevant for the feeding behavior of *O. turicata*.

The lack of a correlation between *O. turicata* phenology and relative humidity (Figure 13) was an expected result. The overall range of humidity at *O. turicata*-active caves was between 43% to 100% and only nine weeks out of 56 weeks had the relative humidity below 60% (Figure 10). *Ornithodoros turicata* is known be active at humidity greater than 60% (Adeyeye and Butler 1991). Therefore, based upon the information that most caves maintain humidity above 60% the majority of the time an argument can be made that estimating *O. turicata* activity in a cave environment that can maintain its relative humidity optimal to *O. turicata* is ill-advised. On the other hand, correlations between *O. turicata* phenology and changes in temperature patterns observed during the study (Figure 14) was similar to previous studies. For example, field evaluation study of CO_2 baited *O. turicata* survey at Gopher tortoise burrow by Adeyeye and Butler (1991) reported the highest *O. turicata* activities during the months of June to October where burrow temperatures ranged from 21.40 to 33.40 °C. In this study, the highest tick activities at *O. turicata*-active caves were observed during the months of June and August where cave temperatures ranged from 21.53 to 30.14 °C (Table 1). Therefore, ambient temperature may serve as a good indicator for tick activities in a cave environment.

Highest overall tick activities observed at the two *O. turicata*-active caves with the highest animal activities in terms of total animal species visited and average visit duration was logical as frequent visits from an animal would have increased the chance of an *O. turicata* population in these caves acquiring a bloodmeal which ultimately increase their population densities. However, the fact that *O. turicata* phenology had no correlations with the number of animals visited nor the average of visit duration at the *O. turicata*-

active cave were unexpected (Figure 15 and 16). A combination of *O. turicata*'s ability to endure prolonged starvation and the tick survey method employed during this study may provide a plausible explanation for such observations. *Ornithodoros turicata* can endure starvation period that measures in years (Francis 1938, Adeyeye and Butler 1989) and the CO_2 baited tick survey method used in this study is designed to attract host-seeking ticks. Therefore, frequent animal activities may equate to constant provisions of bloodmeals to the *O. turicata* population in the cave, facilitating an equilibrium state in active host seeking activities among *O. turicata* population. This equilibrium state may ultimately result in a constant proportion of *O. turicata* in a population to be collected independently of animal activity.

The population structure of *O. turicata* found in cave environments may be more complex than that of non-nidicolous Ixodid ticks. There are three factors which can contribute to relative complexity *O. turicata* population structure. The first factor is their longevity and generation time which may be measured in decades compared to three or less years in the Ixodid ticks (Oliver 1989). These long generation times may allow *O. turicata* to experience different host dynamics that utilize cave environments in different manners than non-nidicolous Ixodid ticks, resulting in *O. turicata* population structures that vary based on specific location and time. The second factor for *O. turicata* population structure complexity is the gonotrophic strategy employed by *O. turicata*. Unlike the single gonotrophic cycle with a large egg mass production strategy employed by Ixodid ticks, *O. turicata* employ multiple gonotrophic cycles with a small egg batch production strategy (Sonenshine and Roe 2014). This gonotrophic cycle strategy may lead to

overlapping generations and differently aged siblings within the population, which then add the complexity of demographical distribution within the population structure. The third factor contributing the *O. turicata* population structure complexity is their ability to utilize the unique advantages that cave environments provide. As observed in this study, each *O. turicata*-active cave maintained relative humidity at an optimal level (<60%) most of the year. In addition, each cave was frequently visited by potential hosts during the year. Finally, each cave provided numerous protective refuge cavities and crevices in its three-dimensional surfaces, which were found to be occupied by *O. turicata* during this study. These caves provided refuges, coupled with the ability to endure a prolonged starvation period measured in years (Francis 1938, Adeyeye and Butler 1989) may allow the population found in a given *O. turicata*-active cave to remain indefinitely, resulting in complex population structures.

Ornithodoros turicata may be a part of complex community structure within in the cave environment based on the observed interactions (i.e., direct and indirect) between the members of the community and the roles they play during this study. For example, the harvestmen aggregation seem to serve as an attractant for some animals as a source of nutrient despite their chemical defense to deter predators (Ekpa et al. 1985). On the one hand, harvestmen aggregation may be beneficial to *O. turicata* in the cave via increased chance of bloodmeal acquisitions from the potential hosts that are attracted to the harvestmen clusters. On the other hand, harvestmen aggregation may be pernicious to *O. turicata* by attracting their shared predator (e.g., canyon wren) to the cave. Also, *O. turicata* may be beneficial to harvestmen population as their act of parasitism can reduce

the host activities at the cave (Figure 27). Indeed, heavy Argasid infestation has been associated with the abandonment of nest by their host (King et al. 1977, Duffy 1983, Justice-Allen et al. 2016). Furthermore, it is plausible that community structure can be driven by indirect effects of the environment. For example, the cave environment may shield both biotic and abiotic factors from the outside environment. These indirect effects may then cascade down to each member of the community, resulting in a unique *O. turicata* community structure at specific *O. turicata*-active cave (Figure 27). Therefore, the *O. turicata* community structure should be assessed while considering both direct and indirect effects on the members of the community as well as the cave environment in which they are found.

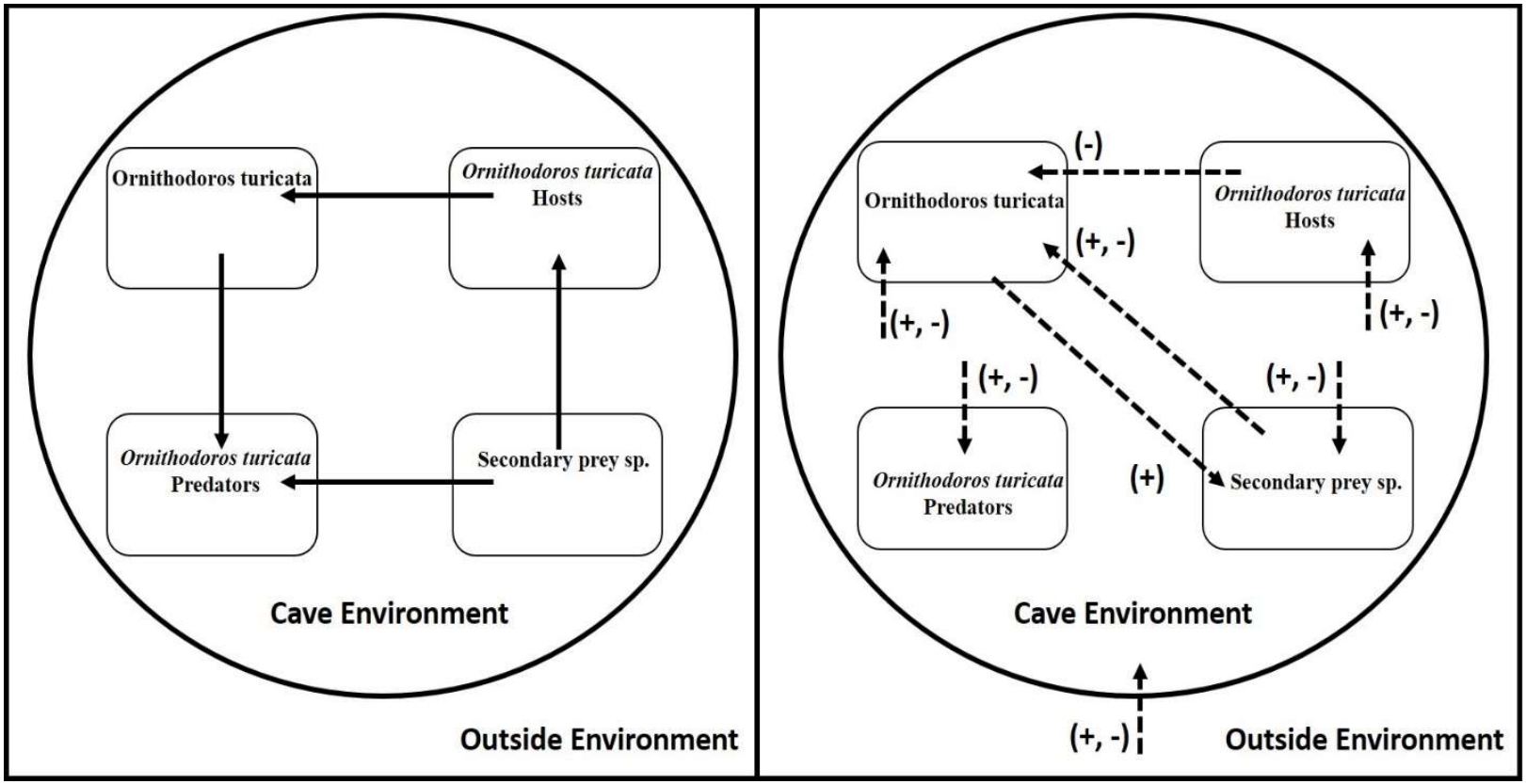

Figure 27. Two hypothetical representations of *Ornithodoros turicata* community interactions at *O. turicata*-active caves at the GCSNA, TX. The community depicted consist of *O. turicata*, *O. turicata* predators (i.e., canyon wren), potential *O. turicata* hosts (i.e., raccoon) and secondary prey species (i.e., Harvestmen). Left: Representation of direct trophic interactions within the *O. turicata* community. Solid arrow denotes the energy flow between organism via predation or parasitism. There are no direct interactions between secondary prey and *O. turicata* found in the cave environment. Right: Representation of indirect interactions within the *O. turicata* community. Dash arrows denote the direction and + indicates a positive effect and – indicates a negative effect. A secondary prey species may have both positive and negative effects on *O. turicata* by attracting potential hosts and predator species, respectively. A host species may have negative effects on *O. turicata* by grooming. Cave environment may have both positive and negative effects on each member of *O. turicata community* via abiotic factors. Both positive and negative influence from the outside environment may cascade through the cave into the *O. turicata* community. Figure generated based on the concept presented in Eubanks and Finke (2014).

Several relevant future studies can be suggested based on the observation made during this study. The first suggested study builds on the foundational information provided assessing the correlation between *O. turicata* phenology and the biotic and abiotic factors in a cave environment. At the conclusion of this study, the effects of biotic and abiotic factors in these cave environments with the microbiomes found in *O. turicata* are unknown. A study examining the longevity and viability of *O. turicata* transmitted pathogens (e.g., *B. turicatae*) in these cave environments would provide a tool in epizootic and endemic risk management of ASFV and TBRF. The second study can be suggested based on the fact that animals that made the most frequent visits and relatively longer durations in this study were previously unknown hosts of *O. turicata*. Specifically, the interaction study between *O. turicata* and raccoons, black vultures, and turkey vultures may shed light on the dispersal strategies employed by the *O. turicata*. Furthermore, the only study addressing *O. turicata* dispersal in the field is the mark and recapture study in gopher tortoise burrows by Adeyeye and Butler (1989). Their study suggested little or no intra-burrow movements of *O. turicata* which also suggest that *O. turicata* dispersal over greater geographical distance is not likely. Therefore, a comprehensive population genetic study using geographically separated *O. turicata* populations may prove vital in solving their dispersal strategies and population structures.

CHAPTER III

DOMESTIC PIG, *SUS SCROFA DOMESTICUS*, ERXLEBEN (ARTIODACTYLA: SUIDAE), IMMUNE RESPONSES AFTER REPEATED *ORNITHODOROS TURICATA* DUGÈS (IXODIDA: ARGASIDAE) CHALLENGES

Introduction

Human tick-borne relapsing fever is a disease in the U.S. caused by several species of *Borrelia* (Dworkin et al. 2002). Over 500 cases of TBRF cases have been reported from 12 western states between 1990-2011 (Forrester et al. 2015). However, these TBRF cases may have been underreported since TBRF is not nationally reportable as there are no standard case definitions (Forrester et al. 2015) in the U.S. Typical symptoms of TBRF include recurring episodes of fever and non-specific malaise (e.g., headache, myalgia, arthralgia, shakings chills and abdominal pains) (Dworkin et al. 2002). In North America, tick-borne relapsing fever *Borrelia* spp. are primarily transmitted by *Ornithodoros* spp (Ixodida: Argasidae) and named based on the tick species (Goubau 1984). For example, *B. parkerii* is associated with tick *O. parkeri*, whereas *B. hermsii* and *B. turicatae* are associated with *O. hermsi* and *O. turicata*, respectively (Rawlings 1995, Dworkin et al. 2008). Generally, TBRF cases occurring at higher altitudes (1,500 to 8,000 feet) in the western and midwestern regions of the U.S. are associated with *O. hermsii* (Dworkin et al. 2002). There has been only one instance where *B. parkerii* spirochete was isolated from *O. parkeri* (Gage et al. 2001). *Ornithodoros turicata*, on the other hand, is often found in prairie dog dens, gopher tortoise burrows, and cave environments and associated with the

TBRF cases occurring in arid regions of the southern U.S. (Fihn and Larson 1980, Rawlings 1995, Dworkin et al. 2002, Dworkin et al. 2008).

In laboratory challenge experiments, *Ornithodoros turicata* can be infected with and ASFV (Hess et al. 1987), which is a highly contagious disease of swine and a global threat to the swine industry (Bech-Nielsen et al. 1995, Costard et al. 2009). First discovered in 1920 in Kenya, the ASFV exists in a sylvatic cycle that involves two species of African warthogs, *Phacochoerus aethiopicus* Pallas (Artiodactyla: Suidae) and *Phacochoerus africanus* Gmelin as hosts and *O. moubata* as the vector (Montgomery 1921). The ASFV in their natural sylvatic cycles does not pose any adverse effect on the hosts (Plowright et al. 1994, Costard et al. 2009). However, either ASFV infected vectors or the sylvatic hosts can transmit ASFV to domestic swine, *Sus scrofa domesticus* Erxleben (Artiodactyla: Suidae) populations with devastating consequences (Costard et al. 2009). Once infected, the mortality rate in *S. scrofa* populations is nearly 100% via acute hemorrhagic fever (Plowright et al. 1994). African swine fever virus can be transmitted via several *Ornithodoros* spp. not only in experimental settings but also in natural settings. Several epidemic ASFV outbreaks have occurred outside of the continent of Africa including Iberian Peninsula, Caucasus regions, South Americas, and Caribbean nations (Hess et al. 1987, Ordas-Alvarez and Marcotegui 1987, Plowright et al. 1994, Bech-Nielsen et al. 1995, Simeón-Negrín and Frías-Lepoureau 2002, Penrith et al. 2004, Costard et al. 2009).

The U.S. is the world's third-largest producer of pork products. Pork and pork-product production in the U.S. are mostly accomplished in large-scale commercial

operations with aggregated and confined facilities where diseases can spread rapidly (Giamalva 2014). In 2015, the U.S. produced more than 110 metric tons of pork products valued at $22 billion personal income and 550,000 U.S. jobs (National Pork Producers Council 2015, USDA 2016). Therefore, an introduction of the ASFV to the U.S. may potentially induce severe financial burdens on the economy. Currently, the ASFV control strategy is limited to the depopulation of affected hosts due to the lack of vaccine and treatment (Costard et al. 2009). In addition to direct financial loss from a depopulation strategy, the ASFV episodes can result in the loss of international trade status, further increasing the financial burden on the affected countries (Arias and Sánchez-Vizcaíno 2002, Simeón-Negrín and Frías-Lepoureau 2002).

Argasid ticks, including *O. turicata*, may be found in peridomestic and domestic settings (Cooley and Kohls 1944), with their ecology overlapping that of feral swine. However, the level of feral swine exposure to *O. turicata* in the U.S. is unknown. The omnivorous behavior, habitat association, and landscape usage of feral swine suggest there are opportunities for *O. turicata*-feral swine interactions (Coombs and Springer 1974, Cushman et al. 2004, Wyckoff et al. 2009, Campbell and Long 2010). This setting poses a concern of rapid spread of ASFV in the U.S. should *O. turicata* serve as the vector of ASFV to feral and domestic pig populations. Indeed, evidence of antibodies generated against *B. turicatae* in feral swine populations in Texas, U.S. reported by Sanders (2011) suggests potential host-vector interactions between *O. turicata* and feral swine. Furthermore, direct interaction between feral swine and domestic swine interactions also have been documented (Witmer et al. 2003, Wyckoff et al. 2009). Therefore, it is

important to investigate the host immune response against *O. turicata* using domestic swine as a model host and explore its potential use in developing *O. turicata* specific surveillance strategy.

Host immune responses to repeated exposure to Argasid tick blood-feeding have been reported. Canals et al. (1990) reported a significant increase of immunoglobulin G (Ig) production among domestic swine when challenged by *Ornithodoros erraticus* Lucas, a vector of ASFV in Spain. Similarly, Wozniak et al. (1995) reported an increase of IgG production among rabbits that were challenged with several *Ornithodoros spp*., including *O. turicata.* To our knowledge, there have been no attempts to investigate domestic swine immune response to *O. turicata* challenge. The primary goal of this study was to examine domestic swine immune responses to repeated *O. turicata* blood-feedings.

Materials and Methods

Ticks (O. turicata) Colony. Adult and late instar *O. turicata* nymphs used in this study were starved for seven months and obtained from a colony maintained at the Tick Research Laboratory at Texas A&M AgriLife Research, College Station, TX, U.S. The colony originated from specimens collected in a natural cavern in Travis County, TX in 1992, and it was maintained under a 14:10 (Light: Dark) photoperiod, 25.0 ± 3.0°C, and 80–85% relative humidity using young cockerels (*Gallus gallus*) as bloodmeal hosts according to procedures approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP No 2014-255).

Host preparation. Four weaned domestic pigs (*S. s. domesticus*) weighing 15-20 kg were acquired from a commercial swine producer. The pigs were reared in a facility that minimizes the exposure to hematophagous ectoparasites or internal parasites, and the pigs were not previously treated for parasites. The pigs were maintained in a facility at the Texas A&M University Veterinary Medical Research Park for the duration of the study in accordance with IACUC-approved AUP No. 2015-0089. This facility was specifically designed to maintain an ectoparasite free environment other than the ticks used in the study. Pigs were quarantined for two weeks during which they were vaccinated for PARAPLEURO SHIELD® P+BE (ELANCO US, Inc. Larchwood, IA, U.S.) and SUVAXYN® RESPIFEND® MH/HPS (ZOETIS Inc. Kalamazoo, MI, U.S.) in accordance with manufacturer instructions. In addition, pigs underwent two positive reinforcement training sessions for sling apparatus where they were encouraged to walk into and rest quietly in a sling (Figure 28).

The sling apparatuses used for this study were locally fabricated with following specifications. The total length and the width of the sling measured 100 cm by 66 cm with five 20 cm diameter holes. Two holes for the pig's forelegs were placed 20 cm from the anterior part of the sling whereas holes for the hind legs were placed 25 cm from the poster end of the sling. One additional hole was placed at the center and immediately in front of the holes for the forelegs to gain access to jugular veins of pigs for blood sample collections (Figure 28). Both sides of the sling had sleeves for 150 cm metal rods to secure the sling and the pig on to the sling rack. The sling rack dimensions were 45 cm wide, 106 cm long, and 100 cm tall. It also had six vertical slots that were 10 cm apart. Next, lateral

sides of the rack had five enclosing bars that were 15 cm apart (Figure 28). Finally, the anterior and the posterior side of the rack had vertical gates to allow pigs to enter and exit.

Tick challenge. All pigs were challenged three times during this study. Timelines and tasks for the tick challenges are outlined in the Table 11. For each tick challenge session, one hundred unfed ticks that had completed four molts (adult and/or late instar nymphs) were placed in a feeding-chamber then randomly assigned to a pig subject. The feeding-chamber was constructed by removing the bottom of a 250ml wide mouth Nalgene bottle (Thermo-Fisher Scientific, Waltham, MA, U.S.) to 4.5 cm from the top and sealing it with fabric with mesh size 2 mm, through which the ticks were allowed to feed (Figure 29). Feeding chambers were secured onto the backs of the pig subjects for 60 min using 3M™ VetRap™ (3M, Oakdale, MN, U.S.) (Figure 28). Each tick chamber was weighted with Metter-Toledo scale Model# AE163 (Metter-Toledo, LLC, Columbus, OH, U.S.) before and after each challenge to assess the post-blood feeding weight increase per 100 ticks. Visual confirmations of engorgements were based on changes in tick color and shape. Tick challenges were conducted on days 14, 28 and 42 of the experiment timeline (Table 11). JMP® Pro 12 statistical software (SAS Co., Cary, NC, U.S.) was used to conduct an ANOVA test and a Tukey's-Honest Significant Difference (HSD) based on an alpha level of 0.05 for all pair-wise combinations test was conducted to determine any significant differences in total ticks fed and post-blood feeding weight increase between challenges, feeding chamber, and pig subjects.

Table 11. A summary of dates and tasks for *Ornithodoros turicata* challenges.

Experiment Days	Task(s)
DAY 01	Quarantine began
DAY 05	Sling training session #1
DAY 06	Pre-challenge blood sample
DAY 09	Sling training session #2
DAY 14	Quarantine end /Tick challenge #1
DAY 27	Post-challenge blood sample #1
DAY 28	Tick challenge #2
DAY 41	Post-challenge blood sample #2
DAY 42	Tick challenge #3
DAY 55	Post-challenge blood sample #3
DAY 62	Post-challenge blood sample #4
DAY 69	Post-challenge blood sample #5
DAY 76	Post-challenge blood sample #6
DAY 104	Post-challenge blood sample #7
DAY 132	Post-challenge blood sample #8

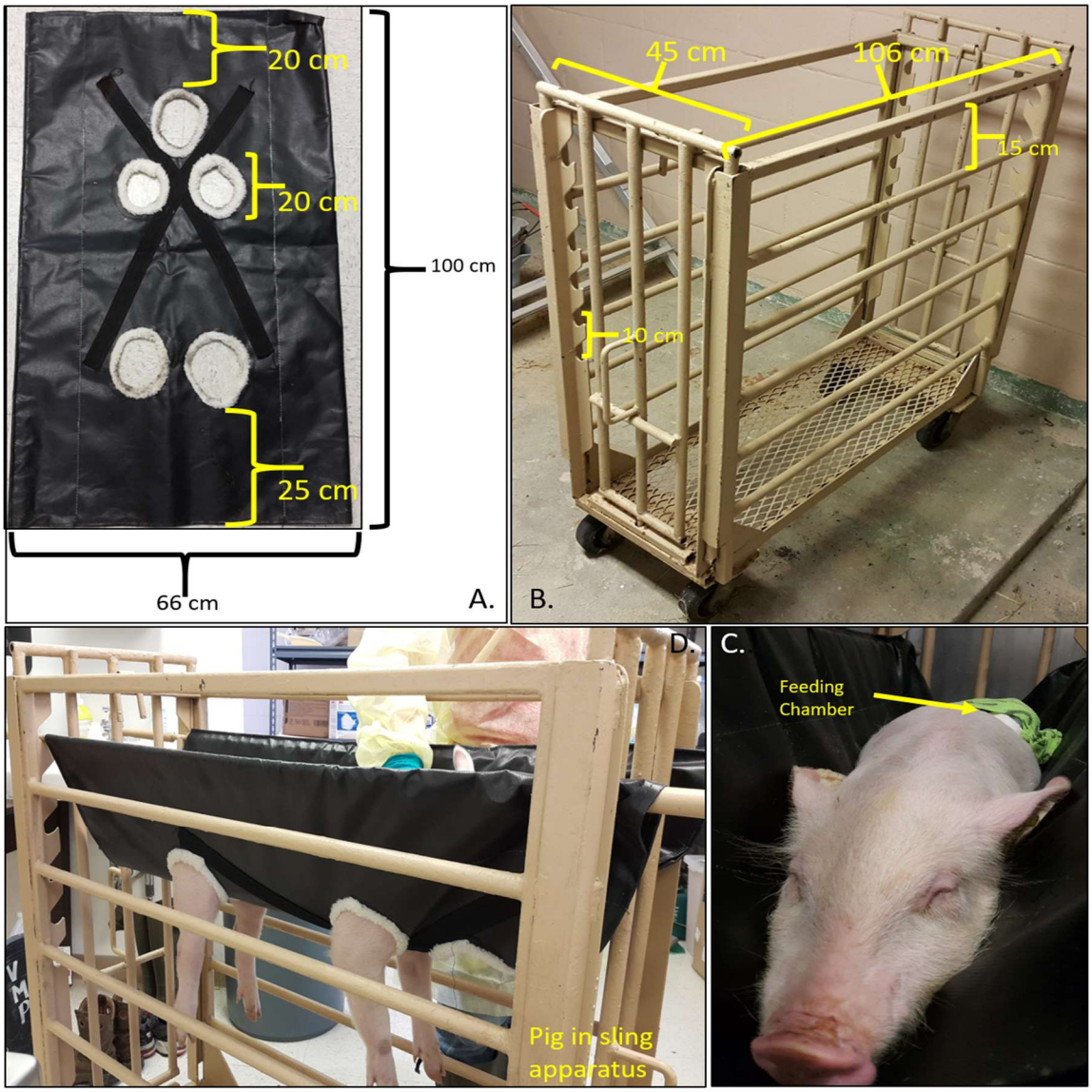

Figure 28. Images of pig sling apparatus used for *Ornithodoros turicata* challenges. Sling (A.) was hoisted up using metal rods and placed on the sling rack (B.). Positive reinforcement and training sessions with sling apparatus allowed pigs to be immobilized during tick challenge without the use of physical or chemical restraint (D.). A feeding chamber was secured using 3M™ VetRap™ (3M, Oakdale, MN, U.S.) for the 60-min tick challenge session (C.).

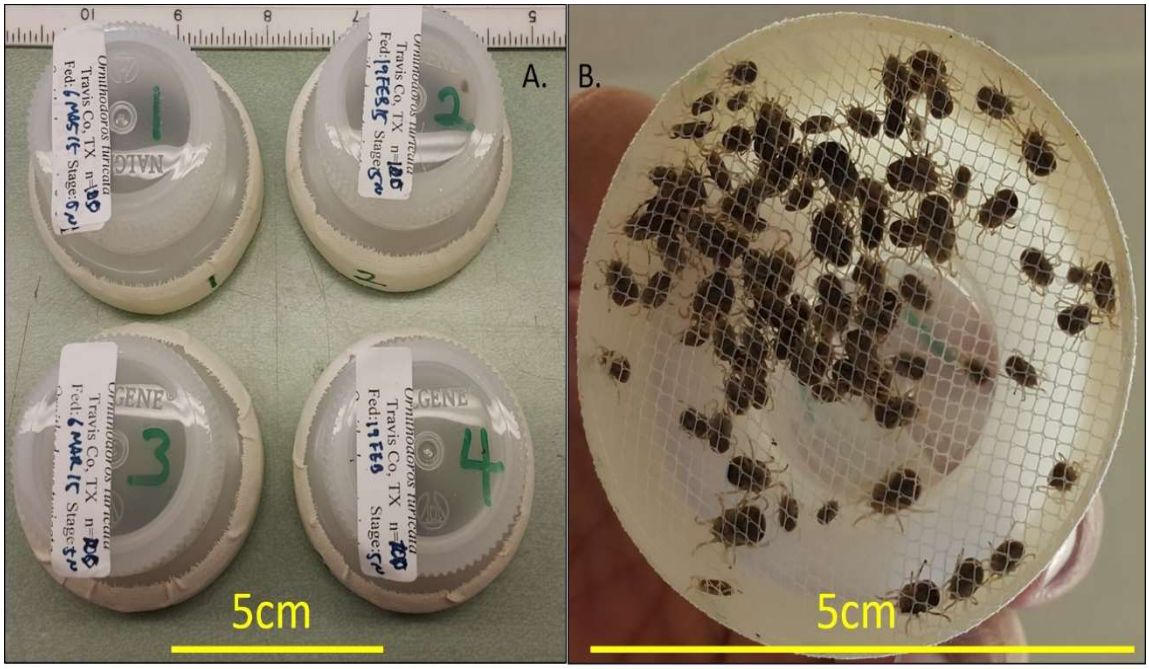

Figure 29. Images of feeding chambers used for *Ornithodoros turicata* challenges. One hundred *O. turicata* that had completed four molts (adult and/or late instar nymphs) were used per pig. Ticks were placed in a feeding chamber then randomly assigned to each pig subject. The feeding chamber was constructed by removing the bottom of a 250ml wide mouth Nalgene bottle (Thermo-Fisher Scientific, Waltham, MA, U.S.) to 4.5 cm from the top (A.) and sealing it with fabric with a 2 mm mesh size, through the which ticks were allowed to feed (B.).

Blood collection, and serum preparation. Nine 10 ml blood samples from each pig were collected over the course of the study. All blood samples were collected by Texas A&M University Veterinary Medical Research Park personnel in accordance with IACUC-approved AUP No. 2015-0089. Table 11 summarizes the blood sample collection dates. Initially, a pre-challenge blood sample was collected on day six from each pig subject which served as the control (baseline). Three post-challenge samples were collected on days 27, 41, and 55 (13 days after each tick challenge). In addition, five blood samples were collected on days 62, 69, 76, 104, and 132 (20, 27, 34, 62, and 90 days post the third tick challenge). The collected blood samples were centrifuged using a Variseal

Model Vs6c centrifuge (Vulcan Tech, New York, NY, U.S.) at a 600-relative centrifugal force (CFM) for 10 minutes and the isolated serum was stored at -20 °C until it was used for analyses.

Tick salivary gland extraction (SGE) and preparation. The ticks used for the tick challenge were also used for SGE production. Tick SGEs served as the antigen for the host serological response study by immunoblotting and enzyme-linked immunosorbent assay (ELISA). The SGEs were made using the modified methods derived from Canals et al. (1990). In summary, each live unfed *O. turicata* specimen was held in place on a glass slide with forceps in the presence of phosphate buffered saline (PBS) – 5 mM Magnesium Chloride ($MgCl_2$), exposing its dorsal plane. A sterile scalpel was used to make three incisions at the posterior end perpendicular to the median plane of the body and each sagittal plane leaving the anterior of the tick's body as the "hinge" point where the dorsal surface of tick can be peeled. Following the incisions, the dorsal surface of the tick was removed using forceps, exposing the salivary gland located at each anterior-lateral side of the tick (Figure 30). Finally, salivary glands were removed using forceps and washed with PBS – 5 mM of $MgCl_2$ (Figure 30). Next, salivary glands were placed in 1.5 ml tube with PBS – 5 mM of $MgCl_2$, and homogenized using a polypropylene pestle (Bel-Art Products, Wayne, NJ, U.S.), briefly centrifuged and supernatants stored at -4 °C until needed. The protein concentration of SGE supernatants were determined using Epoch Microplate Spectrophotometer and Gen5 Data Analysis Software Version 2.00.18 (BioTek, Winooski, VT, U.S.) and bovine serum albumin (Bio-Rad, Hercules, CA, U.S.) as a standard.

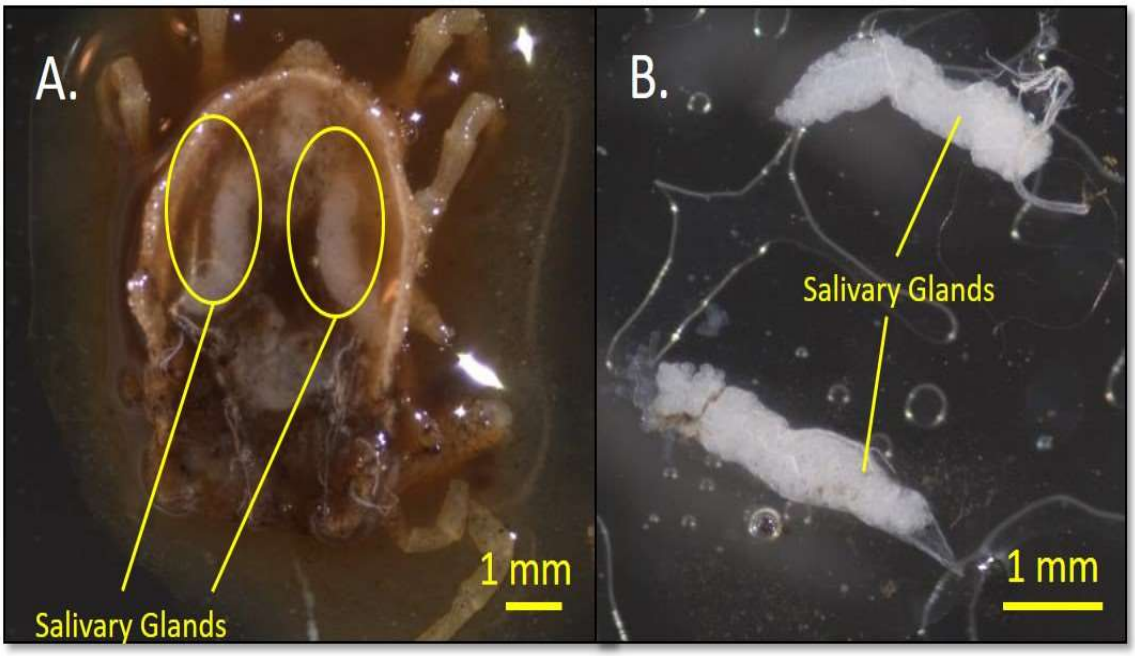

Figure 30. Images of *Ornithodoros turicata* salivary glands. Salivary glands of *O. turicata* exposed (A.) Salivary glands were placed in 1.5 ml tube with PBS – 5 mM of $MgCl_2$, homogenized using a polypropylene pestle (Bel-Art Products, Wayne, NJ, U.S.), briefly centrifuged, and used as the antigen for this study (B.).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Protein Immunoblots Test. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and protein immunoblot (Western Blot) test was performed on each blood sample as described in Lopez et al. (2013) to evaluate the immunogenicity of IgG in post tick challenges. In summary, three µg of protein lysates SGEs were electrophoresed on Mini-PROTEAN TGX precast gels (Bio-RAD, Hercules, CA, U.S.) at 80 volts for 90 minutes to separate SGE proteins, which were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.) at 100 volts for 60 minutes using Mini Trans Blot system (BioRad, Hercules, CA, U.S.). The PVDF membrane was then blocked using I-block™ protein-based blocking reagent (Life Technologies, Grand Island, NY, U.S.)

overnight. Next, the immunoblots were probed with pig sera samples as primary antibodies at a 1:200 dilution for one hour followed by an anti-pig IgG-HRP (Life Technologies, Grand Island, NY, U.S.) as the secondary antibodies at a 1:4,000 dilution for one hour. Finally, Serological reactivity was determined by Amersham Enhanced Chemiluminescence (ECL) Western Blotting System (GE Healthcare Bio-Science Corp., Piscataway, NJ, U.S.).

Enzyme-Linked Immunosorbent Assay. The ELISA protocol described in (Wozniak et al. 1995), using SGE and pre- and post- tick challenge serum samples, were used to evaluate seroconversion and endpoint titers. For all assays, 96-well flat bottom Immulon 2 HB plates (Thermo Electron, Milford, MA, U.S.) were coated with 100µl of coating buffer with SGE protein concentration at1 µg per100 µl overnight at 4°C followed by three washing with PBS-Tween 20 (1X PBS, 0.05% Tween 20) prior to blocking. Each plate was blocked using ELISA diluent (PBS, 0.5% horse serum, 0.05% Tween 20, 0.001% dextran sulfate) at 100 µL per well and incubated for 1 hour at room temperature (RT). Next, each plate was washed three times and 100 µL of pig sera from tick challenges were added to assigned wells at 1:100 dilution and incubated at RT. After one hour of incubation, each plate was removed, washed three times as stated above, and 100 µL of the secondary antibody, HRP- conjugated anti-pig IgG Fc (Thermo Scientific, Waltham, MA), was added to each well at 1:5,000 dilution. After an hour of incubation at RT, each plate was washed three times, and 100 µL of 2, 2'-Azino-di[3-ethylbenzthiazoline-6-sulfonate] (ABTS®) ELISA HRP Substrate, was added to each well and incubated at RT for 15 minutes. Finally, optical density (OD) of each plate was read with absorbance at

405 nm using Epoch Microplate Spectrophotometer and Gen5 Data Analysis Software Version 2.00.18 (BioTek, Winooski, VT, U.S.). Two sets of ELISA were conducted. In the first ELISA, OD reading of pre-challenge sera was compared to post-challenge sera for each pig subject. In the second ELISA, endpoint titer was conducted using pre-challenge sera (diluted 1:100) as baseline against reciprocal dilutions of post-challenge sera with highest OD value from the first ELISA runs (dilutions of 1:100, 1:1000, 1:2000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000, and 1:512,000). All samples were tested in triplicates and two closest OD readings were used to determine mean ($\bar{x}$) and standard deviation (SD). Samples were considered statistically significant if their mean OD reading was more than three times the SD above the mean of negative control (pre-tick challenge sera) ($\mu \pm 3\sigma$, 68-95-99.7 rule, a.k.a. Empirical Rule) (Lopez et al. 2009, Sanders 2011).

Results

Tick challenge. Table 12 summarizes the tick challenge results. The total ticks that fed and weight increase per 100 ticks was not significantly different based on pig subjects (F = 1.556; df =3; P = 0.2739 for total tick fed and F = 0.9568; df=3; P = 0.4584 for weight increase per 100 tick). Similarly, total ticks fed and weight increase per 100 ticks were not significantly different based on feeding chamber (F = 1.8209; df =3; P = 0.2214 for total tick fed and F = 0.20901; df=3; P = 0.8873 for weight increase per 100 tick). In addition, total weight increase per 100 ticks were not significantly different based on challenges (F = 3.7277; df=2; P = 0.0662) However, total ticks fed were significantly

different based on challenges (F = 5.1758; df =2; P = 0.0319). Specifically, total ticks fed during challenge 3 were significantly different from that of challenge 1 (Tukey's-HSD P= 0.0261)

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Test. The *O. turicata* SGE reacted with pig specific IgG Fc region starting with post-challenge sera collected 21 days after the first tick challenge across all pig subjects. Specifically, SGE protein band at 25kDa was observed consistently across all pig sera from the day 41 to end of the study on the day 132 (Figure 31).

Table 12. A summary of *Ornithodoros turicata* fed and weight gains after challenges. Total fed and weight increase per 100 late-instar and adult *O. turicata* used in three blood-feeding challenges at. 25.0 ± 3.0°C, and 80–85% relative humidity. Total ticks fed were significantly different based on challenges (F = 5.1758; df =2; P = 0.0319). Specifically, total ticks fed during challenge 3 were significantly lower than that of challenge 1 (Tukey's-HSD P = 0.0261).

Challenges	Pig #	Feeding Chamber	% Ticks fed	Weight increase per 100 ticks (g)
1	1	2	97	6.19
1	2	1	100	3.74
1	3	4	93	3.10
1	4	3	100	4.46
2	1	3	97	4.23
2	2	4	92	4.56
2	3	2	83	1.90
2	4	1	96	3.91
3	1	1	83	2.31
3	2	2	86	3.12
3	3	4	78	0.74
3	4	3	93	2.36

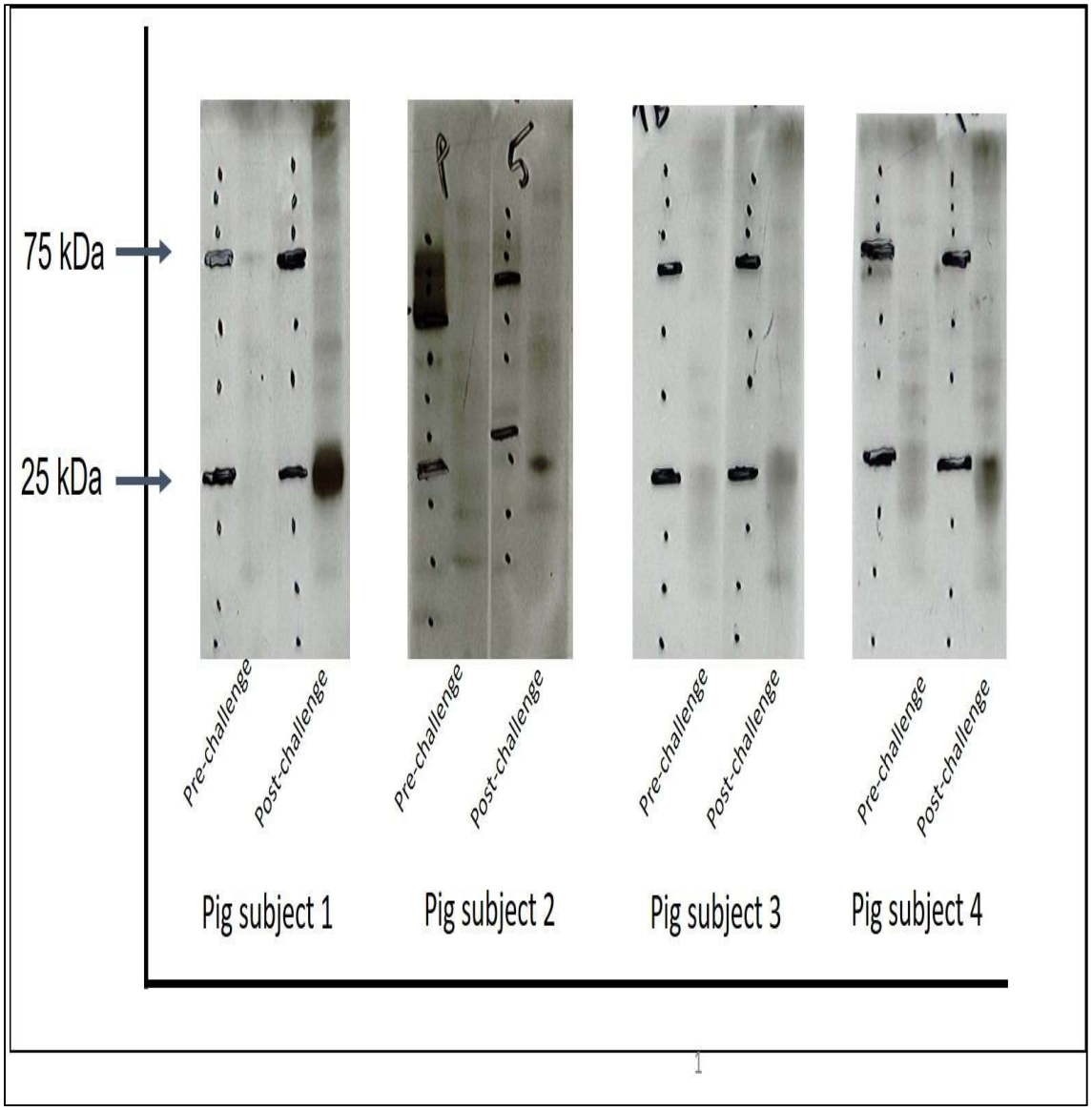

Figure 31. Western Blot results of pig sera three weeks post the first *Ornithodoros turicata* challenge. Several SGE proteins reacted with post-tick challenge pig sera. There were greater reactions observed in all pig subjects for protein(s) size 25 kDa from the day 41 to end of the study on the day 132.

Enzyme-Linked Immunosorbent Assay. Figures 32 to 35 show the ELISA results of pig subjects #01 to #04, respectively. A positive increase in IgG productions (mean of pre-challenge sera + (3xSD)) were detected in all pig subjects starting post-challenge sera

1. In the pig subject #01, a positive increase in IgG productions (mean of pre-challenge sera + (3xSD)) was detected in all post-challenge sera collected during the entire length of study and the highest IgG production was detected from post-challenge sera 3 (Figure 32). In the pig subject #02, a positive increase in IgG productions (mean of pre-challenge sera + (3xSD)) was detected until post-challenge sera 5 and the highest IgG production was detected from the post-challenge sera 4 (Figure 33). In pig subjects #03 and #04, positive increase in IgG productions (mean of pre-challenge sera + (3xSD)) were detected until post-challenge sera 6, and the highest IgG productions were detected in post-challenge sera 2 and 3, respectively (Figures 34 and 35).

The endpoint titer using post-challenge sera with the highest OD reading revealed that the pig subject #01 had greater than 2,000 times of OD value compared to the control (mean of pre-challenge sera + (3xSD) (Figure 36) The pig subjects #02 and #03, OD values were greater than 8,000 and 4,000 times than the control, respectively (Figures 37 and 38). Finally, pig subject #04 had the nearly 1,000-fold increase in OD value when compared to control (Figure 39).

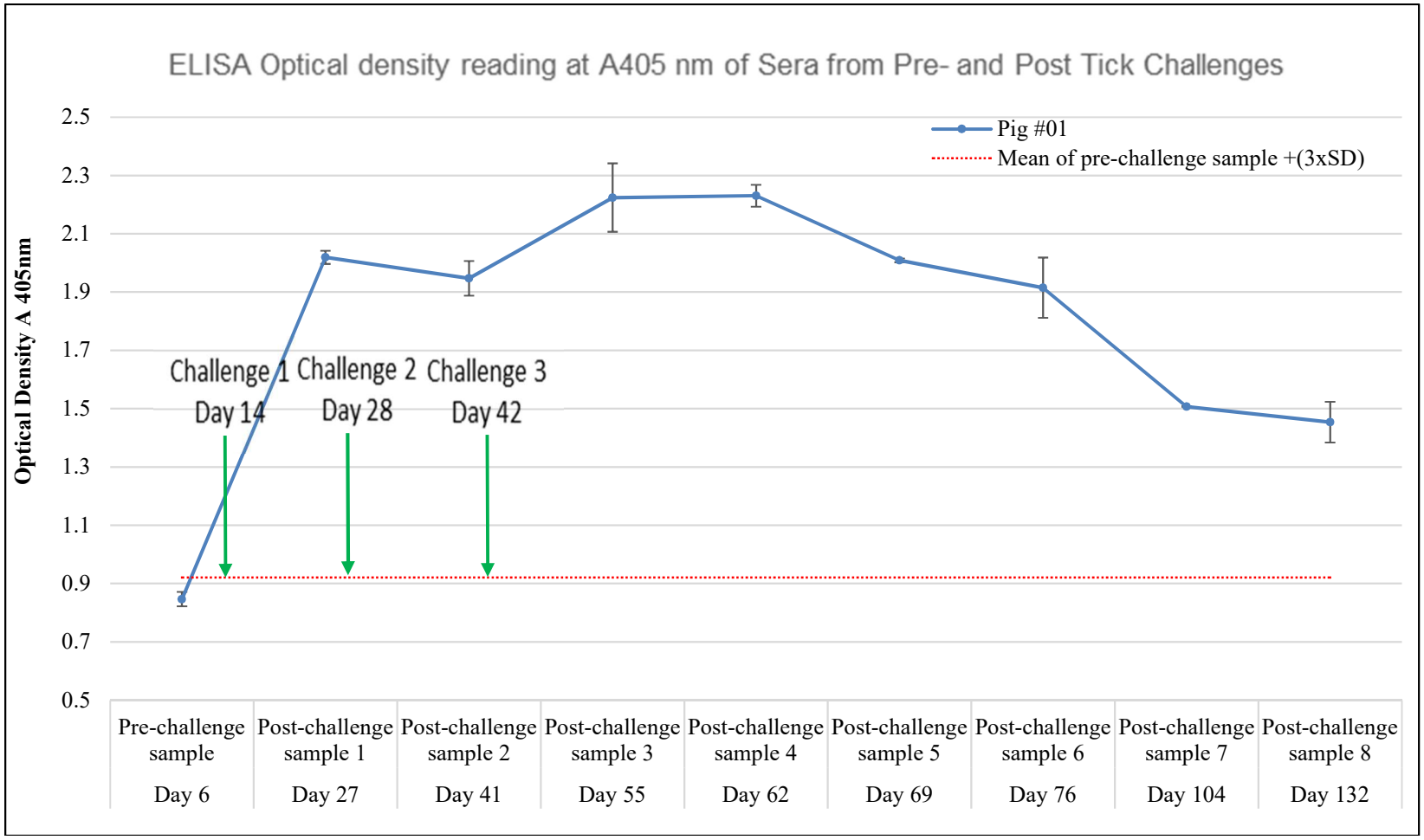

Figure 32. ELISA of pre-and post- *Ornithodoros turicata* challenged pig subject #01 sera. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. A positive increase in IgG production (mean of pre-challenge sera + (3xSD)) was detected as early as the post-challenge serum sample 1 and remained high during the entire study period.

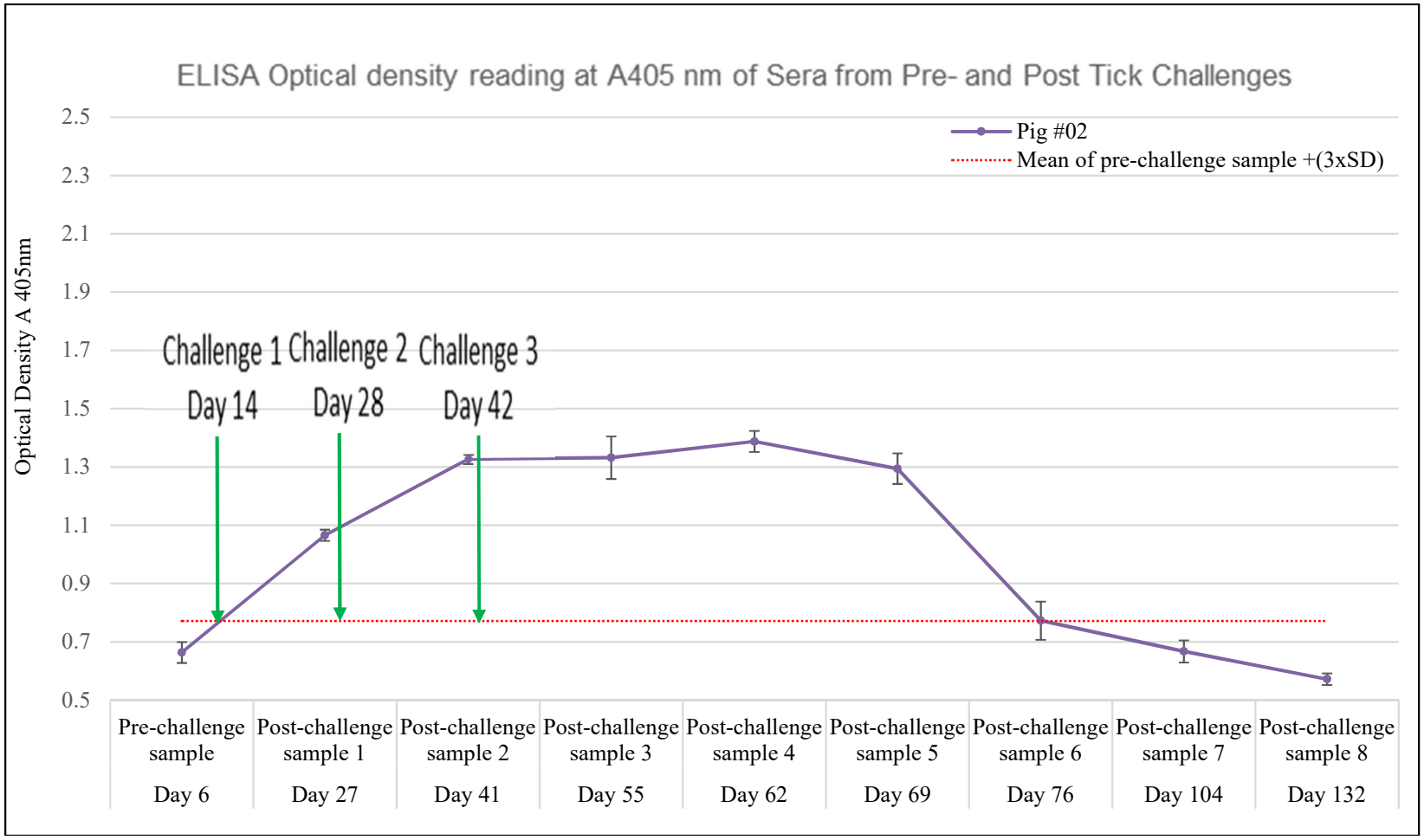

Figure 33. ELISA of pre-and post- *Ornithodoros turicata* challenged pig subject #02 sera. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. A positive increase in IgG production (mean of pre-challenge sera + (3xSD)) was detected as early as post-challenge serum sample 1 and remained high until the post-challenge serum sample 5.

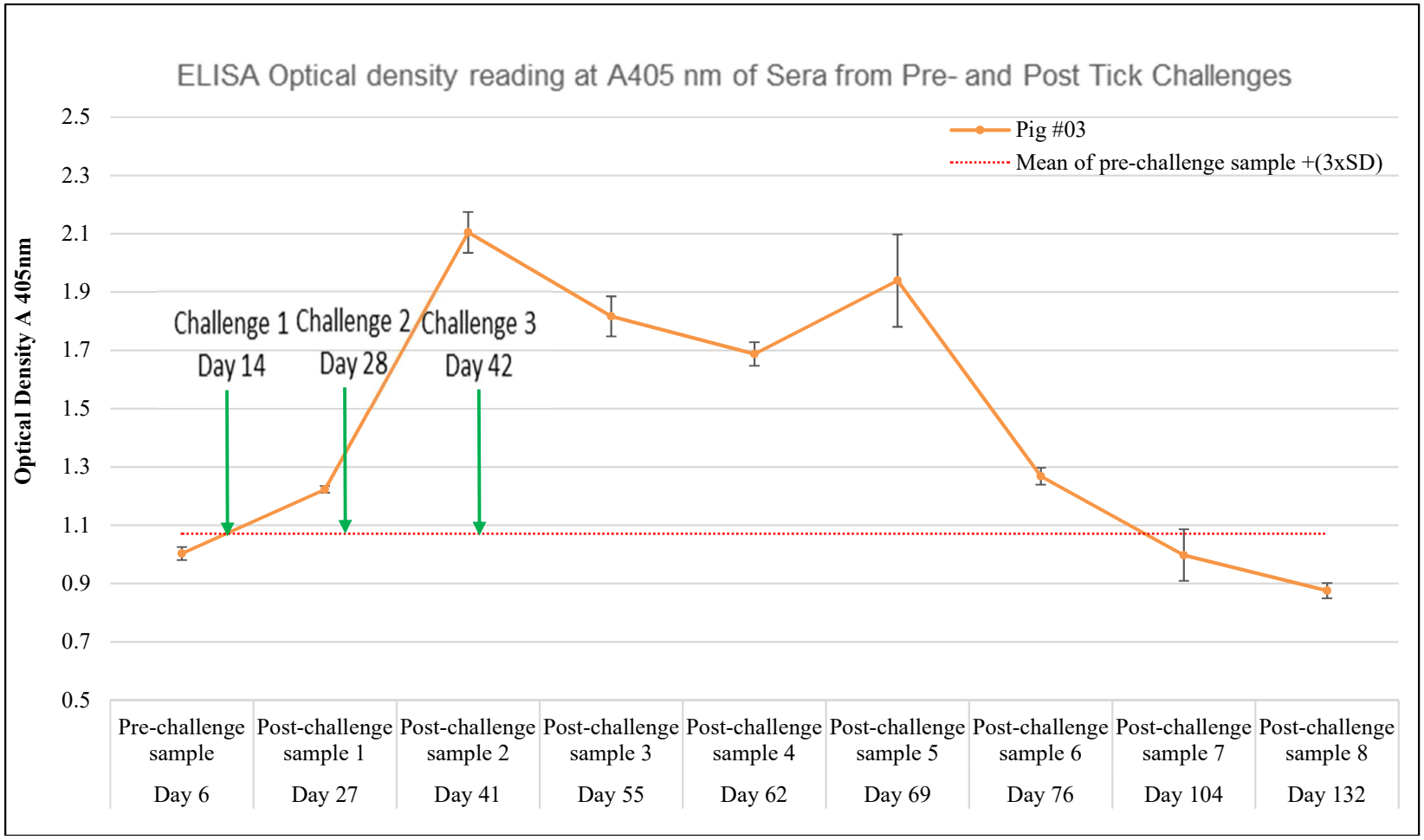

Figure 34. ELISA of pre-and post- *Ornithodoros turicata* challenged pig subject #03 sera. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. A positive increase in IgG production (mean of pre-challenge sera + (3xSD)) was detected as early as post-challenge serum sample 1 and remained high until the post-challenge serum sample 5.

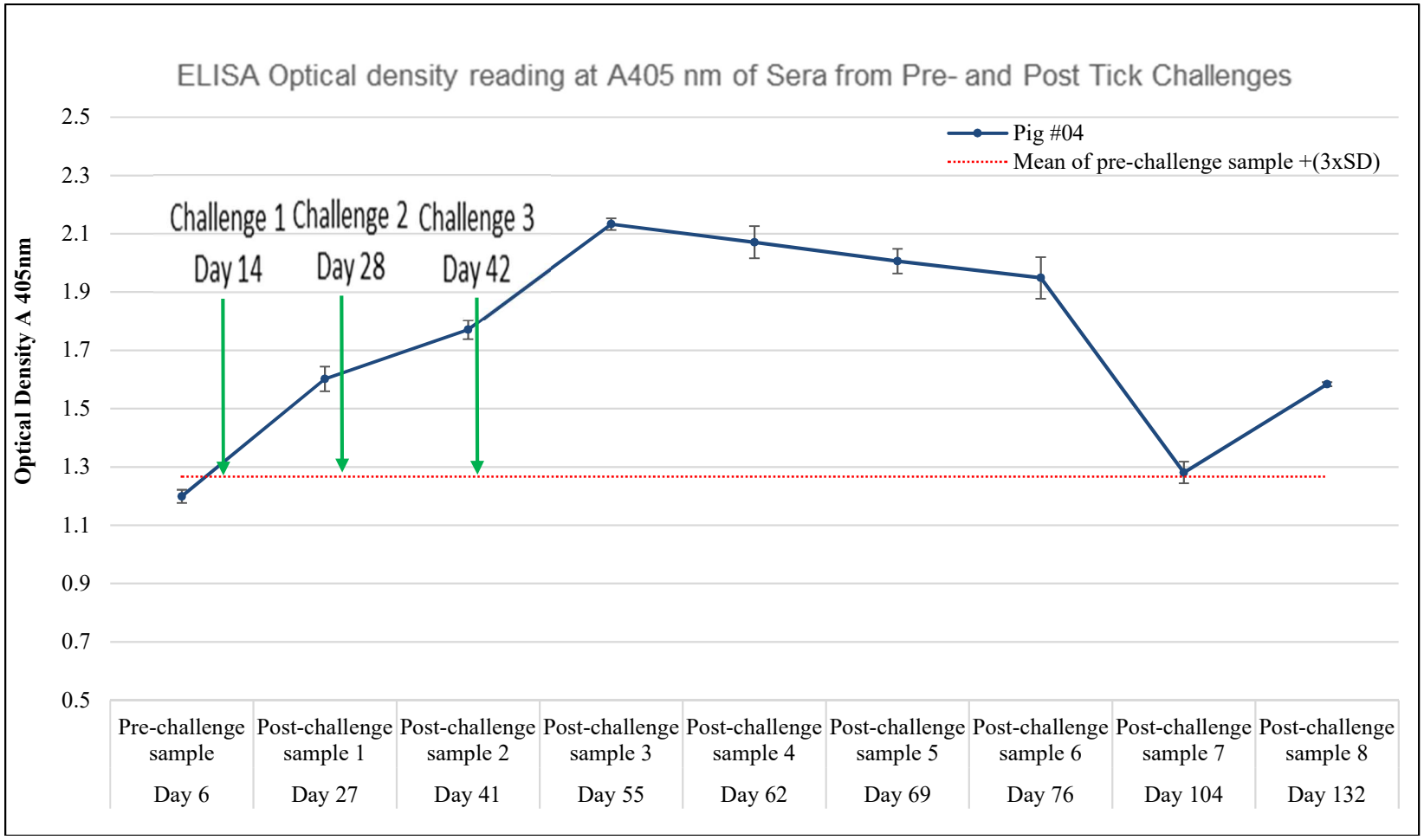

Figure 35. ELISA of pre-and post- Ornithodoros turicata challenged pig subject #04 sera. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. A positive increase in IgG production (mean of pre-challenge sera + (3xSD)) was detected as early as post-challenge serum sample 1 and remained high until the post-challenge serum sample 6.

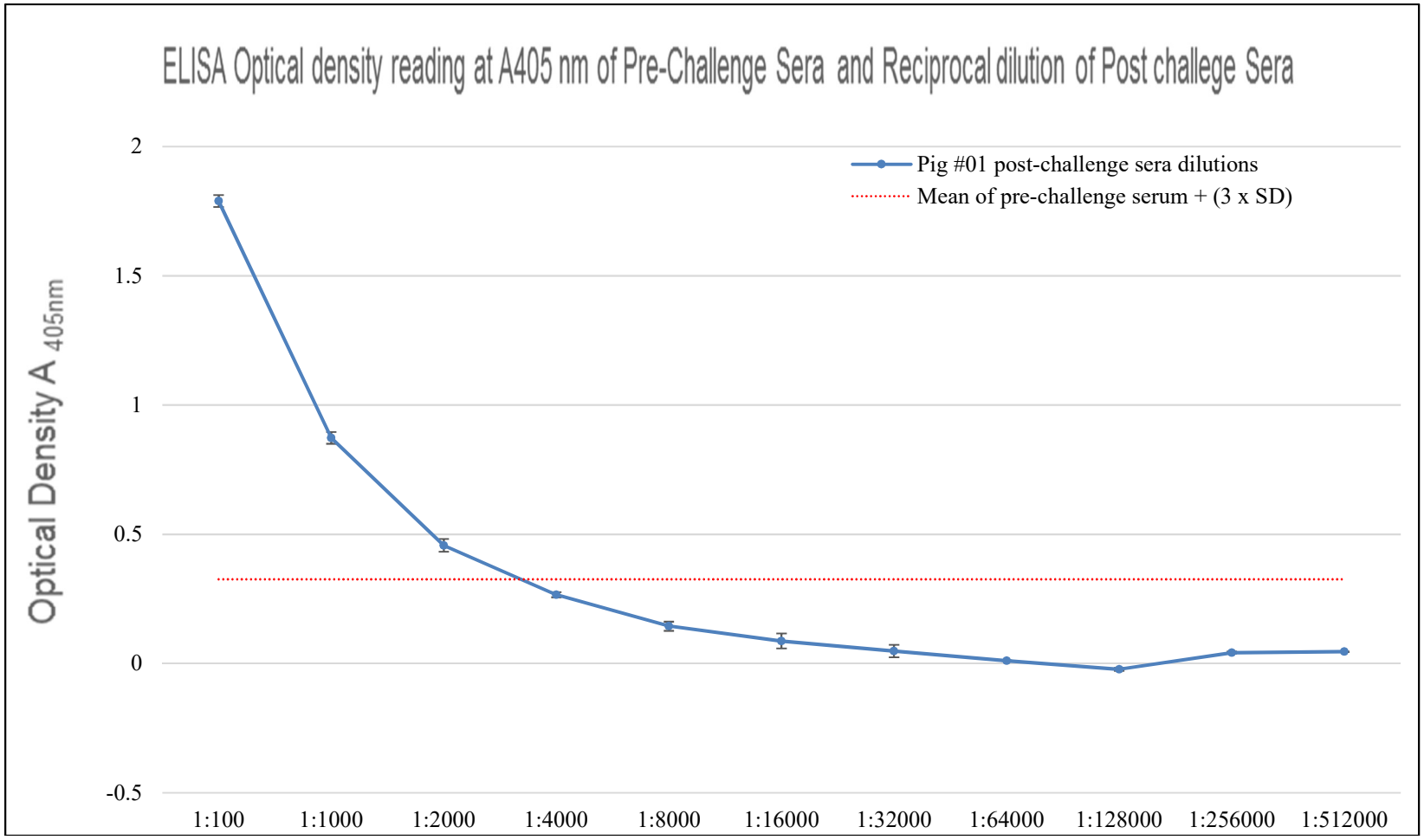

Figure 36. Endpoint titer of post- *Ornithodoros turicata* challenged pig subject #01 serum sample. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. Post-challenge serum sample 3 for pig subject #01 (highest OD value) showed greater than 2000-fold increase in IgG production compared to control (pre-challenge serum sample).

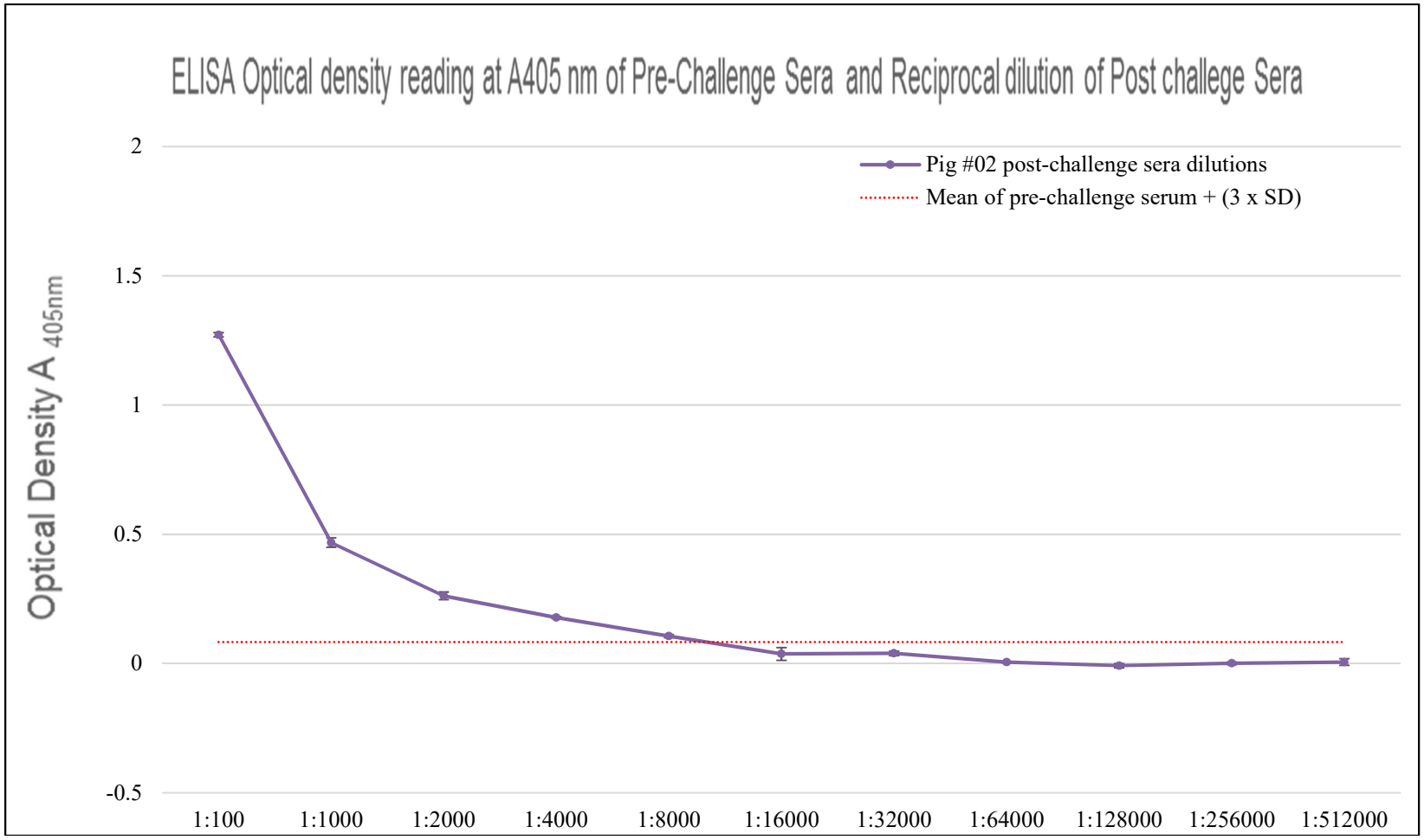

Figure 37. Endpoint titer of post- Ornithodoros turicata challenged pig subject #02 serum sample. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. Post-challenge serum sample 4 for pig subject #02 (highest OD value) showed greater than 8000-fold increase in IgG production compared to control (pre-challenge serum sample).

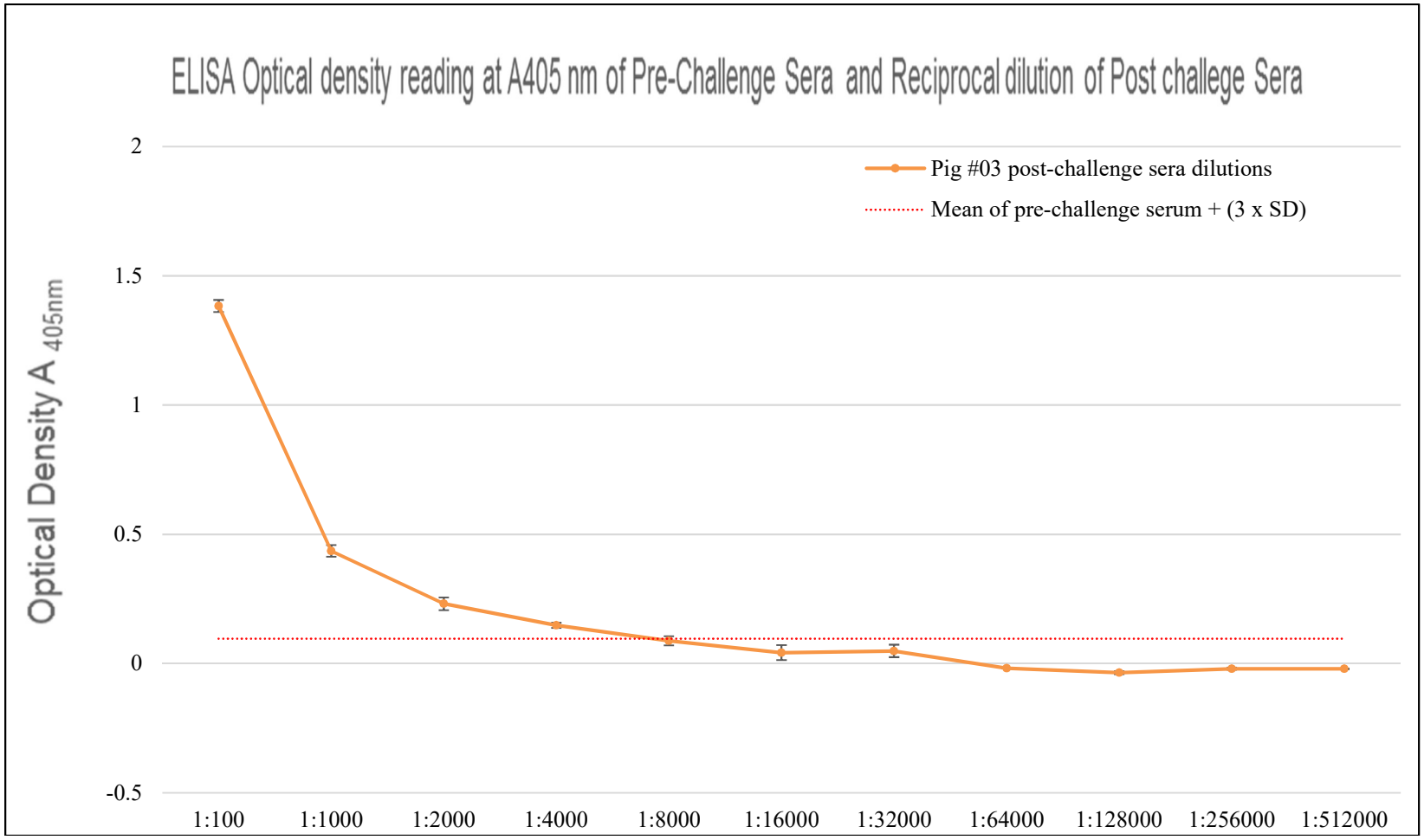

Figure 38. Endpoint titer of post- *Ornithodoros turicata* challenged pig subject #03 serum sample. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. Post-challenge serum sample 2 for pig subject #03 (highest OD value) showed greater than 4000-fold increase in IgG production compared to control (pre-challenge serum sample).

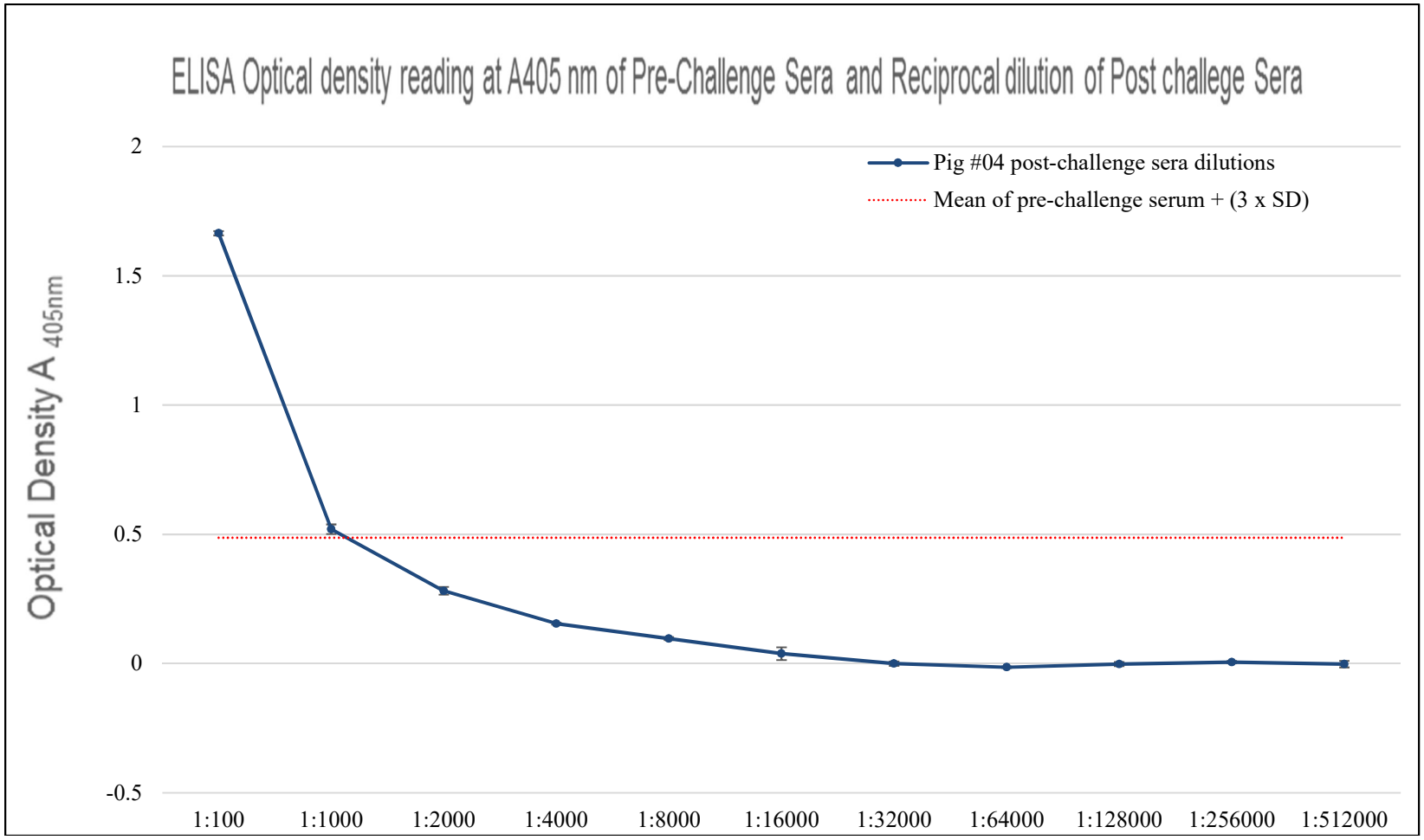

Figure 39. Endpoint titer of post- *Ornithodoros turicata* challenged pig subject #04 serum sample. Total three *O. turicata* challenge sessions in two-week intervals were conducted during the study. Each challenge sessions used 100 late instar nymphs and adults at 25.0 ± 3.0°C, and 80–85% relative humidity. Post-challenge serum sample 3 for pig subject #04 (highest OD value) showed greater than 1000-fold increase in IgG production compared to control (pre-challenge serum sample).

Discussion

Results from this study indicate IgG productions by the post-challenge pigs consistently differed from those of pre-challenge in quantifiable ways. The western blot result showed the presence of anti *O. turicata* SGE pig IgG as early as three weeks post the first *O. turicata* challenges. Similarly, ELISA showed significantly elevated IgG level across all pig subjects just 13 days after the first *O. turicata* challenge. In addition, these elevated host immune responses were quantifiable for several months following the challenges. These observations are in keeping with the finding reported in Canals et al. (1990) and Wozniak et al. (1995). Therefore, pig immune response against *O. turicata* SGEs, coupled with specificity of pig immune response against *B. turicatae,* may serve as vital tools in establishing *O. turicata* distribution in the U.S. Furthermore, the lasting effects of *O. turicata* challenge caused immune responses may provide greater latitudes in developing pig host immune response based *O. turicata* surveillance strategy.

This study revealed several potential concerns which must be addressed prior to host immune response based *O. turicata* surveillance strategies can be developed. The first concern was raised after observing varying degrees of successful feedings and significantly different weight gains by the tick groups observed after tick challenges. The data indicate significantly lower numbers of *O. turicata* successfully fed, and subsequently less weights were gained, during the third challenge compared to those from the first challenge. Initial suspect of such outcome was secretion of coxal fluids by ticks during feedings as a substantial amount of coxal fluid secretions were noted during each challenge session. Perhaps there were discrepancies in how much secreted coxal fluids

were weighted with each fed tick groups after each challenge. However, the influence of feeding chambers, which would have revealed the discrepancies in how much secreted coxal fluids were weighted, did not have a significant impact on the weight gain trends by the ticks. Another suspect on the lack of fed ticks and decreased weight gains by the ticks were the host defense against the ticks that was elicited after each challenge. The host defense against repeated exposures to ectoparasites is a known phenomenon. For example, the study by Reik (1962) reported hypersensitivity to *R.(B). microplus* salivary secretion elicited by heavy tick infestation which resulted in histological changes of the skin at the site of attachment of two species of cattle, *Bos Taurus* L. (Artiodactyla: Bovidae) and *Bos indicus* L. Similarly, Szabó & Bechara (1999) reported repeat exposures to *Rhipicephalus sanguineus* Latreille (Ixodida: Ixodidae) elicited strong skin inflammatory responses from both dogs and guinea pigs. However, such histological changes in the host skin were not observed during this study. This discrepancy raises a question whether or not the host must be exposed to a larger number of *O. turicata* in repeated exposures in order to elicit an immune response. Furthermore, Canals et al. (1990) detected a significant increase in the host immune response using as little as ten *O. erraticus* per challenge; however, this study also did not seek to establish the minimum number of *O. turicata* required to elicit a positive immune response from the host.

The second concern was raised from the fact that that cross-reactivity of host immune response against SGEs tick species other than *O. turicata* was not considered in this study. While the cross-reactivity study was not the central focus of this study, evidence of cross-reactivities in host immune responses against several tick species have been

previously observed (Need et al. 1991, Wozniak et al. 1995, Wikel 1996) suggested host immune responses against whole body extract of hard tick such as, *Amblyomma maculatum* (Koch) (Ixodida: Ixodidae), were found useful in discerning observed cross-reactivity against *Ornithodoros spp.*; however, this method is yet to be tested to increase the pig immune response specify to *O. turicata.*

The third concern was raised based on the varying degrees of host immune responses against *O. turicata* challenges observed during the study. The data from this study regarding the longevity of elevated IgG productions and amplification of IgG productions observed among the pig hosts are inconsistent. For example, IgG level of pig subject #2 return to baseline in the post-challenge sera #6, 34 days after the third tick challenge, but the IgG level of pig subject #1 never return to the baseline even at the end of the study, 90 days past the third tick challenge. Furthermore, the endpoint titer showed that increased IgG level varies between 1000-fold (pig subject #4) to 4000-fold (pig subjects #2 and #3). Therefore, additional observations are needed in order to establish a consistent range of change in pig host immune responses as a result of the *O. turicata* challenges.

Finally, the applicability of immune response of domestic pigs versus that of feral swine is yet to be determined. As mentioned before, potential interactions between feral swine and *O. turicata* pose a threat to further spread of ASFV should the pathogen is ever introduced in the U.S. This study has shown the potential of using domestic pig immune responses to determine its interaction with *O. turicata.* Therefore, future studies comparing the post-*O. turicata* challenge immune responses between domestic pigs and

feral swine must be conducted in order to assess the field applicability of findings from this study.

CHAPTER IV

BLOODMEAL ANALYSIS OF *ORNITHODOROS TURICATA* DUGÈS (IXODIDA: ARGASIDAE) USING DNA-BASED AND STABLE ISOTOPE-BASED TECHNIQUES

Introduction

The utilization of different vertebrates as hosts by arthropod vectors is a fundamental aspect of vector-borne disease ecology (Boakye et al. 1999, Apperson et al. 2004, Keesing et al. 2010, Hamer et al. 2015), and is necessary information to guide the risk assessment and intervention actions to control vector-borne diseases (Boakye et al. 1999, Apperson et al. 2004, Rasgon 2008, Reisen 2010). This concept was observed in a study by Bolzoni et al. (2012), which investigated Tick-Borne Encephalitis Virus (TBEV) cycle in Europe. In their study, contributions of two hosts (e.g., rodent and deer) species that commonly provide bloodmeals to the vector *Ixodes ricinus* L. (Ixodida: Ixodidae) was estimated. This study determined the vector population growth of *I. ricinus* was positively correlated with the population growth of both host species. However, the basic reproduction number (R_0) of TBEV decreased with increasing deer populations, which suggests that deer may act as tick amplifiers, but also divert tick bites from competent hosts (rodents), reducing the R_0 of TBEV.

Numerous bloodmeal analysis techniques are available to examine the host utility and host-feeding patterns of hematophagous arthropods. These techniques take advantage of unique molecular premises to discern different host blood from a broad range of vectors.

The brief history, strengths, and weaknesses of several commonly used bloodmeal analysis techniques including precipitin test, Reverse Line-Blot hybridization (RLB), Polymerase Chain Reaction (PCR), DNA sequencing, and stable isotope analysis are discussed in the following section.

Precipitin test is a serological method dating back to the 1940's, that relies on the antigen-antibody complex (Arnold et al. 1946). A commonly used precipitin method consists of loading a capillary tube with undigested blood from a hematophagous vector (e.g., mosquito), which serves as an antigen, then tests it against antibodies of the blood of various host suspects. The antigen-antibody complex is then visualized as a clot in the capillary tube (Arnold et al. 1946, Tempelis 1975). The precipitin test methods are relatively easy to conduct and similar to the concept of home pregnancy tests. However, precipitin tests are extremely limited in detecting host blood beyond orders/family of suspected hosts (Kent 2009).

Reverse Line-Blot (RLB) hybridization is a method developed to detect human genetic disorders (Gold 2003). The RLB assay relies on host DNA specific oligonucleotide probe hybridization with post host DNA PCR amplicons. Hybridization of PCR products are then colourimetrically visualized (Kent 2009). The RLB can detect DNAs of multiple host blood by utilizing different oligonucleotide probes. Moreover, the RLB can detect multiple host blood in a single bloodmeal extract. However, in ticks that often endure prolonged starvation such as *I. ricinus,* the period between newly molted tick until the next questing activity can significantly affect the consistency of host detection (Cadenas et al. 2007).

The advent of Polymerase Chain Reactions (PCR) and quantitative real-time PCRs (qPCRs) has enabled species level host detection in bloodmeal analyses. PCR techniques in general, amplify DNA sequences of a target gene using the template DNA and gene specific primer in thermocycling steps. DNA amplicons are then visualized using various methods. Conventional PCR visualizes amplicons using dye and gel electrophoresis. Primer-probe based qPCR visualizes amplicons during thermocycling steps via a gene sequence specific probe (Kent 2009). The strength of PCR-based bloodmeal analyses is in their capability to detect specific hosts with high accuracy. However, these techniques are constrained by the availability of host-specific primers (and probe in qPCR) and available reference genomes of host species (Kent 2009). Several downstream post-PCR techniques attempt to circumvent the weaknesses of the PCR-based methods. PCR-sanger sequencing; for example, sequences the DNA base pair post-PCR of amplicons to identify host DNAs from the blood extracts. However, PCR-Sanger sequencing is limited by the size of amplicon it can sequence (i.e., typically less than 500 base pair) and can be cost prohibitive (Kent 2009). The PCR-restriction fragment length polymorphism (RFLP) is useful in separating species that differ by few nucleotides in the gene of interest. This method uses a restriction enzyme to generate different size post-PCR amplicons, which then are used to produce species-specific profiles of the host via gel electrophoresis (Kent 2009). However, PCR-RFLP requires advance knowledge of polymorphisms among host species. Furthermore, techniques mentioned (other than the probe-based qPCR) tend to be unable to discern bloodmeals that consist of multiple host species (Kent 2009).

Amplicon-deep sequencing stems from next generation sequencing technology and is often used in mutagen studies in cancer research (Hall 2007, Shendure and Ji 2008, Kent 2009). Essentially, amplicon-deep sequencing repeats read of nucleotides of the target gene (greater than seven times) during sequencing of amplicons to minimize the errors during sequence assembly of a target gene. Amplicon-deep sequencing can overcome the amplicon size that limits other sequencing methods (i.e., Sanger sequencing) and is highly accurate in sequencing long sequences (Beerenwinkel and Zagordi 2011). Amplicon-deep sequencing can be used not only to detect host species but simultaneously identify pathogens and microbiomes within the vector (Lin et al. 2015, Swei and Kwan 2017). Amplicon-deep sequencing, however, requires significant computing power and may be cost prohibiting for analyzing large sample sizes (Van Vliet 2010). One overall weakness of DNA-based bloodmeal analysis is degradation of DNA in vectors (Kent 2009).

Stable isotope (SI) analysis measures the composition of SI of an organism. Given the stable isotopes most often originate from the diet, SI analysis can be applied to vectors to learn about their prior bloodmeal hosts (DeNiro and Epstein 1981, Vanderklift and Ponsard 2003, Hood-Nowotny and Knols 2007, Rasgon 2008). In this context, SI analysis determines the isotope ratios of target elements, such as carbon and nitrogen, within hematophagous arthropods (Kloft 1992). These isotope ratios are unique based on the host utilization. Because this technique does not measure DNA, it is unconstrained by the DNA degradation that has posed challenges to DNA-based bloodmeal analysis techniques. Therefore, the SI analysis may have greater longevity in determining the bloodmeal

acquired by hematophagous arthropods. Furthermore, SI analysis can potentially identify host after blood has been completely digested. For example, Rasgon (2008) reported in his proof-of-principle experiments the SI analysis not only discerns between blood fed and unfed mosquitoes but also may have identified vertebrate host even after complete digestion of host blood within mosquitos. Also, Hamer et al. (2015) reported DNA-based bloodmeal analysis of *A. americanum* was not able to detect host DNA as early as six weeks post molt; whereas SI analysis of *A. americanum* showed significant results until 34 weeks post molt. However, SI analysis requires SI profiles of hosts in order to maximize its potential. Given that SI profiles of hosts may vary based on the diet of each host, there may be wide variations in the SI signatures of the same host species with different diets across space and time, thereby posing challenges to this method (DeNiro and Epstein 1981, Hobson and Clark 1992, Hood-Nowotny and Knols 2007).

The primary challenge of conducting bloodmeal analysis in ectoparasites enduring starvation periods lasting months to years such as ticks is that the DNA obtained from bloodmeal from previous life stage has been degraded during the molting (Pichon et al. 2005, Léger et al. 2015). This challenge has led groups to consider the alternative methods to infer prior bloodmeal identifications. For example, Önder et al. (2014) used proteomics to consider an additional approach in conducting the tick bloodmeal analysis. Some Argasid ticks such as *O. turicata,* for example, are known to survive for years without a bloodmeal (Francis 1938, Adeyeye and Butler 1989). Furthermore, *O. turicata* is a non-host specific tick with a wide host range, which has up to seven immature stages and may require one or more bloodmeals in each stage before molting to the next (Beck et al. 1986).

Therefore, DNA-based analyses may be an ineffective means to accurately determine the host utilization of *O. turicata* from the field due to DNA degradation and the potential for multiple host blood types acquired during the lifecycle. Nonetheless, there are no studies that have focused on bloodmeal analyses of *O. turicata*. Comprehensive approaches that incorporate both DNA-based analyses and SI-based analyses may be needed in order to understand *O. turicata* host utilization. The primary goal of this study was to compare DNA-based and SI-based bloodmeal analyses on an *O. turicata* cohort that fed on blood from known vertebrate hosts.

Materials and Methods

Ticks (O. turicata) Colony. Adults and late instar nymphs of *O. turicata* used in this study were obtained from a colony maintained at the Tick Research Laboratory, Texas A&M AgriLife Research, College Station, TX, U.S. The colony originated from specimens collected in a natural cavern in Travis County, TX, U.S. in 1992, and has been maintained under a 14:10 (Light:Dark) photoperiod, 25.0 ± 3.0ºC, and 80–85% relative humidity using young cockerels (*Gallus gallus*) as bloodmeal hosts according to procedures approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP No 2014-255).

Cohort preparation. Proposed timelines and protocol overview are outlined in Table 13. Four *O. turicata* cohorts were reared from larvae to the 4th instar nymph stage using different combinations of chicken, *Gallus gallus* L. (Galliformes: Phasianidae), goat *Capra, aegagrus hircus* L. (Artiodactyla: Bovidae), and swine (*S.s. domesticus*) blood.

The first group was labeled “EC”, short for “Exclusively fed on Chicken”, and was reared exclusively on live chickens for four bloodmeals in accordance with IACUC-approved AUP No. 2014-255. The second group was labeled “CG”, short for fed on “Chicken and Goat”, and was reared on live chickens for three bloodmeals and a final bloodmeal on commercially acquired mechanically defibrinated goat blood (Rockland Immunochemicals Inc. Limerick, PA, U.S.) via an artificial membrane. The third group was labeled “CS”, short for fed on “Chicken and Swine”, and was reared on live chickens for three bloodmeals and final bloodmeal on commercially acquired, mechanically defibrinated swine blood (Rockland Immunochemicals Inc. Limerick, PA, U.S.) via an artificial membrane. The fourth group was labeled “ES”, short for “Exclusively fed on Swine”, and was reared solely on commercially acquired, mechanically defibrinated swine blood via an artificial membrane. Once all cohorts completed their final bloodmeal; a five-tick sample unit was harvested from each cohort to serve as the subjects for each DNA-based and SI-based bloodmeal analysis shown on Table 13.

Artificial membrane feeding. A modified blood-feeding apparatus was adapted for this study based on designs reported by Schwan *et al.* (1991), Zheng *et al*. (2015), and Butler *et al*. (1984b). Feeding chambers were made from 50 ml conical polypropylene, screw cap centrifuge tubes (Thermo Fisher Scientific Inc. Waltham, MA, U.S.) cut at the bottom to the length of 4.5 cm. A rectangular strip of Parafilm M® (Bemis Company Inc., Oshkosh, WI, U.S.) measuring 2 cm by 4 cm was used as a membrane by stretching it to maximum capacity over the bottom (the cut side) of each chamber. One-half of a 100 mm glass Petri dish (Kimble Science and Research Products LCC, Rockwood, TN, U.S.) was

filled with 20 ml of blood and suspended in a water bath (Thermo Fisher Scientific Co., Fair Lawn, NJ, U.S.) to maintain the blood temperature at 34.0 ± 2.0ºC (Figure 40). Commercially available, mechanically defibrinated goat and swine blood (Rockland Immunochemical, Limerick, PA, U.S.) was used as a bloodmeal sources for artificial feedings in this study. Each artificial feeding session lasted for 120 min to ensure all ticks in the feeding chamber were given equal opportunities to feed to repletion.

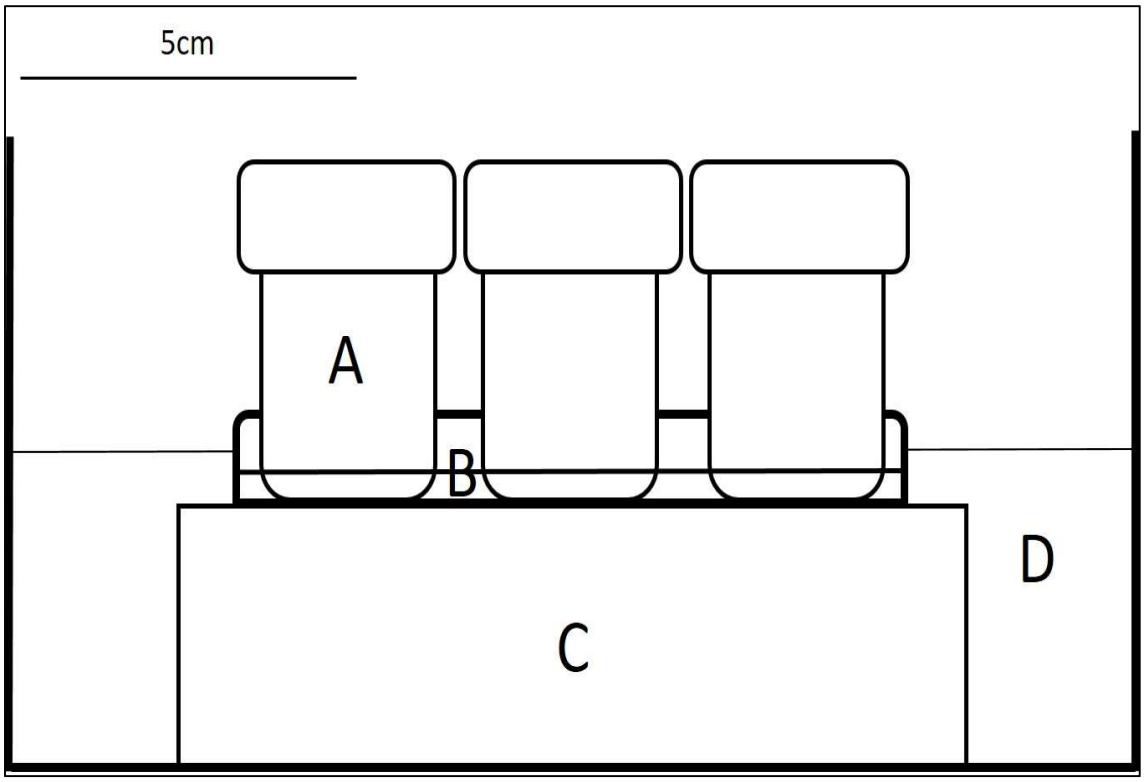

Figure 40. Artificial blood-feeding apparatus for *Ornithodoros turicata* cohorts. Feeding chambers were made from 50 ml conical polypropylene, screw cap centrifuge tubes cut at the bottom to the length of 4.5 cm and sealed with stretched Parafilm M® membrane (A). Feeding chambers were then placed on one-half of a 100-mm glass Petri dish filled with 20 ml of defibrinated blood (B) and placed on top of a test tube rack (C) in a water bath to maintain the blood temperature at 34 ± 2ºC (D).

Table 13. A timeline for the development and sampling scheme of four experimental *Ornithodoros turicata* cohorts fed on different host-blood. All *O. turicata* cohorts were maintained under a 14:10 (Light: Dark) photoperiod, 25.0 ± 3.0ºC, and 80–85% relative humidity

Experiment (days)	*O. turicata* (state)	Note
-	Larvae to 2N	EC, CG, and CS cohort reared to 2N using chicken blood ES Cohort reared to 2N using swine blood
0	2N (engorged)	EC, CG, and CS cohort fed on chicken blood ES cohort fed on swine blood
30	3N (unfed)	Samples collected for qPCR and SI analysis from each cohort
60	3N (engorged)	Final bloodmeal for all cohort EC cohort fed on chicken blood CG fed on goat blood CS fed on swine blood ES fed on swine blood Samples collected for qPCR and SI analysis from each cohort
90	4N 0M (freshly molted)	Samples collected for qPCR and SI analysis from each cohort
120	4N 1M (1 month post molt)	Samples collected for qPCR and SI analysis from each cohort
150	4N 2M (2 months post molt)	Samples collected for qPCR and SI analysis from each cohort
180	4N 3M (3 months post molt)	Samples collected for qPCR and SI analysis from each cohort
210	4N 4M (4 months post molt)	Samples collected for qPCR and SI analysis from each cohort
240	4N 5M (5 months post molt)	Samples collected for qPCR and SI analysis from each cohort
270	4N 6M (6 months post molt)	Samples collected for qPCR and SI analysis from each cohort
330	4N 9M (9 months post molt)	Samples collected for qPCR and SI analysis from each cohort

N= instar nymph, EC= exclusively fed on chicken blood, CG= fed on chicken and goat blood, CS= fed on chicken and swine blood, ES= exclusively fed on swine blood.

DNA Extraction and quantitative real-time Polymerase Chain Reaction (qPCR) analysis. Ticks from each sample unit (n = 5) outlined in Table 13 were used for DNA extraction and qPCR analysis assays. In summary, five ticks from each cohort (EC, CG, CS, and ES) were collected immediately after their last bloodmeal, immediately after a molt (~30 days post last bloodmeal), monthly (~4-week interval) for six times, and at nine months post molt. Before DNA extraction, surface contaminants on each tick were removed by briefly placing the tick in a 50% bleach solution for 15 seconds then wash them with water as outlined in Graham et al. (2012). The whole-body DNA extraction was then made per the manufacturer's instructions using E.Z.N.A. ® Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) with the following modification. In all cases, DNA was extracted from each *O. turicata* by cutting it into two equal segments in a sterile centrifuge tube exposing its midgut content to a lysis buffer solution. Each sample was subjected to lysis overnight then a series of extraction protocols with a final elution of 50 µl. DNA from aliquots of each host blood (15 µl per each host blood) used to feed *O. turicata* treatment groups for this study were also extracted to serve as the positive controls for their respective treatment groups. The host blood DNA extracts were quantified using Infinite® 200 PRO multimode microplate reader (Tecan Group Ltd. Männedorf, Switzerland). Moreover, both water-template and no-template wells served as negative controls.

The qPCR analysis was conducted to amplify and detect the cytochrome b (*cyt*b) gene in the extracted DNAs using host blood-specific primers and probes as previously described (Cupp et al. 2004). The *cyt*b gene was selected as the appropriate molecular

marker for the bloodmeal analyses in this study for two primary reasons. The first reason was the primers targeting the *cyt*b gene are vertebrate-specific and would not amplify *O. turicata* DNA (Cupp et al. 2004). The second reason for selecting *cyt*b gene was because it is a relatively short fragment of DNA (~150 bp); therefore, this small size fragment may still be detectable even in tick samples that have undergone DNA degradation (Hamer et al. 2015). Primers and probes sequences used for the qPCR protocols in this study are listed in the Table 14. The unmodified *cyt*b primers and probes for chicken and goat were used as described in Woods et al. (2009). The primer and probe for the swine blood were designed using Beacon Designer 8.0 software (Premier Biosoft, Palo Alto, CA, U.S.) based on *Sus scrofa* mitochondrion genome (GenBank accession #AF034253.1). Furthermore, the probe of each *cyt*b gene of hosts was tagged with different TaqMan® probe dyes to discern any cross-reaction among the *cyt*b genes of the different hosts (Table 14). The LightCycler® 96 System (Roche Diagnostics Corporation., Indianapolis, IN, U.S.) was used for all qPCR analyses with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 min. Subsequently, the nuclease-free water and the whole blood DNA extracts were used as the qPCR negative and positive control, respectively. The concentrations of primer and probe used for this study are listed in the Table 15. Total 750 reaction wells were tested on DNA extracts from cohort and host-specific primers and probes. Among these, 365 wells were labeled as "unmatched samples", which denoted DNA extracts from *O.turicata* sample units were tested using host-specific primer and probes of blood that was not used to rear them. This was done to assess any cross-reactivity across host blood. The remaining 385

wells were labeled as “matched samples” in which DNA extracts from *O. turicata* sample units cohorts were tested using host-specific primer and probe of blood used to prepare the corresponding cohort. Finally, the qPCR results were deemed positive when a DNA sample cycle threshold (Ct) value was less than 35, and the melting temperature is similar to that of the positive control host blood.

Stable isotope analysis. Stable Isotope analysis was conducted using *O. turicata* from each tick sample unit (n = 5) collected concurrently as those gathered for qPCR to generate comparable results. The Elemental Analysis Isotope Ratio Mass Spectrometry (EA-IRMS) at the SI Geosciences Facility at Texas A&M University, College Station, TX, U.S. was used to analyze individual ticks for carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopic values as described in Hamer et al.(2015). The EA combusted the tick and blood samples at 1200°C, separating CO_2 and N_2 gases, and analyzed on the IRMS. The standard delta (δ) notation: $^{\delta}X = [(Rsample / Rstandard) - 1] X 1000$ where R was the ratio of the heavy to light SI in the sample and standard was used to represent the results. Next, results were referenced according to the Vienna Pee Dee Belemnite (VPDB) carbonate standard for $\delta^{13}C$ and relative to air for $\delta^{15}N$. Finally, the range of $\delta^{13}C$ and $\delta^{15}N$ values of samples for a 2-point calibration and internal laboratory standards every ~12 unknowns were used to measure analytical precision as described in Hamer et al. (2015). Finally, samples from each host blood type used to feed corresponding cohorts served as the exact experimental replicate controls (n = 5 for each host blood type) for the SI analysis.

Table 14. Primer and probe sequences for host-specific *cyt*b gene used as a molecular marker in qPCR assays to identify *Ornithodoros turicata* fed on different host blood.

Host blood	Gene	Amplicon size	Primer and probe sequences	Reference/ GenBank Accession number
Chicken	*cyt*b	162	Forward Primer 5′-CCTCTACAAGGAAACCTCAAACAC-3′ Reverse Primer 5′-GACTAGGGTGTGTCCAATGTAGG-3′ Probe 5′-ROX-CGCCATAGTCCACCTGCTCTTCCTCCA-BHQ-3′	(Woods et al. 2009)
Goat	*cyt*b	125	Forward Primer 5′-TCCTCCCATTCATCATCACAGC-3′ Reverse Primer 5′-TGGTGTAGTAAGGGTGAAATGGG-3′ Probe 5′-ROX-CGCCATAGTCCACCTGCTCTTCCTCCA-BHQ-3′	(Woods et al. 2009)
Swine	*cyt*b	176	Forward Primer 5′-CTACGGTCATCACAAATCTACTATCAG-3′ Reverse Primer 5′-GTGCAGGAATAGGAGATGTACG′ Probe 5′-Cy5-ATCGGAACAGACCTCGTAGAATGAATC-BHQ-3′	This study

Table 15. Primer and probe concentrations for qPCR assays to identify *Ornithodoros turicata* fed on different host blood.

Host	Total reaction volume (µL)	Forward Primer (nM)	Reverse Primer (nM)	Probe (nM)	DNA template (µL)
Chicken	25	400	400	200	4.5
Goat	25	400	400	200	4.5
Swine	25	400	400	50	4.5

Statistical data analysis. The statistical program JMP® Pro 12 statistical software (SAS Co., Cary, NC, U.S.) was used for all statistical analyses. The Exact Cochran-Armitage trend test was conducted to assess any difference in qPCR assay results of each *O. turicata* sample unit (n = 5) based on the experiment days (length of starvation). Chi-squared test was conducted using all *O. turicata* sample unit based on host-specific primer and probe to determine any host blood effects on the qPCR results. The Pillai's Trace multivariate analysis of variance (MANOVA) was used to compare $\delta^{13}C$ and $\delta^{15}N$ values of host blood, all unfed 3rd instar nymphs, all engorged 3rd instar nymphs, sample units from each cohort, and combine sample unit values for all cohort. When MANOVA indicated a significant difference, post hoc test using Tukey's honestly significant differences was conducted to assess in both $\delta^{13}C$ and $\delta^{15}N$ based on an alpha level of 0.05 for all pair-wise combinations.

Results

Host blood DNA quantification. Average quantities of host blood DNA extracts are shown in Table 16. Chicken blood had the highest DNA yield (211 ng/µl, SD ± 41.9 ng/µl), followed by goat blood (17.44 ng/µl, SD ± 3.0) and swine blood (9.26 ng/µl, SD ± 2.84 ng/µl).

Table 16. A summary of DNA concentrations and purities extracted from 15 µl of chicken (*Gallus gallus*), goat (*Capra aegarus hircus*), and swine (*Sus scrofa domesticus*) blood samples (n = 5).

Host blood	Average concentration ng/µl	SD ng/µl	Sample purity 260nm/280nm ratio	SD 260nm/280nm ratio
Chicken	211.72	41.9	1.82	0.01
Goat	17.44	3.0	1.74	0.05
Swine	9.26	2.84	1.72	0.05

Real-time polymerase chain reaction analyses. All 365 "unmatched samples" were qPCR negatives, which denote no significant cross-reactions ($P < 0.0001$, CI = 0, 0.01) among host *cyt*b genes and non-corresponding host primer and probe. The qPCR assay results of the *O. turicata* sample units from EC, CG, and CS cohorts using chicken ctyb gene-specific primer and probe are shown in Table 17. The chicken *cyt*b gene was detected in all tick sample units fed on chicken blood in all EC, CG, and CS cohort during the entire experiment period. The *O. turicata* sample units from EC cohort had the highest overall average qPCR positive of 98%, followed by the sample units from the CG cohort with 76 % and the sample units from the CS group with 60 %. At no time during the entire experiment period was chicken *cyt*b gene not detected in a tick cohort that had fed on chicken at some time previously. There were no significant differences in the qPCR positive/negative results of *O. turicata* sample units based on the experiment day (length of starvation) in EC cohort ($P = 0.10$, Cochran-Armitage trend test) and CG cohort ($P = 0.35$, Cochran-Armitage trend test). However, a significant difference was observed in the qPCR assay results in the tick sample units from the CS cohort based on experiment days ($P < 0.01$, Cochran-Armitage trend test) (Table 17).

Table 17. A summary of qPCR assays (Ct value <35) based on experiment days per *Ornithodoros turicata* tick sample unit (n=5) fed on chicken (*Gallus gallus*) blood.

O. turicata state	Experiment days	EC	CG	CS
		qPCR positives out of 5 samples (%)		
2N (engorged)	0	5 (100)	4 (80)	5 (100)
F 3N (engorged)	60	5 (100)	2 (40)	5 (100)
4N 0M (freshly molted)	90	5 (100)	4 (80)	4 (80)
4N 1M (1 month post molt)	120	5 (100)	5 (100)	2 (40)
4N 2M (2 months post molt)	150	5 (100)	4 (80)	3 (60)
4N 3M (3 months post molt)	180	5 (100)	5 (100)	3 (60)
4N 4M (4 months post molt)	210	5 (100)	2 (40)	1 (20)
4N 5M (5 months post molt)	240	5 (100)	4 (80)	1 (20)
4N 6M (6 months post molt)	270	5 (100)	3 (60)	4 (80)
4N 9M (9 months post molt)	330	4 (80)	5 (100)	2 (40)
Range of % positive per cohort		80-100	40-100	20-100
Mean % positive per cohort		98	76	60
SD per cohort		6.32	22.71	29.81
Mean % positive per all cohort			71	
SD per all cohort			29.68	
Exact Cochran-Armitage trend test		P = 0.1	P= 0.35	P< 0.01

N= instar nymph, EC= exclusively fed on chicken blood, CG= fed on chicken and goat blood, CS= fed on chicken and swine blood.

The qPCR assay results of the *O. turicata* sample units from CG cohort using goat ctyb gene-specific primer and probe are shown in Table 18. The goat *cyt*b gene was detected in each goat blood fed tick sample unit in CG cohort during the entire experiment period. The overall average qPCR positive for the O. turicata sample units from CG cohort was 64.4%. There were no significant differences in the qPCR assay results of *O. turicata* sample units based on the experiment day (length of starvation) in GC cohort ($P = 0.38$, Cochran-Armitage trend test) (Table 18).

Table 18. A summary of qPCR assays (Ct value <35) based on experiment days per *Ornithodoros turicata* tick sample unit (n = 5) fed on goat (*Capra aegagrus hircus*) blood.

O. turicata state	Experiment days	CG qPCR positives out of 5 samples (%)
2N (engorged)*	0	0 (0)
3N (engorged)	60	5 (100)
4N 0M (freshly molted)	90	4 (80)
4N 1M (1 month post molt)	120	2 (40)
4N 2M (2 months post molt)	150	2 (40)
4N 3M (3 months post molt)	180	2 (40)
4N 4M (4 months post molt)	210	2 (40)
4N 5M (5 months post molt)	240	5 (100)
4N 6M (6 months post molt)	270	1 (20)
4N 9M (9 months post molt)	330	5 (100)
Range of % positive per cohort		20-100
Mean % positive per cohort		64.44
SD per cohort		31.27
Exact Cochran-Armitage trend test p<		0.38

* 2N CG cohort not fed on goat blood and excluded from statistical analysis. N= instar nymph, CG= fed on chicken and goat blood.

The qPCR assay results of the *O. turicata* sample units from CS and ES cohorts using swine ctyb gene-specific primer and probe are shown in Table 19. The swine *cyt*b gene was detected in all tick sample units fed on swine blood in both CS and ES cohorts during the entire experiment period. The *O. turicata* sample units from ES cohort had the higher overall average qPCR positive of 82.0 %, followed by the sample units from the CS cohort with 75.6 %. At no time during the entire experiment period, a sample unit failed to detect swine *cyt*b gene. There were no significant differences in the qPCR assay results of *O. turicata* sample units based on the experiment day (length of starvation) in ES cohort ($P = 0.27$, Cochran-Armitage trend test). However, a significant difference was observed among the qPCR assay results in the tick sample units from the CS cohort based

on the experiment days ($P < 0.01$, Cochran-Armitage trend test) (Table 19). Finally, there were no differences between qPCR results of tick sample unit based on the host blood ($\chi^2 = 2.33$, DF = 2, $P = 0.31$) (Table 20).

Table 19. A summary of qPCR assays (Ct value <35) based on experiment days per *Ornithodoros turicata* tick sample unit (n = 5) fed on swine (*Sus scrofa domesticus*) blood.

O. turicata state	Experiment days	CS	ES
		qPCR positives out of 5 samples (%)	
2N (engorged)*	0	0 (0)	5 (100)
3N (engorged)	60	5 (100)	5 (100)
4N 0M (freshly molted)	90	5 (100)	3 (60)
4N 1M (1 month post molt)	120	4 (80)	5 (100)
4N 2M (2 months post molt)	150	4 (80)	3 (60)
4N 3M (3 months post molt)	180	5 (100)	3 (60)
4N 4M (4 months post molt)	210	1 (20)	5 (100)
4N 5M (5 months post molt)	240	3 (60)	5 (100)
4N 6M (6 months post molt)	270	5 (100)	2 (40)
4N 9M (9 months post molt)	330	2 (40)	5 (100)
Range of % positive per cohort		20-100	40-100
Mean % positive per cohort		75.56	82.00
SD Per cohort		29.63	23.94
Mean % positive per all cohort		75.71	
SD Per all cohort		28.99	
Exact Cochran-Armitage trend test p<		$P < 0.01$	$P = 0.27$

* 2N CS cohort not fed on swine blood and was excluded from statics analyses. N= instar nymph, CS= fed on chicken and swine blood, ES= exclusively fed on swine blood.

Table 20. A summary of qPCR assay results based on host blood-specific primer and probe.

O. turicata cohort	Primer-probe	Negative	Positive	Total
Fed on Chicken blood (EC, CG, CS)	Chicken	58	142	200
Fed on Goat blood (CG)	Goat	16	29	45
Fed on Swine blood (ES, CS)	Swine	34	106	140
		108	277	385
	n		385	
	Chi-square DF		2	
	Chi-square value			
	Chi-square P		$P = 0.31$	

Stable isotope analysis. Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for each species of host blood was significantly different ($F = 57.20$; $df = 4, 24$; $P < 0.01$). The post hoc tests showed significant differences in $\delta^{13}C$ based on an alpha level of 0.05 for all pair-wise combinations ($P < 0.01$ each). The post hoc test showed $\delta^{15}N$ was significantly different ($P < 0.01$ each) in all pair-wise combinations except between goat blood and chicken blood (Figure 41). Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for unfed 3rd instar *O. turicata* nymph sample units from each cohort showed significant differences ($F = 4.65$; $df = 6, 32$; $P < 0.01$). The post hoc tests showed no significant differences in both $\delta^{13}C$ and $\delta^{15}N$ based on an alpha level of 0.05 for all pair-wise combinations between EC, CG, and CS cohort. On the other hand, the post hoc tests for $\delta^{13}C$ and $\delta^{15}N$ showed that ES cohort was significantly different from EC, CG and CS cohort ($P < 0.01$ each) (Figure 42). Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for engorged 3rd instar *O. turicata* nymph sample units from each cohort showed significant differences ($F = 29.46$; $df = 6, 32$; $P < 0.01$). The post hoc tests for $\delta^{13}C$ based on an alpha level of 0.05 for all pair-wise combinations showed significant differences ($P < 0.01$ each) except between ES and CS cohort. The post hoc tests for $\delta^{15}N$ showed significant differences ($P < 0.01$ each) except between ES and CS cohort ($P = 0.55$) (Figure 43).

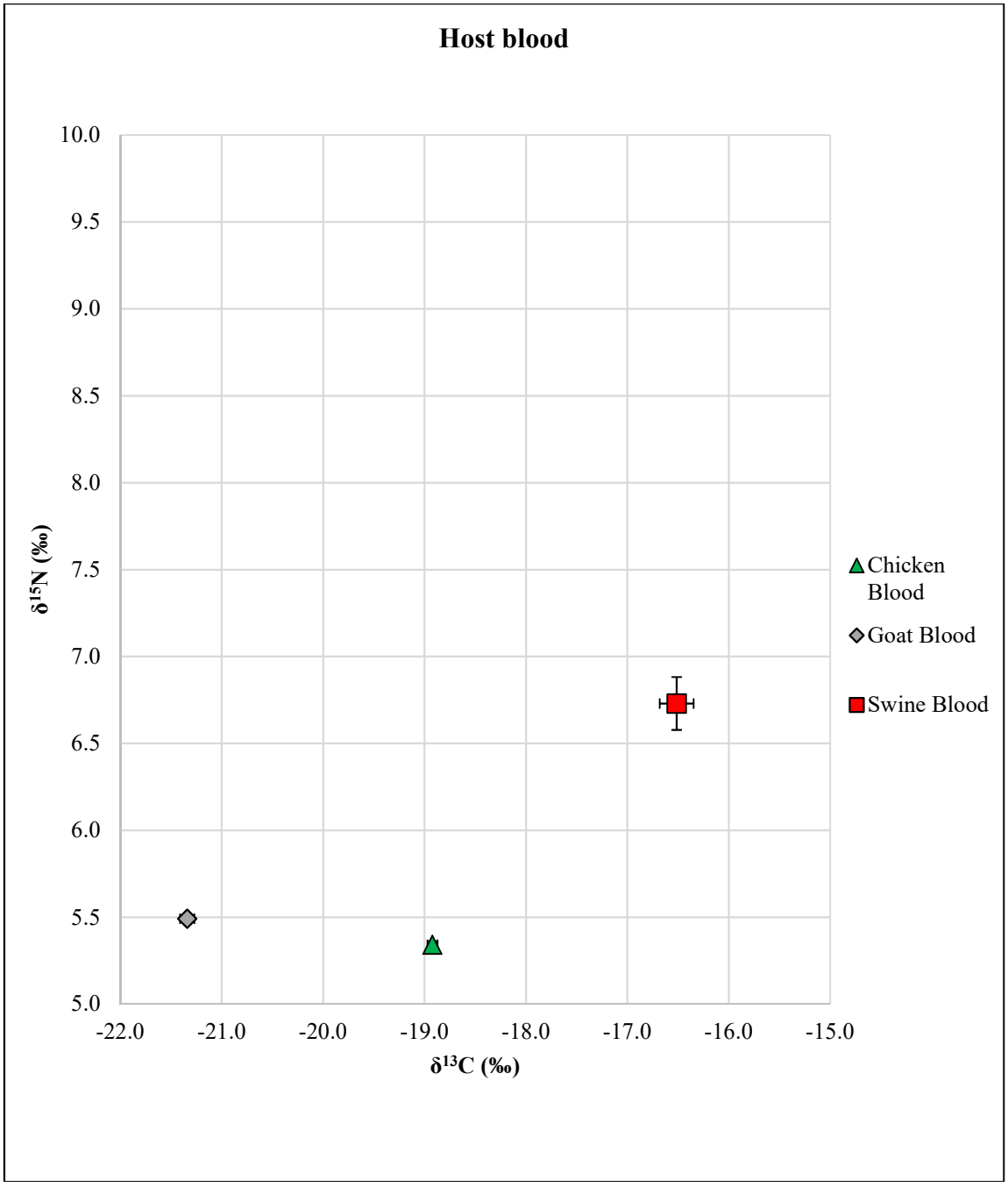

Figure 41. Isotopic results of chicken (*Gallus gallus*), goat (*Capra aegarus hircus*), and swine (*Sus scrofa domesticus*) blood samples (n = 5 each) represented as $\delta^{13}C$ and $\delta^{15}N$. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between host blood ($F = 57.20$; df = 4, 24; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ and $\delta^{15}N$ based on an alpha level of 0.05 for all pair-wise combination showed significant differences ($P < 0.01$ each) except $\delta^{15}N$ between chicken and goat blood.

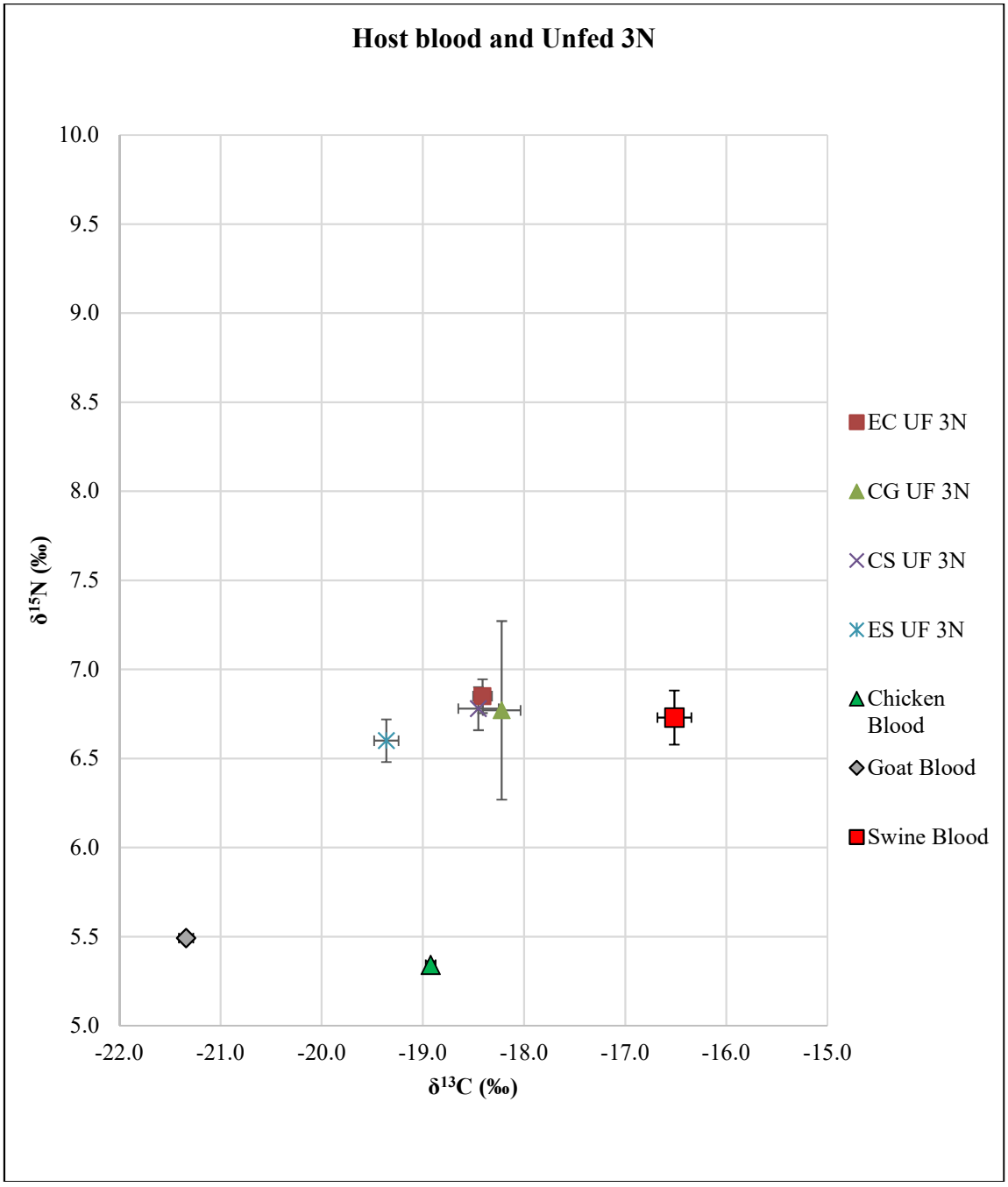

Figure 42. Isotopic results of unfed 3rd instar (UF 3N) *Ornithodoros turicata* nymphs from each cohort sample unit (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y-axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 4.65$; $df = 6, 32$; $P < 0.01$). The post hoc tests (Tukey's HSD) showed no significant differences ($P < 0.05$) in both $\delta^{13}C$ and $\delta^{15}N$ for all pair-wise combinations between EC, CG, and CS cohort. The post hoc tests for $\delta^{13}C$ and $\delta^{15}N$ for ES cohort was significantly different from EC, CG and CS cohort ($P < 0.01$ each). EC= exclusively fed on chicken blood, CG = fed on chicken and goat blood, CS= fed on chicken and swine blood, ES = exclusively fed on swine blood.

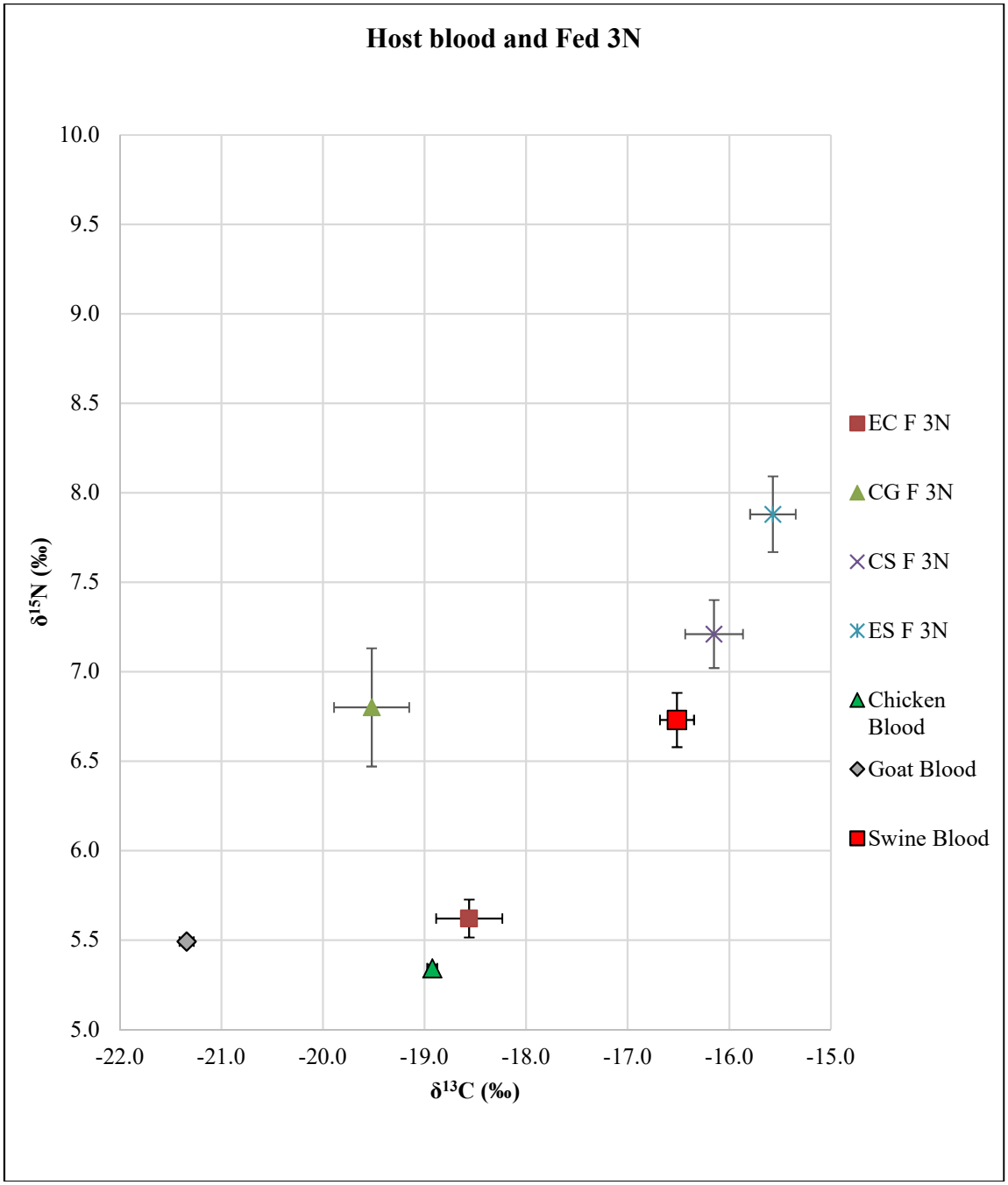

Figure 43. Isotopic results of engorged 3rd instar (F 3N) *Ornithodoros turicata* nymphs from each cohort sample unit (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y-axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 29.46$; $df = 6, 32$; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed significant differences ($P < 0.01$ each) except between ES and CS cohort. All pair-wise comparisons for $\delta^{15}N$ except between ES and CS cohort were significantly different ($P < 0.01$). EC = exclusively fed on chicken blood, CG= fed on chicken and goat blood, CS= fed on chicken and swine blood, ES = exclusively fed on swine blood.

Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for EC cohort 4th instar *O. turicata* nymph sample units based on their post feeding times showed significant differences among sample units ($F = 3.04$; $df = 16, 72$; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences except between engorged 3rd instar nymphs and all post-molt 4th instar nymphs ($P < 0.01$ each). (Figure 44)

Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for GC cohort 4th instar *O. turicata* nymph sample units based on their post feeding times showed significant differences among sample units ($F = 3.10$; $df = 16, 72$; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed no significant differences between ($P < 0.05$) all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and 3, 4, and 6 months post-molt 4th instar nymphs ($P < 0.01$ each). (Figure 45).

Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for CS 4th instar *O. turicata* nymph sample units based on their post feeding times showed significant differences among sample units ($F = 3.91$; $df = 16, 72$; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and all post-molt 4th instar nymphs ($P < 0.01$ each) (Figure 46).

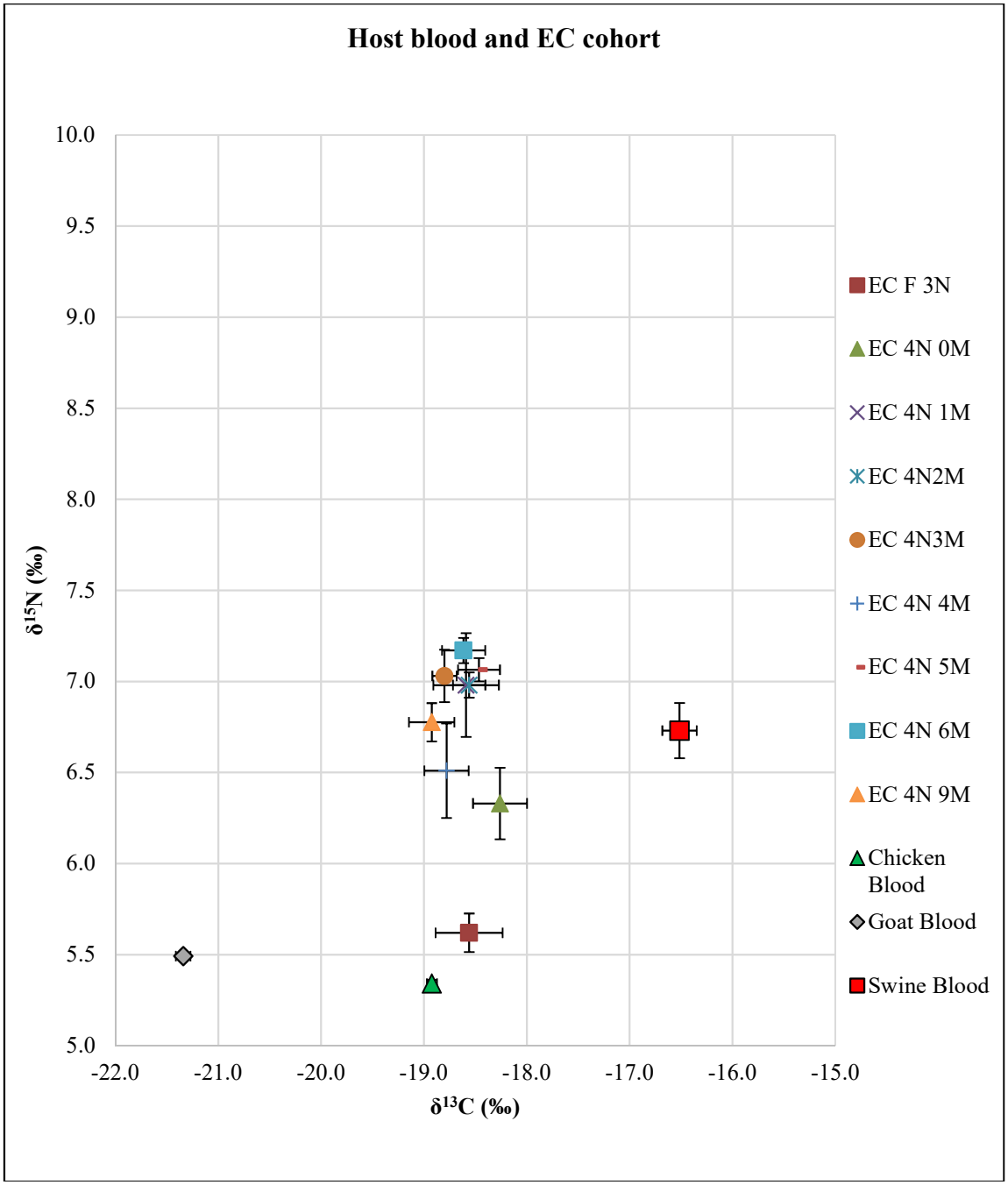

Figure 44. Isotopic results of EC *Ornithodoros turicata* cohort sample unit (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 3.04$; $df = 16, 72$; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except between the except engorged 3rd instar nymphs and all post-molt 4th instar nymphs ($P < 0.01$ each). EC= exclusively fed on chicken blood, 4N 0M= 4th instar nymph immediately after a molt, 4N 1M to 4N 9M= 4th instar nymph 1 month post-molt to 4N 9M= 4th instar nymph 9 months post-molt.

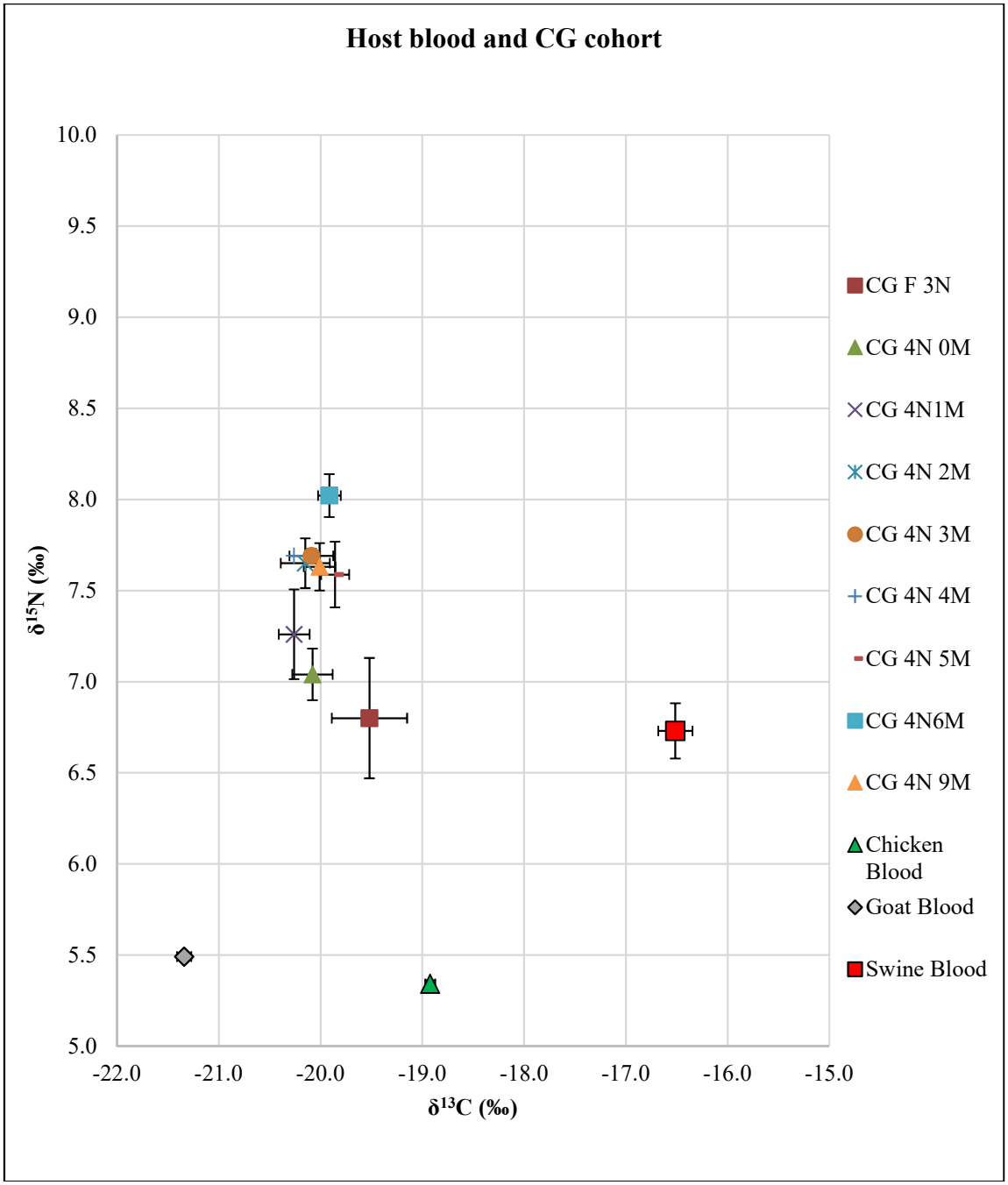

Figure 45. Isotopic results of GC *Ornithodoros turicata* cohort sample unit (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 3.10$; $df = 16, 72$; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and 3, 4, and 6 months post-molt 4th instar nymphs ($P < 0.01$ each). CG= fed on chicken and goat blood, 4N 0M= 4th instar nymph immediately after a molt, 4N 1M to 4N 9M= 4th instar nymph 1 month post-molt to 4N 9M= 4th instar nymph 9 months post-molt.

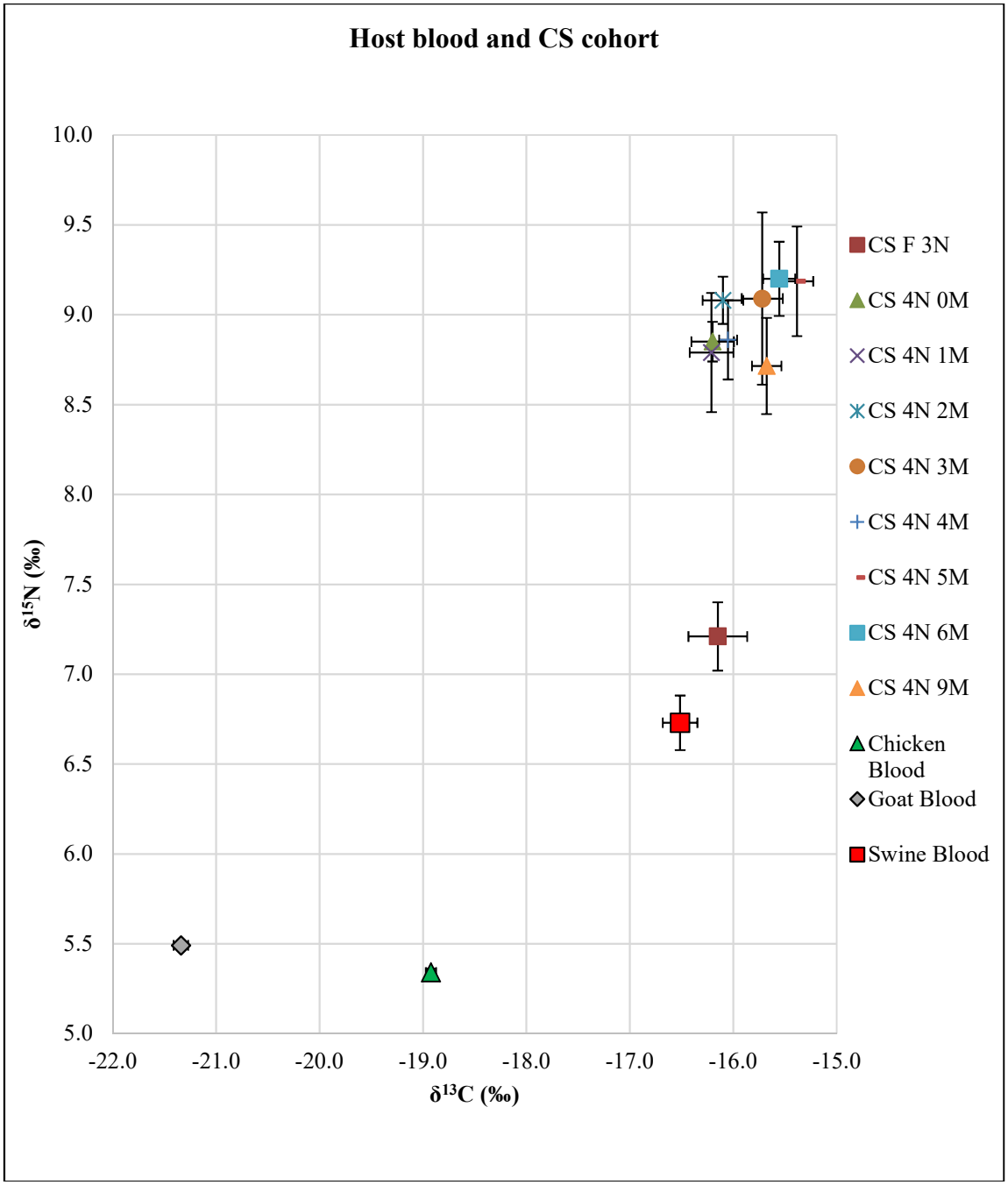

Figure 46. Isotopic results of CS *Ornithodoros turicata* cohort sample unit (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 3.91$; $df = 16, 72$; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and all post-molt 4th instar nymphs ($P < 0.01$ each). CS= fed on chicken and swine blood, 4N 0M= 4th instar nymph immediately after a molt, 4N 1M to 4N 9M= 4th instar nymph 1 month post-molt to 4N 9M= 4th instar nymph 9 months post-molt.

Stable isotope analysis results of $\delta^{13}C$ and $\delta^{15}N$ for ES 4th instar *O. turicata* nymph sample units based on their post feeding times showed significant differences among sample units ($F = 3.22$; df = 16, 72; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for δ15N showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and 1-6 and 9 months post-molt 4th instar nymphs ($P < 0.01$ each) (Figure 47).

The average $\delta^{13}C$ and $\delta^{15}N$ stable isotope values comparing all 4th instar *O. turicata* nymph cohort regardless of their post feeding times (average SI value, n = 45 per cohort) showed significant differences among the cohort ($F = 117.46$; df = 6, 352; $P < 0.01$). The post hoc tests for $\delta^{13}C$ for all pair-wise combinations showed significant differences ($P < 0.05$) between all cohort ($P < 0.01$ each) except between CS and ES cohort. The post hoc test for $\delta^{15}N$ showed significant differences ($P < 0.01$ each) except between CS and ES cohort (Figure 48). The combined $\delta^{13}C$ and $\delta^{15}N$ stable isotope values for all 4th instar *O. turicata* nymph sample units based on their post feeding times showed significant differences ($F = 24.18$; df = 70, 288; $P < 0.01$). The post hoc tests for $\delta^{13}C$ and $\delta^{15}N$ were identical to the sum of all the post hoc tests from the previous results. However, there appear to be three clusters of $\delta^{13}C$ and $\delta^{15}N$ values observed (shown by dotted oval shapes) that represented EC, CG, and CS+ES cohort, respectively (Figure 49).

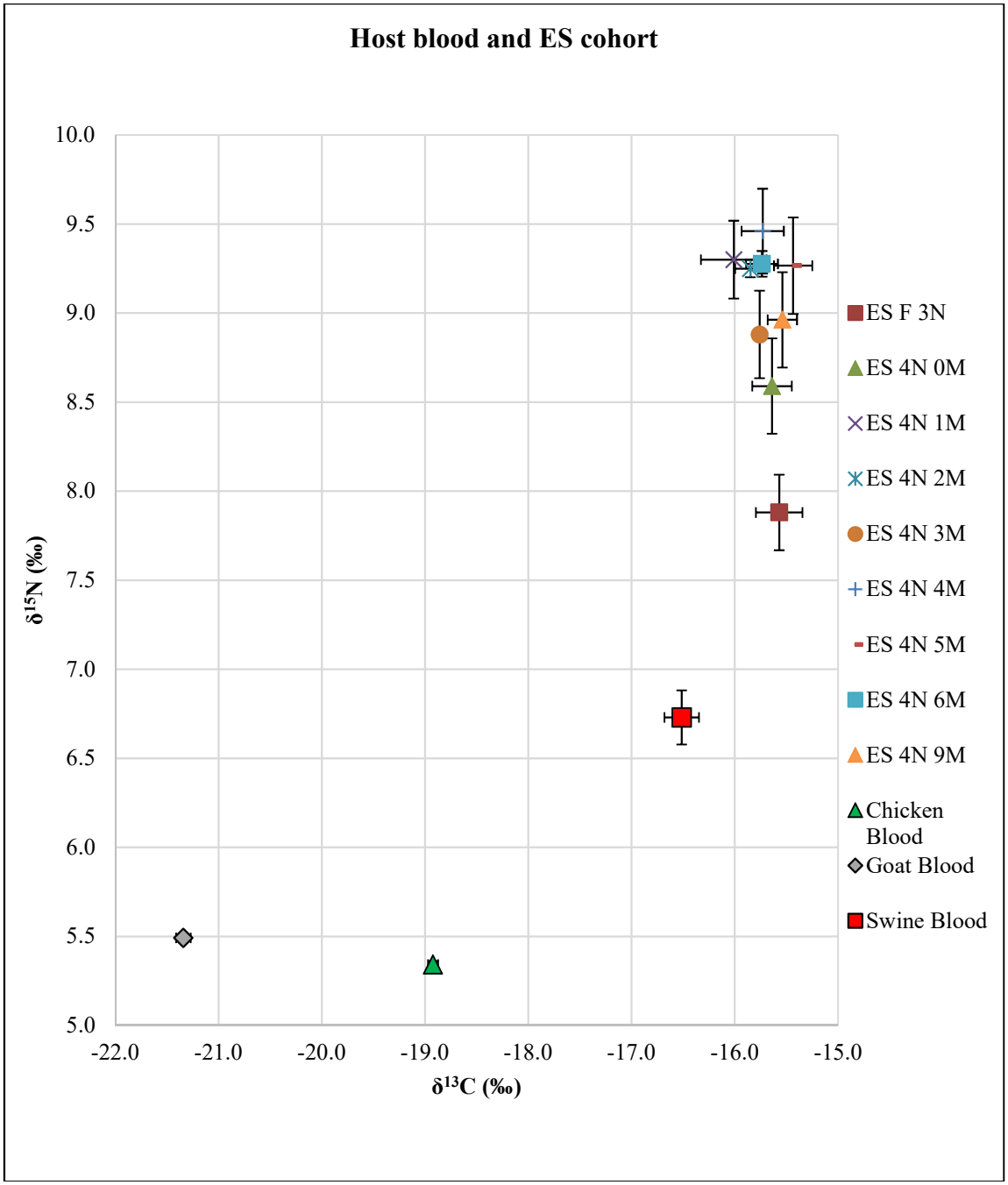

Figure 47. Isotopic results of ES *Ornithodoros turicata* cohort sample unit (n=5 each) represented as δ13C and δ15N superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 3.22$; $df = 16, 72$; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ for all pair-wise combinations showed no significant differences ($P < 0.05$) between all sample units. The post hoc test for $\delta^{15}N$ showed no significant differences ($P < 0.05$) except engorged 3rd instar nymphs and 1 - 6 and 9 months post-molt 4th instar nymphs ($P < 0.01$ each). ES= exclusively fed on swine blood, 4N 0M= 4th instar nymph immediately after a molt, 4N 1M to 4N 9M= 4th instar nymph 1 month post-molt to 4N 9M= 4th instar nymph 9 months post-molt.

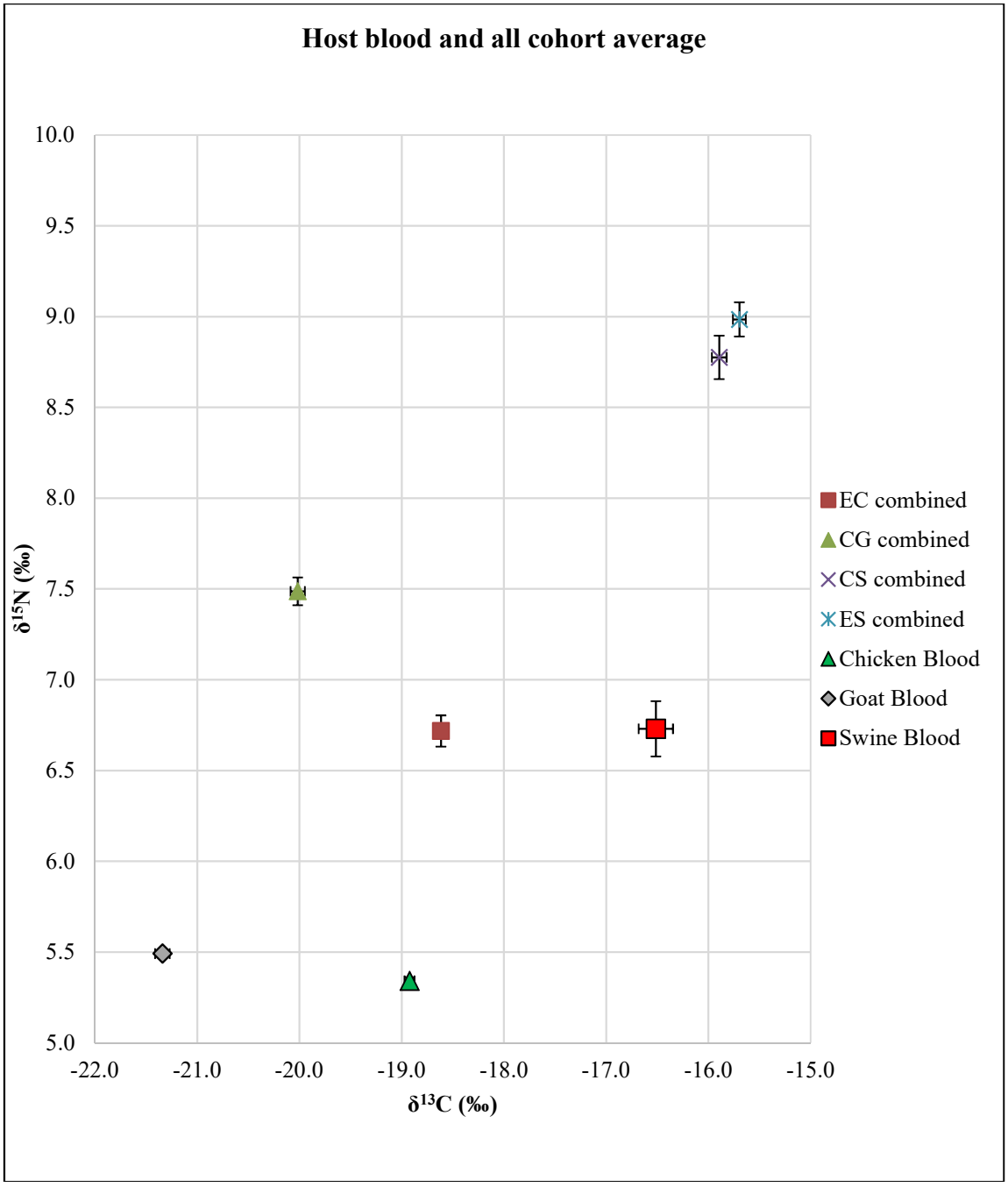

Figure 48. Isotopic results of all *Ornithodoros turicata* cohort sample units regardless of post molt time (n = 45 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 117.46$; df = 6,352; $P < 0.01$). The post hoc tests (Tukey's HSD) for $\delta^{13}C$ all pair-wise combinations showed significant ($P < 0.05$) differences between all cohorts ($P < 0.01$ each) except between CS and ES cohort. The post hoc test for $\delta^{15}N$ showed significant differences between all cohort ($P < 0.01$ each) except between CS and ES cohort. EC= exclusively fed on chicken blood, CG= fed on chicken and goat blood, CS= fed on chicken and swine blood, ES= exclusively fed on swine blood.

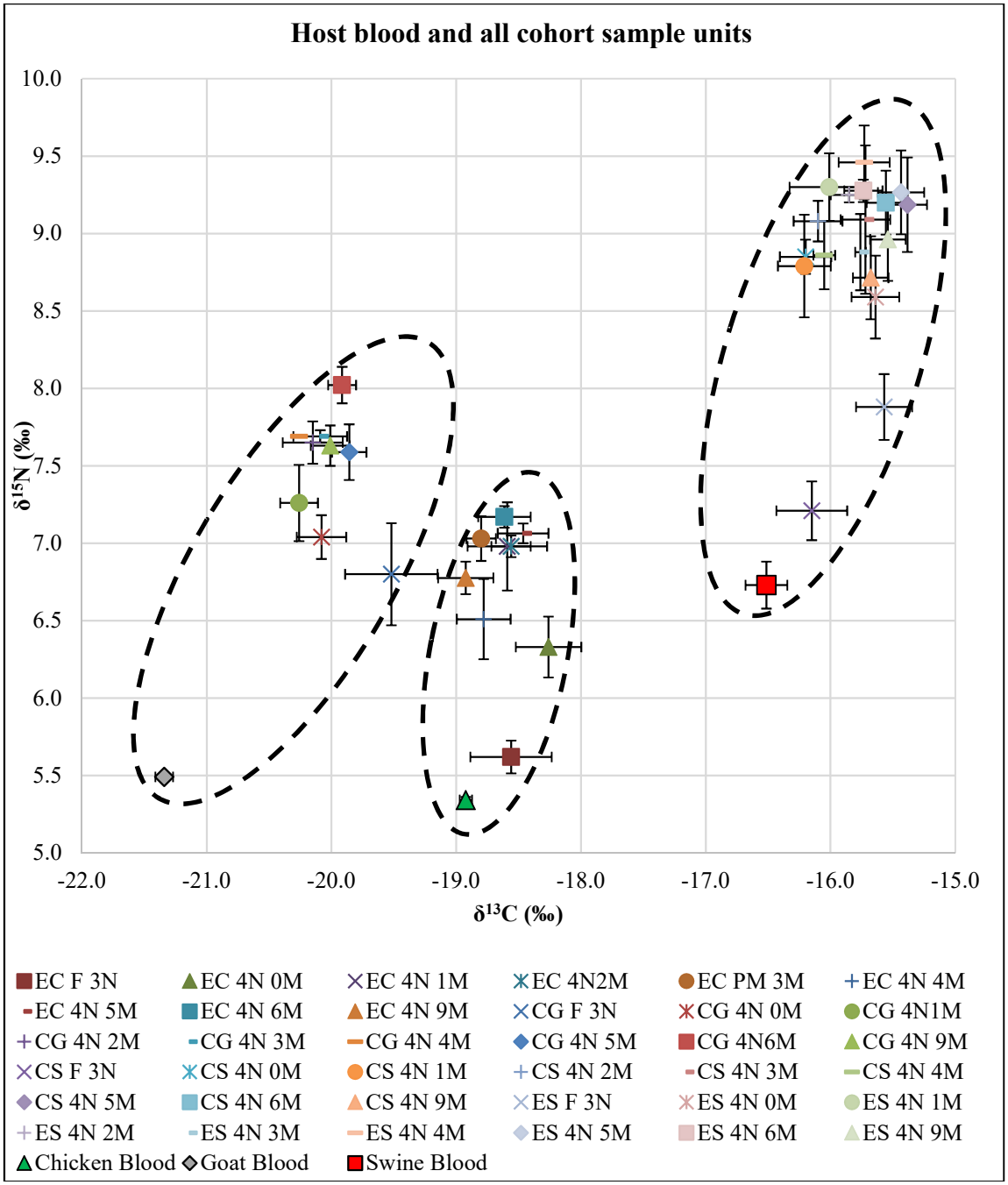

Figure 49. Isotopic results of all *Ornithodoros turicata* cohort sample units based on post-molt time (n=5 each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y- axes error bars represent SEs around means. The Pillai's Trace MANOVA indicated significant differences between cohort ($F = 24.18$; df = 70, 288; $P < 0.01$). Dotted Oval shapes encircle $\delta^{13}C$ and $\delta^{15}N$ values for EC, CG, and CS+ES cohort. EC= exclusively fed on chicken blood, CG= fed on chicken and goat blood, CS= fed on chicken and swine blood, ES= exclusively fed on swine blood. 4N 0M= 4th instar nymph immediately after a molt, 4N 1M to 4N 9M= 4th instar nymph 1 month post-molt to 4th instar nymph 9 months post-molt.

Discussion

Bloodmeal analysis studies for Ixodid ticks became increasingly common in the past two decades (Kirstein and Gray 1999, Pichon et al. 2005, Cadenas et al. 2007, Humair et al. 2007, Allan et al. 2010, Hamer et al. 2015, Léger et al. 2015). On the contrary, bloodmeal analysis studies for Argasid ticks are seldom found (Boctor 1972, Minoura et al. 1985, Chinzei and Minoura 1987, Gill et al. 2004, McCoy et al. 2010). This study reports the first in-depth bloodmeal analysis of an Argasid tick, *O. turicata*, using DNA-based and SI-based techniques. Both DNA-based and SI-based bloodmeal analysis techniques in this study accurately detected *O. turicata* cohorts fed on different host blood. The host-specific *cyt*b genes were detected during the entire experiment period of 330 days using the DNA-based techniques. The SI analysis generated distinctive $\delta^{13}C$ and $\delta^{15}N$ values for each host blood as well as the *O. turicata* cohort that fed on different host blood. Therefore, the results of this study suggest both DNA-based and SI-based bloodmeal analyses may be useful tools in assessing host-vector interactions and determining host utilization for *O. turicata*.

Detectable levels of a host-specific ctyb gene within *O. turicata* fed on a single or multiple hosts were detected in *O. turicata* sample units across all cohorts regardless of host blood type during the entire 330 experiment days (Tables 17 to 19). This observation suggests processing and storing host blood in *O. turicata* may be drastically different from Ixodid species. The implication is based on the difference in the longevity of host-specific *cyt*b genes detection in this study in comparison to the longevity of *cyt*b gene detections reported in other similar studies. For example, the study by Hamer et al. (2015) which also

conducted host-specific primer and probe qPCR-based bloodmeal analysis on the Ixodid species, *A. americanum,* reported the qPCR failed to detect host-specific *cyt*b as early as six weeks post-feeding in their adult sample units. Exploring the difference in the bloodmeal digestion process between *A. americanum* and *O. turicata* may allow elucidation of a plausible inference.

The bloodmeal processing in both Ixodid and Argasid species are composed of three phases. Hemolysis takes place during the first phase which occurs immediately upon feeding and lasts 2 to 15 days. The second phase, also called the "rapid" digestion take place in the midgut of ticks and can last from several weeks to 3 months. Finally, the third phase, also called the "slow" digestion occurs mainly in the apical branches of diverticula and can last for years (Sonenshine and Anderson 2014). The difference between Ixodid and Argasid tick digestion process is in the third digestion phase. In Ixodid ticks bloodmeal digestion occurs in a uniform manner, and the ingested bloodmeal is evenly stored and consumed at a steady rate in the midgut as well as in the diverticula (Sonenshine and Anderson 2014). On the other hand, bloodmeal digestion in the third phase of Argasid ticks occurs with an uneven rate because a substantial amount of bloodmeal is stored in the peripheral regions of midgut diverticula with no digestive activity (Sonenshine and Anderson 2014). This slow and uneven digestion of bloodmeal allows Argasid ticks to endure starvation that could last for years as observed in *O. turicata* (Davis 1941). Hence, this feature may be the reason for the discrepancies in the longevity of *cyt*b gene detections using qPCR observed in the *A. americanum* and *O. turicata*.

The detectability *O. turicata* fed on multiple hosts varied depending on the bloodmeal analysis techniques employed. Stable isotope analysis technique could not be used to discern the difference between single-host and multi-host blood fed *O. turicata* cohorts. For example, there were no differences between the overall $\delta^{13}C$ and $\delta^{15}N$ values of the CS and ES cohorts (Figure 48). Moreover, engorged 3rd instar nymphs from CS and ES cohorts showed no significant difference in their $\delta^{13}C$ and $\delta^{15}N$ values (Figure 43), despite each cohort being fed different host blood previously, further strengthening the argument the last bloodmeal *O. turicata* acquired determines the outcome of the SI analysis. In contrast, the qPCR analysis could be used to detect all host-specific *cyt*b gene correctly across all *O. turicata* cohorts fed on multiple host blood when using probes specific for each host. In other words, for cohorts that have fed on two host species (e.g., CG, CS), DNA from both hosts was detected using individual assays each with host-specific primer/probe sets. Additionally, *O. turicata* sample units from CG and CS cohorts were able to maintain a detectable level of chicken *cyt*b gene over the course of two molts and starvation periods exceeding nine months (Table 17). This ability may be due to previously mentioned "slow" digestion phase in *O. turicata*. Indeed, the rate of biochemical processes (i.e., no digestive activity) in peripheral regions of midgut diverticula can slow down the digestion of the bloodmeal, thus, prolonging the overall bloodmeal consumption (Sonenshine and Anderson 2014). Nevertheless, the physical capacity of peripheral regions of midgut diverticula that store a previous bloodmeal may also force subsequent (and newly) acquired bloodmeal to be kept in the medial regions of midgut where active digestion occurs (Sonenshine and Anderson 2014). This "blocking"

of storage space by the previous bloodmeal may allow residual bloodmeal from earlier feedings to remain the entire tick lifespan of *O. turicata*, enabling qPCR analysis to detect multiple host *cyt*b genes.

The duration of starvation could influence the outcomes of each type of bloodmeal analysis techniques. For example, qPCR results for the CS cohort using chicken-specific primer and probe showed significant differences in qPCR results based on the experiment days (length of starvation) (Table 17). Similarly, qPCR results for the CS cohort using swine-specific primer and probe also seem to be influenced by the duration of starvation (Table 19). There was no logical explanation for this since the qPCR results of other groups, such as the ES cohort, which also reared in using swine blood was not affected by the duration of starvation endured. Naturally, the number of ticks used for each sample unit may be a suspect for inconsistency observed in qPCR results. There were only five ticks per sample tick unit and perhaps increasing the sample size may reduce the inconsistency observed in qPCR results for future studies. However, no sample tick unit failed to retain detectable host *cyt*b gene level all together at any time during the entire experiment period

Another plausible explanation for the apparent influence of starvation period on the outcomes of each type of bloodmeal analysis technique can be inferred based on the relatively low swine blood DNA extracted during this study (Table 16). Chicken blood which consists both immature and mature nucleated erythrocytes yielded the highest quantity of DNA in the extract. In contrast, goat and swine blood (and other mammals) are known to have immature nucleated erythrocytes that become anucleated once matured,

attributing to relatively low DNA extract yield (Lazarides 1987, Jones 2015). While this study did not examine the proportion of *cyt*b gene within the total DNA extract of host blood, an inference can be made based on the ubiquitous presence of the *cyt*b gene in vertebrates as part of their mitochondria, in that the relative proportions of *cyt*b gene in the chicken, goat, and swine blood would be similar to that of total DNA extract (Borst and Kroon 1969, Kocher et al. 1989, Chiu et al. 2003, Cupp et al. 2004). Therefore, *O. turicata* CS cohort, which fed on swine blood once, may not have had the chance to acquire and maintain the adequate amount of swine *cyt*b gene throughout the entire experiment period compared to *O. turicata* ES cohort, which had four opportunities to feed on swine blood. Moreover, the fact that the host blood type had no effects on the overall qPCR results of all tick sample units (Table 20) further denigrates the significance of different qPCR results seen in CS cohort. Therefore, an argument can be made that the inconsistency observed in qPCR results based on the length of starvation seen in CS cohort may not be attributed to a single reason but due to combinations of low sample number, lower DNA extract yield in swine blood, and uneven rate of bloodmeal digestion of *O. turicata*.

Starvation duration influenced the outcomes of SI analysis differently than that of qPCR analysis. First, patterns of increased $\delta^{13}C$ and $\delta^{15}N$ values in engorged 3rd instar nymphs in each cohort compared to their corresponding host blood were observed. This observation could be due to SI (e.g., nitrogen) being enriched (DeNiro and Epstein 1981, Minagawa and Wada 1984, Vanderklift and Ponsard 2003). The increase of $\delta^{13}C$ and $\delta^{15}N$ values in engorged *A. americanum* were also observed in the study by Hamer et al. (2015).

However, the SI analysis failed to provide conclusive evidence for the SI fractionation, which occurs due to nutrient stress such as starvation. Such physiological stresses cause nitrogen fractionation via changes in the rate of amino acid consumptions, uric acid formations, and secretions (Hobson and Clark 1992, Vanderklift and Ponsard 2003). Indeed, Hamer et al. (2015) reported changes in $\delta^{13}C$ over time in *A. americanum* fed on chicken; however, data from this study was inconclusive to make such inference. This may be due to the inconsistent digestion rate in *O. turicata* mentioned above.

The applicability of the bloodmeal analysis techniques used in this study must be carefully considered in the contexts of *O. turicata* biology and ecology. For example, the longevity of *O. turicata* ticks may generate a population structure that inadvertently interferes with the accurate assessment of *O. turicata* host utilization. The longevity of a non-nidicolous tick, such as *A. americanum*, is typically less than three years, and overlapping generations found in their population structure may rarely consist of more than two generations (Apanaskevich and Oliver Jr 2014). In this case, tick-host feeding patterns observed in the population may closely resemble the actual host utilization as the host population dynamic may not change drastically within the typical generation time of the tick species in question. On the other hand, the longevity of nidicolous ticks, such as *O. turicata* may be measured in decades (Francis 1938, Davis 1941). Thus, ticks may outlive their hosts or live through the drastic changes in host population dynamics. Consequently, overlapping *O. turicata* generations in their population structure may consist of several generations. In this case, bloodmeal analysis may not accurately depict

the host utilization of *O. turicata* as older generation ticks may have had exclusive access to the host that are no longer available to younger generation ticks.

In summary, challenges of studying the host utilization by *O. turicata* stem from complexity in their biology and ecology. A comprehensive understanding of vector ecology- including at its forefront the patterns of host utilization- must be known in order to most efficiently manage vectors in nature. The bloodmeal analysis techniques outlined in this study are promising tools for determining the host utilization of *O. turicata.* Specifically, the DNA-based bloodmeal analysis results from this study underscored the feasibility to discern multiple-host utilization by *O. turicata* and applicability of *cyt*b gene as a host-specific molecular marker, and future studies could expand this work to include naturally-relevant host taxa. On the other hand, SI-based bloodmeal analysis was able to accurately distinguish host blood, *O. turicata* cohort fed on different host blood, and nitrogen enrichment in *O. turicata* post bloodmeal consumption.

CHAPTER V

CONCLUSIONS

This dissertation provided information foundational to *O. turicata* ecology with respect to habitat-host-vector interactions. The results from this dissertation may guide future studies in the area of *O. turicata* dispersal strategies/population genetics, pathogen maintenance/transmissions, and host utilizations.

The study of *O. turicata* cave environments in GCSNA, TX provided several inferences in the correlations between *O. turicata* phenology and abiotic factors and animal activities. The study revealed not only that peak *O. turicata* activities correlate with the months with the highest average temperatures, but also that the relative humidity and temperature profiles among four *O. turicata*-active caves in GCSNA remain similar in their values and pattern changes throughout the year. Furthermore, activities of 20 vertebrate species, of which only seven were the previously known hosts of *O. turicata,* at *O. turicata*-caves were observed, extending potential host range of *O. turicata*.

Nevertheless, the animals which spent the most time in the cave such as black vultures, canyon wrens, opossums, raccoons, ring-tailed cats, and turkey vultures were not known hosts of *O. turicata* (Cooley and Kohls 1944, Donaldson et al. 2016). Furthermore, this study noted several indirect interactions between *O. turicata* and other invertebrate species (i.e., harvestmen) found in the cave, revealing the potential for complex interactions within the *O. turicata* community structure found in cave environments.

The study of host immune responses to repeated *O. turicata* challenges in this dissertation provided promising data for an indirect *O. turicata* surveillance via host animal's immune response against *O. turicata* SGE. This finding can be a great use to assess vector-host interactions between *O. turicata* and feral swine populations in Texas. However, future studies are needed before the field applications of indirect *O. turicata* surveillance via host immune response against *O. turicata* SGE can be implemented. The data from this dissertation are inadequate to determine the minimum number of *O. turicata* required to elicit host immune response, the degree of cross-reactivities in host immune responses among SGE of *O. turicata* related ticks, and level of comparable immune responses against *O. turicata* SGE between domestic and feral swine. Therefore, future research investigating the specificity and sensitivity of feral swine immune response to *O. turicata* challenge is warranted.

The bloodmeal analysis of *O. turicata* fed on chicken, goat, and swine blood using qPCR showed that *O. turicata* could retain detectable fragments of a host gene (*cyt*b) beyond 330 days through multiple moltings. Also, the qPCR-based bloodmeal analysis could discern *cyt*b genes of multiple hosts in *O. turicata* with multiple host bloodmeals. The SI analysis data from this study revealed that different host blood types generate discernable isotopic signatures. This offers a bloodmeal analysis method that appears to be unhindered by host blood digestion and DNA degradation in hematophagous vectors. However, SI analysis data fell short in discerning the *O. turicata* with multiple host bloodmeals, providing grounds for future research. Also, SI signature of a host blood can be altered significantly based on the diet of the host (DeNiro and Epstein 1981, Hobson

and Clark 1992, Hood-Nowotny and Knols 2007). Therefore, research to examine the multitrophic-level cascade effects of host diets on the SI signatures of *O. turicata* that feed on the secondary or tertiary consumers of the food-web found in their habitats is needed.

Two lingering questions are remaining regarding interactions between habitat-host-vector interactions of *O. turicata*. The first question is their dispersal strategies. At the conclusion of this dissertation, there are no data (e.g., evidence of long-term attachment on the host) which could shed light on dispersal strategies employed by *O. turicata* that could cover the greater distances (e.g., From Texas to Florida). If there are no long-distance dispersal strategies, subsequent questions, "How did the *O. turicata* population in Florida get there?" or "Did *O. turicata* originate in Florida and moved westward across U.S.?", can be asked. To answer these questions, future research that focuses on the *O. turicata* population genetics must be conducted.

The second question derived from this dissertation is the community structure and interactions among microbiome within *O. turicata*. The data from this dissertation showed the intricate direct and indirect interactions among the members of the *O. turicata* community and their environment. However, *O. turicata* can play two roles (i.e., vector and reservoir) in the natural history of vector-borne disease due to their long generation time, endurance over starvation, and ability to transovarially transmit pathogens (Davis 1941, Dworkin et al. 2002, Cutler 2010). Therefore, *O. turicata* as an individual can be viewed as a platform in which a microbiome community can establish their unique structure. Future studies examining the microbiome community within *O. turicata* in the context of direct and indirect interactions among the members of the *O turicata*

community found in cave environments can provide a comprehensive life history of vector-borne diseases.

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