ABSTRACT

Title of Dissertation: A NOVEL IXODES SCAPULARIS PROTEIN DICTATES TICK HEMATOPHAGY AND CUTICLE INTEGRITY, IMPACTING TICK DEVELOPMENT

Shraboni Dutta, Doctor of Philosophy, 2023

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Ticks are prevalent throughout the world and are capable of transmitting a variety of pathogens (e.g., bacteria, protozoa, and viruses) to humans. Incidence rates for tick-borne diseases (TBD) are also increasing globally, and effective vaccinations to combat tick infestations and TBD transmission remain a critical unmet need. Of the six major tick genera that spread human illnesses worldwide, Ixodes ticks are the most prevalent. Specifically, *Ixodes scapularis* (also known as the blacklegged or deer tick) is an obligate blood-feeding arthropod that transmits several human and animal pathogens that include *Borrelia burgdorferi* sensu lato complex – the causative agent for Lyme disease. Unlike many hematophagous insects and soft ticks, *I. scapularis* (hard ticks) remain attached to their hosts for several days and are capable of uptaking bloodmeals that are 100 times greater than their initial body weight. A large and

nutrient-dense bloodmeal is essential for their sub-adult and adult development processes and fecundity. However, the molecular and cellular processes that regulate tick blood feeding (hematophagy) and development have not been extensively elucidated. Therefore, our major objective is to characterize tick molecular components that are critical in the tick parasitism and life cycle in order to develop new strategies to combat tick infestations and spread of tick-borne diseases.

Herein, we describe the structural and functional properties of a newly identified *I. scapularis* protein isolated from the partially fed nymphal ticks. Although the protein displays minor homology to proteins of known functions, structurally, it resembles some features of arthropod Odorant Binding Proteins (OBP). Therefore, we refer to this protein as, *Ixodes* Gut OBP (IGOBP). We show that the knockdown of *IGOBP* via RNA interference in ticks results in impaired blood feeding (hematophagy) and significantly decreases their post-fed weights. In addition, systemic IGOBP knockdown gives rise to aberrant phenotypes, significantly reduces tick molting rate, and compromises the structural integrity of the cuticle, specifically the flexible alloscutum components. Notably, IGOBP knockdown has profound effects on the molting efficacy and fitness of females than males. This is likely due to the fact that female adults consume a greater volume of bloodmeal than male adults, necessitating a more pronounced expansion of the alloscutum. Subsequently, our RNA sequencing data identifies multiple genes whose expressions are regulated by IGOBP. The underlying mechanism of possible IGOBP or associated gene functions may aid in identifying future targets for anti-tick vaccines. In summary, our studies characterized a novel *I. scapularis* protein revealing that the protein is essential for tick hematophagy and development. To the best of our knowledge, this is the first characterization of a tick odorant-binding protein (OBP), using structural and functional genomic

tools that unearthed the unique and possibly multifunctional role of IGOBP in vector biology and parasitism. We anticipate that the presented data will enhance our fundamental understanding of tick biology and contribute to the development of potential anti-tick measures.

A NOVEL IXODES SCAPULARIS PROTEIN DICTATES TICK HEMATOPHAGY AND CUTICLE INTEGRITY, IMPACTING TICK DEVELOPMENT

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2023

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Dedication

For my family and friends who have always supported me and wanted the best for me.

For my parents, who have always believed in me and fought against all odds to pave this path for me.

Acknowledgements

Firstly, I would want to thank my advisor, Dr. Utpal Pal, for his guidance and providing me the opportunity to explore so many fascinating projects. Your advice enabled me to think critically and effectively. I appreciate your assistance throughout my time in UMD.

Secondly, I would like to thank the members of my committee, Drs. Daniel Nelson, Georgiy Belov, Adriana Marques, and Zhengguo Xiao. I am grateful for your time, direction, and insight that shaped my thought process and improved the impact of my dissertation project.

I also want to thank Dr. Nelson and his lab members at IBBR (Dr. Amanda Altieri and Daniel Kemboi) and Dr. Juan Anguita, Ikerbasque Research Professor, CIC bioGUNE for their collaborative efforts that were crucial for some of the data presented in this dissertation.

I would also like to thank all of my former and current lab members. I would like to thank Dr. Vipin Singh Rana in particular, who not only helped and guided me with his vast knowledge but was also a wonderful friend. Thank you for being there for me through the ups and downs. It has been a pleasure working with you on various projects. I am also grateful to have had Sandhya and Shelby as colleagues, as they were always there to listen, to cheer me up, and provide support. I am fortunate to have you all as my friends and I could not have asked for more supportive lab mates. I would like to thank Dr. Chrysoula Kitsou and Kathryn Nassar for their help and support in lab maintenance and lab management. I appreciate your efforts in running this lab efficiently. In addition,

I would like to thank the Department Staff and Faculty, as well as my fellow graduate students, for their assistance and for making this workplace so welcoming.

I would like to thank my family and friends for always being there for me and for encouraging me to achieve my goals. I am thankful for my parents' love, support, and sacrifices. Maa and baba, you have taught me to be determined, honest and humble. Words cannot fully describe your contributions to this accomplishment. I would like to express my gratitude to my grandparents (Dadu and Nanu, Dadu and Dida, and Sejodadu) for their love and blessings. I'd like to thank my grandfather, Dadu, for his love and support. I know you are excited for my graduation, and I wish Dida were still alive! I want to thank my parents-in-laws. Your constant love, support and understanding (not to mention your delicious home cooked meals) have made my journey a lot easier. I would like to thank my nephews (Trenav and Audric) for their adorations for their ‘Kaki.’ Spending time with them have been my stress relievers.

I want to thank my younger sister, Misti. I could not have imagined my life without you. Thank you for always being by my side, for listening to all my boring stories and for loving me unconditionally.

I cannot thank my husband, Dipankar, enough for always being there for me. You have always given me the best advice and pointed me in the right directions. Thank you for patiently listening to my daily stories and putting up with my stress. I could not have accomplished this work without your love, guidance, and support.

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List of Abbreviations

AA	Amino acid
AChE	Acetylcholinesterase
ATR	Acquired Tick Resistance
BSK	Barbouer Stoener Kelly medium
CD	Circular dichroism
cDNA	Complementary DNA
^{o}C	Degrees Celsius
CO2	Carbon-Dioxide
CaCl2	Calcium Chloride
CHBD2	Chitin Binding Domains
CPR	Cuticular Proteins
CPAPs	Cuticular proteins analogous to peritrophins
CSPs	Chemosensory proteins
CYTP450	Cytochrome p450
DNA	Deoxyribonucleic Acid
DEET	N, N-Diethyl-Meta-Toluamide
E	Ecdysone
20E	20-hydroxyecdysone
EcR	Ecdysone receptor
ELISA	Enzyme linked Immunosorbent Assay
EM	Erythema Migrans
EST	Expressed Sequence Tag
ETH	Ecdysone-triggering hormone
GPCRs	G protein coupled receptors
GST	Glutathione S-transferase
HIS	6x Histidine Tag
IR	Infrared Radiation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IgE	Immunoglobulin E
JH	juvenile hormone
kDa	Kilo Dalton
LB	Luria Bertani medium
LD	Lyme Disease
MS	Mass Spectrometry
µl	Microliter
µM	Micromole
USP	Ultraspiracle
mm	Millimeter
MF	Methyl farnesoate
NADA	N-acetyl dopamine

NBAD	Beta-alanyl dopamine
nM	Nanomole
nanoDSF	Nano scale differential scanning fluorimetry
OBP	Odorant Binding Protein
ODE	Odorant Degrading Enzymes
ORs	Odorant Receptors
Orco	Odorant Receptor Coreceptor
ORF	Open reading frame
ORNs	Olfactory Receptor Neurons
OSNs	Olfactory Sensory Neurons
PBS	Phosphate-Buffered saline
PBS-t	PBS + 0.05% Tween 20
PBPs	Pheromone-Binding Proteins
PCR	Polymerase chain reaction
PPO	Prophenoloxidase
PTTH	Prothoracicotropic hormone
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcriptase PCR
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
TBE	Tick-borne encephalitis

Chapter 1: Introduction

1.1 Ticks

Ticks are obligate hematophagous arthropods that feed on almost any vertebrate host, including mammals, birds, reptiles, and amphibians. During their acquisition of a bloodmeal, they can acquire pathogens from infected hosts, which can be transmitted to naïve animals on their next meal. Ticks spread numerous pathogens and are considered highly effective vectors, second only to mosquitoes, and are responsible for serious diseases in humans and animals, including Lyme disease, ehrlichiosis, rocky mountain spotted fever, human granulocytic anaplasmosis, tularemia, tick-borne encephalitis, and babesiosis (Eisen and Eisen 2018b). Tick infestation also poses several serious health-associated complications in livestock such as allergic reactions, paralysis, and major blood loss, which ultimately results in significant economic losses, such as loss of optimal milk production in cattle (Jongejan and Uilenberg 2004). Even though ticks thrive in tropical climates, they are present in most parts of the world, including Antarctica (Barbosa et al. 2011).

Although the origin of ticks is unclear, a 99 million old amber fossil provides evidence that ticks fed on the blood of feathered dinosaurs, indicating that they have existed at least since the Mesozoic era (Peñalver et al. 2017). Origin, evolution, and divergence occurred throughout the Mesozoic era (~225 million years, Mya) simultaneously with the divergence of modern birds and placental mammals during

the late Cretaceous period (120–92 Mya), with notable evolutionary changes and distributions occurring during the Tertiary period (65-5 Mya) (José de la Fuente 2003 (Kitsou, Fikrig, and Pal 2021). Analysis of phylogenetic relationships suggests that ticks are related to the predatory mites Holothyrida, which feed on the bodily fluids of deceased arthropods (Dobson and Barker 1999; Kitsou, Fikrig, and Pal 2021).

1.1.1 Classification

Ticks are classified as Arachnida under the subphylum Chelicerata of the phylum Arthropoda. Ticks belong to subclass Acari and the suborder Ixodida. They differ from other arthropods in that they lack a head, thorax, and antennae and have fewer body segments. They feature scissors-like chelicerae and Haller's organs; the latter are unique sensory organs on their front legs. Chelicerata includes ticks, mites, scorpions, horseshoe crabs and spiders whereas, Arachnida includes spiders, ticks, and mites. Adult arachnids have eight legs, which is the most distinguishing characteristic between them and insects (3 pairs). Ticks and mites are very closely related (Lozano-Fernandez et al. 2019). In their process of development, eggs deposited in the environment transform into six-legged larvae, eight-legged nymphs, and eight-legged adults (Burton 2021).

There are around 900 known tick species in the world, all of which belong to one of three families: Ixodidae, Argasidae, or Nuttalliellidae (Dantas-Torres, Chomel, and Otranto 2012). Approximately 80% of the known tick species worldwide are Ixodid ticks or hard ticks (703 species) (Dantas-Torres, Chomel, and Otranto 2012).

Hard ticks are then subdivided into the seven most wide-spread genera, including, *Ixodes, Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Rhipicephalus, and Rhipicephalus* (formerly *Boophilus*) (Jongejan and Uilenberg 2004). As the name implies, hard ticks feature a sclerotized dorsal hard scutum. In adult males, the whole dorsal body surface is composed of the hard scutum, whereas in nymphs and adult females, only small parts of their dorsal body surface are made of scutum. Unlike Argasidae or soft ticks, the body of ixodid ticks does not cover the mouthparts and hence is distinct from the rest of the body. Hard ticks are referred to as slow feeders because they remain attached to their hosts for several days in order to finish their bloodmeal. They only feed once in each life stage, have one nymph instar, and mature females die after laying thousands of eggs all at once (Jongejan and Uilenberg 2004).

Argasid ticks lack hard dorsal scutum and have a soft leathery cuticle; hence they are referred to as soft ticks (Daniel E. Sonenshine and Roe 2013). Their mouthpart or capitulum cannot be differentiated from their body parts as their body surface covers their anterior parts. Argasidae includes 183 species and some of them have more than one instar in the nymphal stage (Jongejan and Uilenberg 2004). Soft ticks take bloodmeal multiple times at each life stage, each lasting several minutes. Nuttalliellidae shares characteristics from both Ixodidae and Argasida and include only one living species in South Africa (Daniel E. Sonenshine and Roe 2013).

Depending on the habitat, ticks can be divided into nidicolous and non-nidicolous. Most of the argasid ticks are nidicolous as they shelter in caves, burrows, nests and prefer to live in close proximity to their hosts (Daniel E. Sonenshine and

Roe 2013; Coons and Rothschild 2008). Ixodid ticks are mostly non-nidicolous meaning they live in open environments, such as forests, bushes, meadows, and in semi-desserts. The Ixodidae family is further divided into metastriate and prostriate ticks. Metastriate ticks, such as those belonging to the *Dermacentor* or *Rhipicephalus* genera, have relatively tiny mouthparts and instead rely on saliva-secreted cement or adhesives compounds to securely attach to their hosts (Francischetti et al. 2009). Prostriates, such as members of the *Ixodes* genus, have longer mouthparts that are barbed and depend more on this physical mechanism and structure to remain securely connected to their hosts (Francischetti et al. 2009).

Although many ticks can transmit human pathogens, Ixodes sp. ticks are one of the most predominant disease vectors that are most diverse, and distributed globally, and yet are genetically distinct (Jia et al. 2020). Ixodes genus is represented by many closely related ticks of which three species, namely *I. scapularis* (black-legged or deer tick), *I. ricinus* (sheep tick), and *I. persulcatus* (taiga tick) are most widespread and are responsible for most human infections in North America, Europe, North Africa, and Asia, respectively. These ticks belong to the Ixodidae family of hard-bodied ticks. They are obligate blood-feeders that target vertebrate hosts such as small rodents, birds, reptiles with humans being accidental or dead-end hosts. *I. scapularis* is the principal vector of *Borrelia burgdorferi*, the pathogenic agent of Lyme disease in the northeastern United States. In addition to transmitting *B. burgdorferi*, blacklegged ticks also transmit *A. phagocytophilum, Ehrlichia muris, Babesia microti, and the Powassan virus* (Eisen and Eisen, 2018). According to the

Centers for Disease Control and Prevention, incidence rates for tick-borne diseases have been steadily rising with approximately 476,000 new estimated cases of Lyme disease reported per year in the United States alone (CDC 2022a). Lyme disease pathogens thrive in nature vector-host cycle where naïve ticks acquire the bacteria after biting infected hosts and then spread the bacterium to naive hosts when they feed again. Over the last two decades, the geographic ranges of *I. scapularis*, Lyme disease, and other tick-borne diseases (TBD) have grown. Moreover, alpha-gal syndrome is a growing public health concern resulting from tick bites. It is a severe allergic reaction to a sugar (galactose-α-1,3-galactose) triggered by the bite of certain tick species, such as, *Amblyomma americanum* (lone star ticks) (Crispell et al. 2019b; "Alpha-Gal Syndrome Factsheet | Ticks | CDC" 2022).

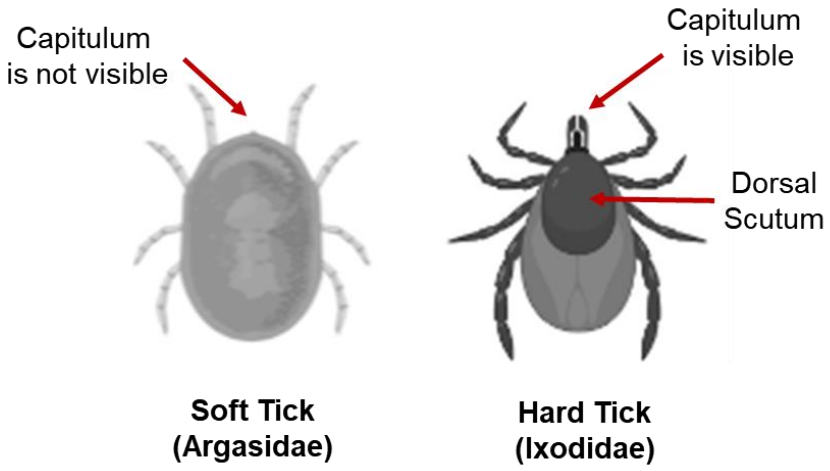


Figure 1.1. Illustration of soft ticks and hard ticks (created in Biorender.com). Soft ticks do not have dorsal hard scutum and their mouthparts or capitulum cannot be seen from the top. Hard ticks have dorsal hard scutum and their capitulum is visible from the top.

1.1.2 Life Cycle

The tick lifecycle consists of four stages – eggs, larvae, nymphs, and adults. At every stage except for the egg, they must uptake a full bloodmeal in order to molt to the next stage. Once eggs hatch into larvae, they attach to small animals and acquire a bloodmeal until full repletion. Fully fed larvae usually drop off their hosts after feeding and undergo intermolt development lasting for 3-4 weeks. Once larvae molt to nymphs, they find a new host and acquire a subsequent bloodmeal. Nymphs drop off the host after repletion and molt into either male or female adults. While adult *Ixodes* males feed infrequently and may take a small bloodmeal they can mate with a female in the presence or absence of hosts. In contrast, females must consume a full bloodmeal that can weigh 100 times more than their own body weight, which is necessary in order to lay a few thousand mature eggs before they die (Kiszewski, Matuschka, and Spielman 2001). In the case of argasid ticks, they have multiple nymphal stages and lay eggs in batches. The life cycle of ixodid ticks is normally around 2 years, but some of the argasid tick species' life span can be extended up to 10 years. Some argasid and ixodid feed on specific hosts, whereas other highly adapted and widely spread species, such as *I scapularis* (or related ticks) can feed on any available vertebrates including small rodents, birds, lizards, and large mammals where humans are usually accidental hosts (shonenshine Daniel E. Sonenshine and Roe 2013).

Ixodid ticks have one host, two hosts, or three hosts life cycles (Daniel E. Sonenshine and Roe 2013). One host feeder lives and feed on the same host in all

three stages. In two host species, larvae and nymphs feed on the same host, and adult feed on different hosts. In three host species (such as, *I. scapularis*), they drop off the hosts after each bloodmeal and feed on different hosts in each stage ("CDC - DPDx - Ticks" 2019).

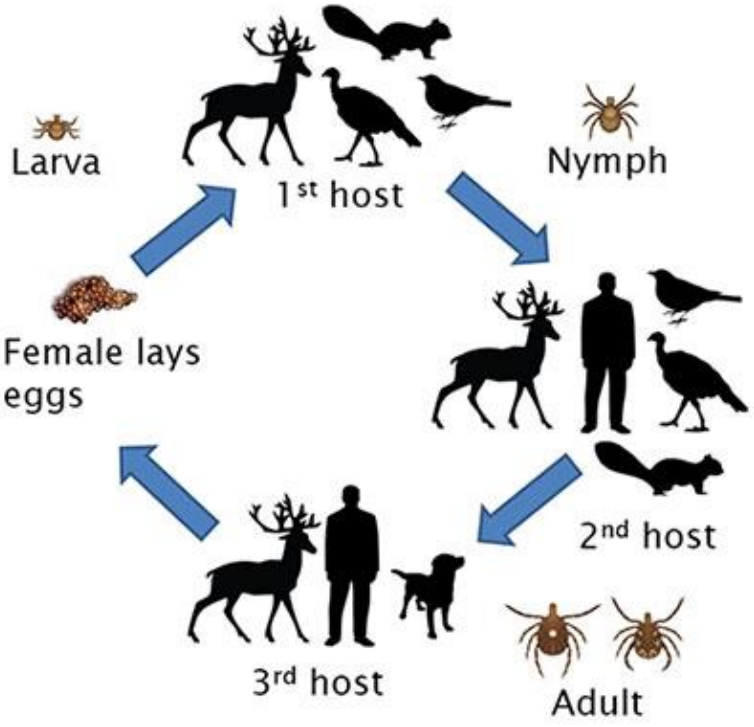


Figure 1.2. Three hosts tick life cycle. The figure is adapted from ("FS1281: Lone Star Ticks in New Jersey: Risk, Ecology, and Prevention (Rutgers NJAES)" n.d.). The diagram shows the lifecycle of the lone star tick (Ixodidae). They have four life stages: egg, larvae, nymph, and adult. They uptake bloodmeal at every stage except egg and need a new host at each stage of their life cycle.

1.1.3 Feeding Behavior

Ixodid ticks are unique in terms of their feeding behavior. They feed for several days on a single host and consume a large amount of blood, distinguishing them from

other blood-feeding arthropods such as argasid ticks, mites, and mosquitoes. Ixodid ticks start feeding with ‘questing behavior’, an activity characterized by the search for a host (Daniel E. Sonenshine and Roe 2013). Ticks crawl onto tall grass or shrubs, where they wait for animals to approach near them. They discover hosts by detecting various odors such as CO2, ammonia, and other factors using their sensory Haller's organs that are located on the first set of legs. Once they detect hosts nearby, they attach to their host by grasping fur, skin, or clothes (in the case of humans) by their legs. As ticks must remain attached for extended periods of time without being discovered, the next crucial step is to locate a suitable attachment site on the host skin. Once they locate the attachment site, they pierce the host skin and create a small hole using small knife-like appendages called chelicerae (Sonenshine and Roe, 2013). The ticks then insert their feeding apparatus, called hypostome, and secretes cement compounds which function as adhesives to securely attach to the hosts’ skin, as well as inject bioactive compounds such as vasodilators, immunosuppressants, anticoagulating agents via their saliva (Šimo et al. 2017c).

After attaching to a host, Ixodid ticks feed slowly for the initial few days, followed by rapid intake of blood at the final stages. For example, Ixodid larvae and nymphs feed for 3-5 days, whereas adult females feed for 7-8 days (Franta et al. 2010a). Slow phase of feeding continues from day 2 to day 6 for mated females, followed by a period of rapid engorgement lasting 24-48 hours (Franta et al. 2010a). During this last period of feeding before detachment, they consume two-thirds of the bloodmeal and expand their body mass by a factor of one hundred (Franta et al.

2010b). This massive increase in their body size is possible due to the concurrent synthesis of new cuticles along with the feeding period, which allows their exoskeleton to expand to accommodate the vast volume of the host's blood (Peter C. Flynn and Kaufman 2011; P. C. Flynn and Kaufman 2015). In addition, they concentrate their bloodmeal in the gut and eliminate excess water through their salivary glands (Šimo et al. 2017c). After consuming their bloodmeal, larvae, and nymphs undergo molting and ecdysis (shedding of the old cuticle) whereas adult females begin oviposition and egg laying.

1.2 Tick (I. Scapularis) Genome

I. scapularis genome is ~2.2 Gb which, is relatively large compared to many other sequenced insect genomes with ~69% of repetitive DNA (De et al., 2023). The genome contains 13 pairs of autosomes and two sex chromosomes (XX in female and XY in male) (Ullmann et al. 2005; Gulia-Nuss et al. 2016a). Their genome consists of 27 % highly repetitive and 39% moderately repetitive DNA (Ullmann et al. 2005). In early 2004, *I. scapularis* genome project was initiated and was the first sequencing project for a Chelicerata member (Hill and Wikel 2005a). With a shotgun and clone-based technique, the whole genome was sequenced with approximately 6-fold coverage of the genome followed by assembly and annotations (Pagel Van Zee et al. 2007; Gulia-Nuss et al. 2016a). In 2008, sequencing centers released full genome sequence data (IscaW1, 2008) in a bioinformatics resource center for invertebrate vectors of human pathogens named VectorBase and available through NCBI as well.

The assembly IscaW1 represents 57% of the genome (1.2Gbps) and predicts 20,486 protein coding genes and 4,439 non-coding RNA. ~60% of *I. scapularis* genes have arthropod orthologs, 50% of the remaining genes have homologs and 20% are unique (Gulia-Nuss et al. 2016b). Despite past seminal efforts, only 57% of the genome was covered and featured incomplete annotations. Recently, higher-quality genome sequencing has been conducted using a high molecular weight DNA that was extracted from a single female *I. scapularis* tick (De et al., 2023). The genome was obtained with greater than 90-fold coverage and improved annotations and re-annotations featuring thousands of new protein-coding genes and a BUSCO score of nearly 99%, resulting in a higher-quality reference genome (De et al., 2023).

1.3 Lyme Disease

Lyme disease, also referred to as Lyme borreliosis, is caused by the pathogenic spirochetal bacteria *B. burgdorferi* sensu lato complex and is the most common tick-borne illness in the Northern hemisphere (Bockenstedt and Wormser 2014). *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, and *B. afzelii*, together referred to as *B. burgdorferi* sensu lato, are the three major species of *Borrelia* responsible for most of the human Lyme disease cases in Europe and Asia, whereas in the United States, *B. burgdorferi* s.s. is the only causative agent of Lyme disease (Tilly, Rosa, and Stewart 2008). Ticks of the genus *Ixodes* transfer *B. burgdorferi* between hosts, including small animals, reptiles, and birds, as well as humans, who are incidental hosts. In the United States, *I. scapularis* and *I. pacificus* transmit Lyme disease pathogens, while *I.*

ricinus and *I. persulcatus* spread in Europe and Asia, respectively (Tilly, Rosa, and Stewart 2008). The Centers for Disease Control and Prevention (CDC) estimates the yearly incidence of Lyme disease in the United States is at least 10 times higher than the total reported cases (Kuehn 2013). Lyme disease is presently difficult to diagnose, especially at preliminary stages when antibiotics are most effective, and vaccination to prevent the disease in humans is not available. An infected tick bite might result in local inflammatory symptoms that cause erythema migrans (EM), a skin rash that is one of the first visible symptoms of Lyme disease, although 30% of the patients fail to display the EM rash. If left untreated, individuals might develop severe and chronic inflammatory complications, such as Lyme arthritis and carditis (Radolf et al. 2021).

1.4 Tick-Host Interactions

Tick bites produce robust immunological responses in the host, such as hemostasis, inflammation, vasoconstriction, and wound healing, which may interrupt tick feeding and result in tick rejection by hosts (Šimo et al. 2017c). Consequently, ticks have developed several strategies that allow them to remain attached to their hosts for several days without being detected. Tick saliva contains pharmacological and bioactive compounds with cytolytic, vasodilator, anticoagulant, anti-inflammatory, and immunosuppressive properties, supporting ticks to counteract host defenses and extend the duration of blood feeding (Šimo et al. 2017c). Ticks may frequently infest natural permissive hosts, such as wild mice and even laboratory mice, without inducing symptoms of resistance (Anderson et al. 2017c). Intriguingly,

non-natural hosts such as guinea pigs and rabbits are tolerant of an initial tick infection but develop acquired tick resistance (ATR) if they are repeatedly infested with ticks (Narasimhan et al., n.d.; Anderson et al. 2017a). ATR may cause severe local inflammation and erythema, which impairs tick feeding and ultimately blocks TBD transmissions (Karasuyama, Miyake, and Yoshikawa 2020). ATR is associated with reduced tick weights, prolonged feeding duration, impaired molting, tick detachment, and tick death. ATR may also exist in humans since repeated tick exposures are linked with tick-associated itching and a decreased prevalence of Lyme disease (Burke et al. 2005).

Tick bite causes an inflammatory response in the host skin leading subsequently to the infiltration of various immune cell types (e.g. lymphocytes, neutrophils, basophils, eosinophils) at the tick-host interaction sites(Krause et al. 2009a; Kitsou, Fikrig, and Pal 2021). Infiltration of neutrophils, dendritic cells, and mononuclear cells has also been reported at tick bite sites in humans as well as the increase in pro-inflammatory cytokines (Glatz et al. 2017). However, these primary immune responses decrease substantially at human tick bite sites beyond 24 h of tick attachment and are thought to be due to the immunosuppressive properties of tick saliva (Glatz et al. 2017). Ticks secrete similar but unique saliva proteins every 24 hours, which is most likely part of their immune evasion tactics (Kim et al., 2016).

1.5 Tick Anatomy

Externally, the body of a tick consists of three regions: the mouthpart or capitulum, the idiosoma or body, and the legs (Daniel E. Sonenshine and Roe 2013). In contrast to soft ticks, hard ticks have sclerotized scutum on their dorsal surfaces. During feeding, the bodies of ticks, particularly larvae, nymphs, and adult female ticks, enlarge substantially. Most of the male tick's body is coated in scutum, preventing them from stretching (Daniel E. Sonenshine and Roe 2013). The mouthparts or capitulum of a tick consist of two palps, two chelicerae, and one hypostome that resembles a barbed needle. Ticks utilize their mouthparts to puncture the skin of their hosts and consume their bloodmeal. Palps do not play a direct function in feeding, although they may play a role in gustation or chemosensation (Daniel E. Sonenshine and Roe 2013). Chelicerae are scissor-like appendages that cut through the skin of the host, and the hypostome bears backward-pointing barbs, making it difficult to remove ticks without injuring the skin (“How Ticks Work” 2007). Six legs are seen on larvae, whereas nymphs and adults have eight legs. The front pair of legs contain Haller's organs, which serve as the ticks' sense organs.

The tick's internal circulatory system is open and filled with hemolymph, which bathes the tick's organs. Their internal organs consist of synganglion, salivary glands, and the digestive system (foregut, midgut, hindgut) amongst others. The central nervous system of synganglion consists of a combined brain and ventral nerve chord (Daniel E. Sonenshine and Roe 2013). Tracheae are dispersed throughout the tick's body and function as connective tissues, a mechanism for gas exchange, and organ

supports, as well as aiding tick body enlargement during blood feeding (Daniel E. Sonenshine and Roe 2013; Starck et al. 2018a). The tick's trachea is exposed to the environment via a pair of spiracles located on its exoskeleton. In Ixodidae, the spiracle is located posterior to coxa IV, but in Argasidae, it is situated between coxae III and IV (Basu and Charles 2017).

1.5.1 Salivary Glands

Tick salivary glands have vital functions both when attached and detached from hosts. These include the release of saliva containing immunomodulatory substances, cement for strong attachment to hosts, removal of surplus fluid and bloodmeal concentration, and protection from dehydration by collecting atmospheric moisture (Šimo et al. 2017c). Each side of a tick has a pair of salivary glands composed of acini (clusters of cells). Female Ixodid tick salivary glands contain three acinus types (I, II, III), whereas male tick salivary glands contain four acini kinds (I-IV) (Šimo et al. 2017c). During feeding, the size of salivary glands increases significantly (up to 25-fold) due to the expansion of acini II and III, while the number of cells remains constant (Sauer et al. 1995). Type III acini are most abundant, and it is likely that the majority of feeding fluids are carried by this acinus type and the lumen greatly expands due to the fluid intake from hemolymph (Sauer et al. 1995). Type II and III acini contain membrane-bound granules, which may be the origin of bioactive substances and cement compounds (Sauer, Essenberg, and Bowman 2000). Additionally, type III epithelial cells aid in the removal of excess water and ions from

salivary glands back to the host (Šimo et al. 2017c). Type I acini, on the other hand, actively absorb humidity from the environment by releasing a highly hygroscopic fluid rich in Na+, K+, and Cl onto the surface of the mouthparts that are subsequently ingested along with the absorbed moisture (Šimo et al. 2017c) (Daniel E. Sonenshine and Roe 2013). Only male ticks possess type IV acini, which may play a role in reproduction via spermatophore transfer to female ticks during mating (Sauer, Essenberg, and Bowman 2000).

1.5.2 Gut and Blood Digestion

Tick digestive tract is comprised of the foregut, midgut, and hindgut, where the midgut forms the major part and several infoldings to fit inside the ticks' whole body (Caperucci, Mathias, and Bechara 2009). The unfed tick gut contains a small lumen and a layer of epithelium cells formed by resting digestive cells and stem cells (Franta et al. 2010b). In feeding ticks, the gut lumen starts to expand, and digestive epithelium cells enlarge in volume (Franta et al. 2010b). Unlike other blood-feeding arthropods, tick blood digestion occurs intracellularly (Franta et al. 2010b). Erythrocytes are broken in the lumen and hemoglobin gets internalized by digestive epithelium cells and gets further digested by multiple cysteine and aspartic proteases (Sojka et al. 2013a). Heme gets liberated and detoxified in an organelle called hemosomes. Peptide fragments are further digested into oligopeptides, dipeptides, and amino acids and are released into the cytoplasm (Sojka et al. 2013a). Hemoglobinolytic activity remains relatively low until the second day of tick feeding

(Sojka et al. 2013a). Enzymatic activity increases from day 4-6 of tick feeding and most of the enzymes are abundant in the fully fed ticks suggesting maximum hemoglobin lysis activity at that phase (Sojka et al. 2013a). On the fourth day of feeding in female ticks, digestive cells increase in size further and balloon-shaped apical side of the cells reaches the lumen and the thin lower part stays attached to the basal membrane (Starck et al. 2018a). These cells are filled with numerous vesicles which contain enzymes to process hemoglobin. In fully engorged ticks, digestive epithelium cells get stretched significantly, and the hemoglobinolytic activity peaks at this phase (Sojka et al. 2013a; Franta et al. 2010c).

1.6 Tick Sensory Organs and Their Roles

In insects, exterior appendages such as antennae and maxillary palps serve as sensory organs. These organs are coated with sensilla, which resemble olfactory hairs. Each sensillum includes olfactory sensory neurons (OSNs/ORNs) that react to stimuli through various odorant receptors (Nakagawa and Vosshall, 2009). Ticks, unlike insects, lack external antennae. Instead, ticks have a structure called Haller's organ in the fore tarsus of their fore legs (Sonenshine and Roe, 2013). Haller's organ has two sensory areas, the anterior pit and the posterior capsule (Foelix and Axtell, n.d.; Zellforsch, n.d.). The anterior pit has 6-7 setiform sensilla that are exposed to the environment, whereas the capsule has setiform and pleomorphic sensilla that are exposed via a thin slit (Zellforsch, n.d.). In *Drosophila*, each sensillum contains 1-4 olfactory receptor neurons (ORNs), that are bathed in the

sensilla lymph and each ORN expresses between one and three odorant receptors (ORs) in addition to the odorant receptor coreceptor (Orco) Orco83b (Sato and Touhara* 2008). Orco83b is an obligatory component, which together with ORs functions in response to stimuli. Odorants or pheromones enter the porous sensillum and interact with ORNs either directly or by odor ligand-odorant binding protein (OBP) complex (Leal 2013b). This interaction activates odorant receptors (ORs) and causes spontaneous action potential followed by degradation of odor molecules by odorant degrading enzymes (ODE) (Leal 2013b).

Compared to the *Drosophila* olfactory system, tick sensory systems are poorly understood. Genomic and transcriptomic analysis of the tick genome and tick Haller's organ respectively suggest that ticks lack OBPs (Carr et al. 2017a; Gulia-Nuss et al. 2016a; De et al. 2023). Ticks also lack OR genes, although they do possess gustatory and ionotropic glutamate receptors, which are not exclusive to Haller's organ (Carr et al. 2017a; De et al. 2023). However, foreleg-specific GPCRs, secondary messenger proteins, and ODEs have been found in ticks (Carr et al. 2017a).

Animals, especially many vertebrates release different volatile compounds through their skin, sweat, urine, and breath, which act as attractants for bloodsucking arthropods. Some of these compounds are lactone, alcohols, ketones, aldehydes, and phenols. Ixodes ticks have specific types of sensillum which are called single-walled-wall pore (SW-WP) sensilla and are exclusively olfactory (Daniel E. Sonenshine, Taylor, and Carson 1986). *I. scapularis* SW-WP sensilla of capsule detects phenols, which are found in tick extracts, deer urine, and glands (Josek et al. 2021). Synthetic

compounds mixture of phenol, aldehyde, and carboxylic acid cause excitation in three ORNs. However, alcohol inhibits the activation for a short period of time (Josek et al. 2021). On the other hand, sensory cells of *I. ricinus* were able to respond in presence of lactone components of mammalian hair odor and steer wool odor (Leonovich 2004). Neurons sensitive to ammonia were detected in *R. sanguineus*, which might play role in ticks' host seeking behavior (Josek et al. 2021) (Haggart and Davis 1980). Sex pheromone 2,6-diclorophenol (2,6-DCP) was first identified in *A. americanum* and generally helps ticks to find their mates (D. E. Sonenshine 2004). CO2 is an attractive stimulus for most of the blood-feeding arthropods. As one example, female mosquitoes detect CO2 with the help of ORNs present in the maxillary palps (Tauxe et al. 2013). These neurons comprise of gustatory receptors, which are normally involved in taste. Ticks are also attracted to CO2 and blowing air through the mouth, ticks move their front legs upward and downward as insects do with their antennas. Also, CO2 traps are used to collect ticks from fields (Kensinger and Allan 2011). Although transcriptomic analysis of American dog ticks' Haller's organ did not identify any gustatory receptors specific to forelegs, it is observed that ticks with Haller's organs came to close proximity of CO2 significantly greater than the ticks without Haller's organs (Morales 2019; Carr et al. 2017b).

Tick Haller's organ can also detect host body heat as Carr et.al. reported that the capsule detects radiant heat emitted from the warm surface at 37^0C (Carr and Salgado 2019). Thermotaxis, which is a directed movement in response to a heat gradient, in lone star tick, *A. americanum* towards the source of heat was impaired

when capsules were blocked (Carr and Salgado 2019). Thermotaxis was also absent when the surface of the heat source was covered with aluminum foil, which blocks infrared radiation (IR) (Carr and Salgado 2019). IR light is detected via transient receptor potential cation channels (TRP) that are sensitive to changes in temperature in insects and snakes (Mitchell et al. 2017). Ticks also have a similar capacity to detect IR through their Haller's organs as experimental removal of the organ significantly abrogates tick's sensitivity to IR but not their ability to detect visible light (Mitchell et al. 2017). Additionally, Haller's organ-specific transcriptomics analysis shows the presence of several TRPs indicating their potential roles in IR sensing (Mitchell et al. 2017).

Ticks also have eye-like appendages named ocelli, found on the tick body just above the second pair of legs. By blocking ocelli, ticks were unable to move toward visible light but didn't affect their ability to sense infrared light (Mitchell et al. 2017). It is unknown whether tick Haller's organ plays role in hygroreception, but it is observed that ticks are able to detect and avoid wet surfaces through their front legs (Kröber and Guerin, n.d.). Ticks are very sensitive to dehydration. To compensate for any water deficits, they absorb water through hygroscopic salivary gland secretion (Šimo et al. 2017a).

N, N-diethyl-meta-toluamide (DEET) is a very commonly used repellent that is effective on a wide variety of arthropods, including ticks. However, the actual mode of action of DEET is not clear. Studies show that DEET inhibits the responses of odorant receptors that require the co-receptor Orco83b in *Drosophila*, thereby

masking the detection of host odors by ORs (Ditzen, Pellegrino, and Vosshall 2008). However, Afify et.al. suggested that DEET does not interact with odorant receptors in the mosquito, *Anopheles coluzzii*, but rather masks volatile odor molecules and inhibits odor perception by reducing the amount of odorants available to ORNs (Afify et al. 2019). DEET is also effective in repelling ticks, and Haller's organs are thought to play a role in detecting DEET in the environment (Carr et al. 2017b). For example, the removal of the Haller's organs (via removal of the first pair of legs) in *D. variabilis* ticks, mitigated the repellent activity of DEET, however, the detailed mechanism remains unknown (Carr et al. 2017b). RNA sequencing shows that the expression of the detoxifying enzyme, Cytochrome p450 (CYTP450) and neurotransmitter, acetylcholinesterase (AChE) are strongly reduced in presence of DEET suggesting that the DEET is an inhibitor for CYTP450s and AChE in ticks (Koloski et al. 2019a). DEET has been also reported as inhibitor for CYTP450s in *Aedes aegypti* (Ramirez et al. 2012). CYTP450 oxidizes lipophilic molecules and generates reactive oxygen species (ROS), which is further degraded by glutathione-S-transferase (GST) and superoxide dismutase (SOD), thus acting as odorant degrading enzymes (ODEs) (Leal 2013a; Koloski et al. 2019b). In fact, the *I. scapularis* genome contains multiple gene products belonging to the GST family (De et al. 2023). Analysis of transcriptomic data derived from tick leg shows the presence of odorant ODEs in the fore tarsi which includes CYTP450, glutathione-S-transferase (GST), and superoxide dismutase (SOD) (Carr et al. 2017b). ODEs play critical roles by rapidly removing odor molecules from receptors in order to retain olfactory

sensitivity for new odor molecules (Koloski et al. 2019b; Leal 2013a). Thus, DEET may suppress odor responses in ticks by inhibiting ODEs such as CYTP450s which, leads to desensitization of neurons thereby reducing sensitivity to new odor molecules (Koloski et al. 2019b).

1.7 Insect Odorant Binding Proteins

Arthropod sensory system plays significant roles in host-seeking, feeding, mating, egg-laying, and detection of predators among other important behaviors. Solubilization of external odor molecules, their binding to the membrane receptor, and transport of the signals to the sensory neurons are the first steps in olfactory recognition (Leal 2013a). Odorant Binding Proteins (OBPs) and Chemosensory proteins (CSPs) are two primary families of small soluble proteins in insects that transport ligands, such as pheromones, host odors, and other organic substances, to the receptors in the antennal sensillum (Paolo Pelosi et al. 2014). The first OBP was discovered in a moth (*Antheraea polyphemus*) antenna in the 1980s followed by the identification of OBPs in vertebrates and nematodes (Paolo Pelosi et al. 2014; Vogt and Riddiford 1981). This moth protein is found in relative abundance in the antenna of male moths, and since it binds to pheromones, it is known as Pheromone-Binding Protein (PBPs). Genome sequencing efforts revealed 52 OBPs in *Drosophila*, 69 in the malaria vector *Anopheles gambiae,* and 109 in the cockroach species, *Blatella germanica* (Sun, Xiao, and Carlson, n.d.). OBPs are diverse and share ~15-20% of amino acid sequence identities among the species (P. Pelosi et al. 2006). However, a

single class of soluble olfactory proteins is identified in vertebrates. Vertebrate OBPs differ significantly from insect OBPs as they belong to the lipocalin superfamily consisting of β-barrel and α-helix structures (P. Pelosi et al. 2006). Despite having different structures, all three of the above-mentioned olfactory classes have hydrophobic ligand binding pockets (Paolo Pelosi et al. 2014).

1.7.1 OBP Structure

OBPs are small, soluble proteins that usually contain six cysteines (Sun, Xiao, and Carlson, n.d.). The relative locations of these cysteines are constant across insect species, and in general, they create three disulfide bridges at positions C1-C3, C2-C5, and C4-C6 that make OBPs more constrained and stable (P. Pelosi et al. 2006). Some members of the OBP family designated C-plus OBPs and C-minus OBPs, have more or less than six cysteines (P. Pelosi et al. 2006) (Paolo Pelosi et al. 2018). Insect OBPs have six alpha-helical domains and a hydrophobic pocket that forms a compound with hydrophobic ligands (Rihani, Ferveur, and Briand 2021). The PBP from *Bombyx mori* is the first described OBP structure. *B. mori* PBP (BmorPBP) is found in high concentration in the male antenna sensillum, where it binds to the female pheromone bombykol and carries it to the receptors of the sensillum (Sandler et al., 2000; (Rihani, Ferveur, and Briand 2021). The presence of a longer C-terminus in BmorPBP induces conformational changes at acidic pH, resulting in the release of ligands from the ligand-binding pocket due to the inclusion of a seventh alpha helix (Horst et al., 2001). In LUSH, an OBP from *Drosophila*, the C terminus of the protein

was refolded into its core and is a component of the alcohol-binding pockets; however, the conformational shift in LUSH is not pH-dependent. It exhibits the same crystal structure at pH 4.6 and 6.5 and continues to bind to alcohols at pH 4.6 (Kruse et al. 2003a). Nonetheless, this phenomenon is not found in all OBPs. The cockroach *Leucophaea maderae* OBP has a shortened C-terminus, which inhibits the formation of the seventh alpha helix (Lartigue et al., 2003). Consequently, their binding pocket remains open in the absence of the additional helix (Lartigue et al., 2003). *Apis melifera* OBP (ASP1) has a shorter C-terminus than BmorPBP but a longer C-terminus than *L. maderensis* PBP. Their medium-length C-termini do not create additional alpha helices but instead fold back into the binding pocket (Lartigue et al., 2004a). To date more than 20 insect OBP structures have been identified, whereas only 3 CSP structures have been analyzed (Paolo Pelosi et al. 2018; Rihani, Ferveur, and Briand 2021). CSPs are smaller than OBPs and are more conserved (40-50%) across various species, as compared to OBPs (Paolo Pelosi et al. 2018). CSPs contain 4-cysteines, which produce two disulfide bridges between adjacent residues, as opposed to three interlocking disulfide bridges seen in insect OBPs (Paolo Pelosi et al. 2018; Tegoni, Campanacci, and Cambillau 2004). As a result, they are less stable than OBPs, but their structure is more flexible, enabling them to interact with a wider range of ligands of varying shapes and sizes. The first crystal structure of a CSP from *Mamestra brassicae* (MbraCSP1) may expand to accommodate three molecules of 12-bromo-dodecanol in its binding area (Paolo Pelosi et al. 2018; Campanacci et al.

2003). OBPs and CSPs are resistant to organic solvents and extremely stable at high temperatures (Pelosi et al., 2018b).

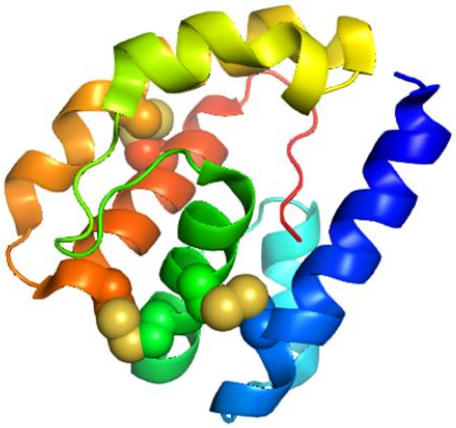


Figure 1.3. Structure of *Drosophila* odorant binding protein, LUSH at pH 6.5 (Kruse et al. 2003b). The crystal structure of LUSH is retrieved from PDB and visualized using PyMOL. This protein has six alpha helices (blue for helix 1 and dark orange for helix 6) and three disulfide bridges, which are represented by spheres.

1.7.2 Binding cavities and ligands

Odorant binding proteins are abundant in insect lymph and vertebrate nasal tissue extracts (Paolo Pelosi and Maida 1990). In general, odorants are hydrophobic molecules with limited solubility in aqueous environments such as in the sensilla lymph. Therefore, OBPs are required to bind and transport odorants from the sensillum pore wall to the receptors on dendritic membranes (P. Pelosi et al. 2006). In BmorPBP, the residues flanking the binding pocket for bombykol are mostly hydrophobic, with the exception of the Ser56 residue, which forms hydrogen bonds with the hydroxyl group of bombykol (Sandler et al. 2000). Due to the protonation at

acidic pH, the His residues His-69, His-70, and His-95 may be involved in the pH-dependent conformation change of BmorPBP (Sandler et al. 2000). This results in the shift of the loop from residues 60 to 69 and the destabilization of the BmorPBP and bombykol complex, triggering the release of the ligand by the formation of a seventh alpha helix that enters the binding pocket (Horst et al. 2001; Tegoni, Campanacci, and Cambillau 2004; Sandler et al. 2000). However, the molecular surface of BmorPBP lacks hydrophobic regions and is comprised of 14 glutamates, 9 aspartates, 14 lysines, and 1 arginine which may explain why BmorPBP is exceptionally soluble in aqueous conditions at low pH (Wojtasek and Leal 1999; Horst et al. 2001). Similar to BmorPBP, the wall of the binding pocket of AgamOBP20, an OBP from the malaria mosquito *Anopheles gambiae*, is comprised mainly of hydrophobic residues, such as 1 Glycine, 4 Isoleucine, 5 Methionine, 4 Leucine, 2 Phenylalanine, and 1 Proline (Ziemba et al. 2013). Two polar residues, Thr55 in helix 3 and Ser66 in helix 4, are positioned at one end of the cavity to potentially make multiple hydrogen bonds with other polar ligands (Ziemba et al. 2013). However, pH changes do not induce conformational changes or ligand binding in AgamOBP20, likely due to the absence of histidine residues (Ziemba et al., 2013).

In contrast to BmorPBP and AgamOBP, the binding cavity of LmaPBP has a substantial number of polar or charged residues, allowing for the binding of hydrophilic H3B2 (3-hydroxy-butan-2-one), one of the principal components of the *L. maderae* pheromone mix (Lartigue et al., 2003; Lartigue et al., 2004). pH-dependent mechanism for pheromone binding and release has been seen in various lepidopteran

PBPs. In contrast to Lepidoptera, the majority of dipteran OBPs lack a C-terminal segment that is not sufficiently long to form a new helix and compete with the ligand for the binding cavity (Rihani, Ferveur, and Briand 2021). The C-terminus regions of AaegOBP1 cover the binding pockets like a "lid" (Leite et al. 2009). AaegOBP1 and another mosquito OBP, AgamOBP1, are likely to undergo a pH-dependent conformational change that disrupts hydrophobic and polar contacts between the C-terminus and surrounding helices. This causes the loop to migrate away from the binding pocket, leaving it exposed to solvents (Wogulis et al. 2006).

1.7.3 OBPs in Chelicerata

In general, biochemical characterization of Chelicerata proteins is still in its early stages. While ionotropic receptors (IRs) and gustatory receptors (GRs) are abundant in the genomes of non-insect arthropods, odorant receptors (ORs) are absent. In fact, Pelosi et al. concluded that OBPs are not present in any non-insect arthropods, but present in all species of insects (Paolo Pelosi et al. 2014). However, members of the CSP family have been found in Crustacea, Myriapoda, and Euchelicerata (Paolo Pelosi et al. 2014)(Vizueta, Rozas, and Sánchez-Gracia 2018). Only one CSP sequence was found in *I. scapularis* (Paolo Pelosi et al. 2014). Polypeptides belonging to the OBP class of putative semiochemicals in Chelicerata are known as OBP-like proteins because they share 15% or less of their sequence with insect OBPs (Renthal et al. 2017; Vizueta, Rozas, and Sánchez-Gracia 2018). OBPs from mites, members of the subphylum Chelicerata, have been characterized,

and the crystal structure of OBP from *Varroa destructor* has been determined until very recently (J. Zhu et al. 2021; Amigues et al. 2021a; Wu et al. n.d.). Like insect OBPs, Chelicerata OBPs include six conserved cysteines, but their positions cause them to create different patterns of disulfide bridges (J. Zhu et al. 2021; Amigues et al. 2021a). Identification of two OBP-like protein sequences was first reported in ticks by Renthal et al. in 2017. Two OBP-like expressed sequence tag (EST) expression was 2- to 3-fold greater in the forelegs of lone star tick, *A. americanum* (Renthal et al. 2017) compared to the other legs and palps. However, genomic and transcriptome analysis of the *I. scapularis* tick genome and the Haller's organ of *D. variabilis* indicate that ticks lack OBPs (Carr et al. 2017a; Gulia-Nuss et al. 2016a; De et al. 2023). High OBP divergence and limited research may explain the small number of detected OBPs in ticks and non-insect arthropods. In fact, the most recent genome study on *I. scapularis* has uncovered 16 novel ionotropic glutamate receptors (De et al., 2023). Although not exclusive to Haller's organ, ticks possess gustatory receptors (Carr et al., 2017). However, foreleg specific GPCRs, secondary messenger proteins, and ODEs have been found (Carr et al., 2017).

1.7.4 OBP's other functions besides chemosensation

The fundamental role of OBPs in olfaction is supported by research demonstrating that the majority of these proteins are expressed in antennae (Pelosi et al., 2006). In addition, OBPs are involved in hygroreception and taste perception. OBP59a is identified in the humidity-detecting sensilla of the *Drosophila* antenna,

and Obp59a-deficient mutants respond abnormally to humidity (Sun et al., 2018). OBP49a, OBP57d and OBP57e are expressed in the *Drosophila* taste organs, labellum of the fly head and legs, respectively. OBP49a discourages flies from being attracted to sugar by decreasing the action-potentials of sugar-activated neurons with bitter compounds, whereas OBP57d and OBP57e influence taste perceptions and their host (plant) preferences (Sun, Xiao, and Carlson, n.d.; Matsuo et al. 2007; Jeong et al. 2013). However, the increasing numbers of OBPs in organs other than chemosensory sensilla suggest that they have other functions (Rihani, Ferveur, and Briand 2021; Paolo Pelosi et al. 2018). PregOBP56a, which is expressed on the oral disk of the blowfly *Phormia regina*, may participate in the solubilization and transfer of fatty acids to the midgut during feeding (Ishida, Ishibashi, and Leal 2013). The gut transcriptomes of *Culex nigripalpus* and *Rhodnius prolixus* also included OBPs (Ribeiro et al. 2014; Smartt and Erickson 2008). The elevated expression of one of the OBPs in female *C. nigripalpus* after a bloodmeal suggests a potential role in blood feeding (Smartt and Erickson, 2008). Symbiotic bacteria in the tsetse fly larval gut and *Drosophila* boosts the expression of Obp6 and OBP28a, resulting in an increase in the fly's immune system (Benoit et al. 2017). OBPs are also discovered in mosquito saliva. The D7 protein in *Aedes aegypti* saliva is a member of the OBP superfamily and binds biogenic amines, probably serving as an anti-inflammatory protein (Calvo et al. 2009). Additional OBPs are found in mosquito ovaries. In the ovaries of the mosquito *A. aegypti*, OBP45 expression is greatest 48 hours after blood feeding, indicating a function in oocyte maturation (Paolo Pelosi et al. 2014; Costa-

da-Silva et al. 2013). In addition, other OBPs are expressed in *B. dorsalis* and *D. melanogaster* fat bodies, although their roles are unclear (Rihani et al., 2021).

1.8 Tick Cuticle and Molting

1.8.1 Cuticle Structure and Composition

A common characteristic of arthropods is the presence of an integument or exoskeleton. Exoskeleton provides stability, mechanical protection, desiccation prevention, and attachment sites for muscles, hence facilitating their mobility. The cuticle, which is produced by the epidermis, forms the exterior skeleton or integument of the tick body (Hackman 1982). Cuticle consists of two major layers: the outer epicuticle and the inner procuticle. Exocuticle refers to the outside parts of the procuticle, which are often sclerotized (Hackman 1982). The degree of sclerotization or hardening of the cuticle varies between hard and soft ticks (Hackman, 1982). The outer epicuticle acts as a permeability barrier and is composed of long-chain hydrocarbons, fatty acid ester, and alcohols but does not normally contain chitin (Fabritius et al., 2009). Chitin microfibrils and protein however are the major components of the inner procuticle (Fabritius et al. 2009; Hackman 1982). Chitin is a linear homopolymer comprising 1–4 linked N-acetylglucosamines and primarily exists in three distinct forms based on the arrangement of the chitin chains; alpha chitin (antiparallel chains), beta-chitin (parallel chains), and gamma-chitin (presence of both parallel and anti-parallel chains) (K. Y. Zhu et al. 2016; Fabritius et al. 2009). After cellulose, chitin is the second most abundant polysaccharide in nature and the

structural component of the exoskeleton of arthropods such as chelicerates, insects, crustaceans, and myriapods (Fabritius et al., 2009) and alpha chitin is the most predominant of three crystalline forms. In a widely accepted model for chitin organization in arthropod cuticles, 18 to 25 chitin molecules are stacked antiparallel in nanofibrils (Moussian 2010; Fabritius et al. 2009). Clusters of nanofibrils are integrated into the protein matrix. In each layer, the chitin protein fibers are horizontally oriented in the same direction (Fabritius et al. 2009). These layers rotate helically around the axis of the cuticle to generate the twisted plywood-like structure (Fabritius et al. 2009).

A second sort of structural arrangement occurs when layers are stacked along the vertical axis of the epidermal surface in a unidirectional fashion (Fabritius et al. 2009). This helicoidal pattern of chitin arrangements was initially observed for crustacean cuticle, and later research has shown that insect cuticle also follows this pattern (Moussian 2010; 2013). Stacking heights vary in exocuticle and endocuticle, as well as among other arthropods, most likely related to the number of layers or the rotating angle of the fibers (Fabritius et al. 2009).

Cuticle is secreted by the single layer of cells present beneath the cuticle and the pore canals are perpendicular channels that extend from the epidermal cell layer surface to the epicuticle (Riddiford 2009); (Locke 1961). During molting, epidermal cells actively synthesize and deposit endocuticles at the apical surface of the epidermis, while pore canals transport substances such as wax to the surface of the

cuticle and may allow contact insecticides to enter the insect body (Riddiford 2009; Locke 1961; Dennell 1943).

Within the cuticles are chitin bundles embedded in a matrix of structural proteins that contain chitin-binding domains. Cuticle-associated proteins are classified into several categories based on the presence of certain sequence motifs. Many cuticular proteins (CPRs) have the Rebers and Riddiford (R&R) consensus motif (Muthukrishnan et al., 2020a). CPRs may be classed as RR1, RR2, or RR3 depending on the extended consensus regions. RR1 proteins are often found in soft cuticles, while RR2 proteins are typically found in hard cuticles (Muthukrishnan et al., 2019). Resilin is one of the R&R motif-containing proteins that gives excellent flexibility during cuticle expansion as well as, for flying and jumping (Moussian, 2013). Although the R&R consensus is generally conserved throughout arthropods, the flanking regions of cuticular proteins do differ. Cuticular proteins analogous to peritrophins (CPAPs) are an additional type of cuticular proteins that are mostly cysteine-rich and include one to three chitin binding domains (CHBD2) (Muthukrishnan et al. 2019). Factors such as the ratio of chitin to protein, the diversity of cuticular proteins, and the degree of protein crosslinking are key differentiators between soft and hard cuticles (Muthukrishnan et al. 2020).

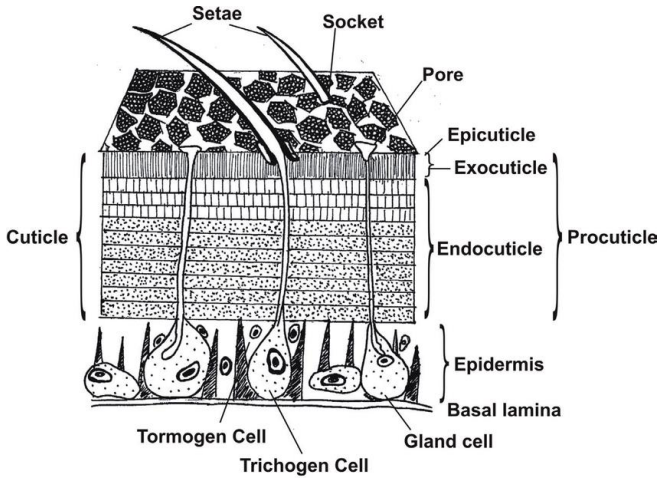


Fig. 1.3 Structure of arthropod exoskeleton. The figure is adapted from (Kaleka, Kaur, and Bali 2019). Exoskeleton consists of the cuticle, epidermis, and basal lamina. The outermost layer is the cuticle and consists of the upper epicuticle and inner procuticle layers. Epidermis is a single layer of cells that secrete cuticles. Basal lamina or basal membrane is present beneath the epidermis and consists of protein, collagen, and glycoproteins. Trichogen cells generate hair-like projections named setae. Pore canals traverse through the cuticle and transport cuticular compounds and enzymes from the epidermis to the upper surface of the procuticle (Bhubanananda Adhikari 17:40:09 UTC).

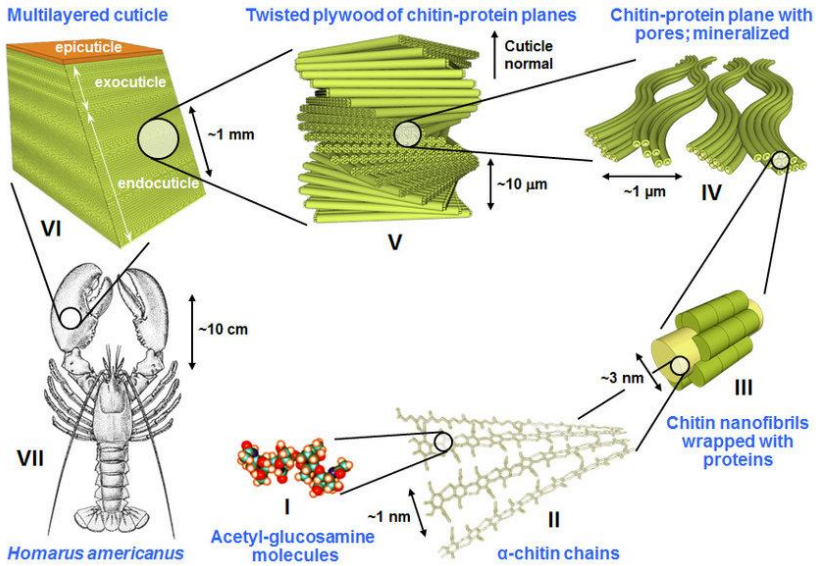


Fig 1.4. Structural organization of the lobster cuticle. The figure is adapted from (Nikolov et al. 2016). N-acetyl-glucosamine molecules (I) are incorporated into the alpha-chitin chains (II) and 18 to 25 chitin molecules are stacked antiparallel in nanofibrils and the cluster of

nanofibrils are wrapped in protein matrix (III). In each layer, the chitin protein fibers are horizontally oriented in the same direction (IV). These layers rotate helically around the axis of the cuticle to generate the twisted plywood-like structure (V). (VI) shows three layered cuticle structures.

1.8.2 Cuticle plasticization

There are periods in the arthropod life cycle when the cuticles grow at a much faster rate than average; this phenomenon is known as plasticization. In 1962, Cottrell (1962a) and Bennet-Clark (1962) independently reported cuticle plasticization in blow flies, *Calliphora erythrocephala* and kissing bugs, *Rhodnius prolixus* (Bennet-Clark 1962; Cottrell 1962). In most insects, plasticization happens after ecdysis. For example, newly emerging blow flies increase their body size by swallowing air while the abdominal cuticle of the kissing bug expands within an hour after ecdysis (Cottrell 1962; Reynolds 1974). Similarly, several species undergo plasticization during their bloodmeal. In the instance of the hematophagous insect *R. prolixus*, the abdomen swells to accommodate a massive quantity of bloodmeal (Bennet-Clark, 1962). Although the abdominal cuticle undergoes fast alteration, it does not damage the structure of the cuticle (Ianowski et al. 1998; Hackman and Goldberg 1987). Bennet-Clark hypothesized that certain blood factors promote plasticization (Bennet-Clark, 1962). Later, it was shown that the cuticle may expand even without a bloodmeal and that the nervous system controls cuticle plasticization (Maddrell, n.d.; Ianowski et al. 1998; Reynolds 1980; Núñez 1963).

5-hydroxytryptamine (5-HT) neurotransmitter produced from abdominal nerve terminals induces plasticity in *R. prolixus* demonstrated that damage to the nerves prevented the abdomen of fed *Rhodinus* from expanding (Maddrell, n.d.; Núñez 1963). Different stimuli, including a ringer solution meal and contact with warm surfaces, may induce plasticity in *Rhodinus,* indicating that the abdominal wall begins to prepare for expansion prior to consuming a bloodmeal (Ianowski et al., 1998). Reynold proposed that a change in pH increases hydration and ultimately reduces noncovalent intermolecular interactions (Reynolds, n.d.). Enhanced cuticle flexibility in an acidic pH range is a result of increased proton transport in the subcuticular region and subsequent hydration. In addition, the pH of the plasticized cuticle is acidic, which provides more support for the notion (Reynolds, 1975).

As hematophagous arthropods, tick alloscutum undergoes plasticization during blood consumption. Kaufman et al. showed that a shift in pH accompanies an increase in hydration during feeding in *Amblyomma hebraeum* alloscutum, possibly resulting in the breaking of hydrogen bonds in the chitin-protein matrix (Kaufman et al., 2010). Okura et al. first studied tick plasticization and observed that following copulation, female Ixodid ticks *H. longicornis* expand their cuticle (Okura et al. 1996). They also reported that the synganglion extracts from non-mated females and hemolymph from mated females may induce plasticization when injected into partially fed non-mated and mated females, respectively (Okura et al., 1997b). This indicates the presence of active compounds in synganglion extracts and hemolymphs that may induce plasticization.

Octopamine, noradrenaline, and dopamine could also induce cuticular plasticization, however, 5-HT had no effects in this process. Interestingly, Zhong et al. found the importance of 5-HT biosynthesis in *H. longicornis* ticks. They showed that the symbiont Coxiella (CHI) derived chorismate upregulates 5-HT synthesis in *H. longicornis* which in turn affects intake of the tick bloodmeal. Thus reduced colonization of Coxiella causes a reduction in blood acquisition (Zhong et al. 2021). As blood intake and cuticle plasticization are very related to each other, it is possible that the 5-HT might play roles in cuticle plasticization as well as in ticks. However, it is important to note that plasticization is not observed in soft tick *O. moubata* during feeding (Kaufman, Flynn, and Reynolds 2010).

1.8.3 Alterations in tick cuticle during feeding

During feeding, cuticles must stretch in order to accept a massive amount of bloodmeal relative to the size of the ticks. Female hard ticks feed for seven to ten days. The engorgement phase may be classified in the following ways: 1) a period of slow feeding, and 2) a phase of fast feeding. During the first phase of slow feeding, the weight of *I. ricinus* grows from 2 mg to around 50 mg (Lees, 1952). In addition, due to the synthesis of endocuticles, the thickness of the cuticle surface grows from 50 microns to 105 microns (Lees, 1952). During the fast-feeding period, their body weight grows to around 200 mg (Lees, 1952) (Andersen and Roepstorff, 2005a). During this time, cuticle production ceases and the cuticle is stretched to around 50 microns in thickness (Lees, 1952). Due to the quick feeding rate of soft ticks, their

cuticle is preformed and stretches while feeding (Lees, 1952). Cuticle structure is significantly altered during cuticle formation as epidermal cells become hypertrophied and pore canals are split due to stretching (Lees, 1952).

Female *I. ricinus* has almost twice the body length of a male. Once female ticks start to feed, their body length increases moderately from 4 mm to 6.9 mm and remains the same during this partially filled stage (Dillinger and Kesel 2002). Once they complete their bloodmeal in about 7-12 days, their body length increases greatly from 6.9 mm to 12.4 mm (Dillinger and Kesel 2002). The increase in body size exclusively happens in the alloscutum. The hard scutum in the dorsal part and other body regions does not grow. Alloscutum possesses sheet-like (lamellar) structures, which unfold as alloscutum increases during feeding, as demonstrated by SEM images by S.C.G. Dillinger and colleagues (Dillinger and Kesel 2002). Endocuticle grows during feeding as a result of chitin and protein deposition by epidermal cells and at the completion of the feeding, their body surface area expands by 15 times (Lees 1952a; Peter C. Flynn and Kaufman 2011).

Although some studies imply that the endocuticle develops solely during the slow phase of feeding, others indicate that the endocuticle grows during both slow and fast feeding (Svend Olav Andersen and Roepstorff 2005b; Peter C. Flynn and Kaufman 2011; Lees 1952b). Dillinger and Kesel (2002) suggested that the presence of a rubber-like protein, resilin, allows alloscutum to stretch and expand. Resilin deposition can be observed during the slow phase of feeding in the folds of alloscutum as well as, in the joints of the legs, pedipalps, setae, and along the edge

between the alloscutum and the scutum. Although protein extraction from cuticle is challenging due to protein crosslinking, two cuticle proteins from *I.ricinus* have been identified (Svend Olav Andersen and Roepstorff 2005b). Both proteins contain R&R motif for chitin binding. In addition, the protein mixture from the cuticle extraction fluoresces under UV light, indicating the presence of resilin and the amino acid dityrosine and trityrosine (Svend Olav Andersen and Roepstorff 2005b). The authors hypothesized that the randomly coiled shape of resilin protein enables the protein's elasticity. Crosslinking by tyrosine residues, on the other hand, makes the cuticle stable even during significant distension. However, the precise mechanism causing cuticle distension is unknown; other potential explanations include changes in the cuticular pH (Andersen and Roepstorff, 2005) (Kaufman et al., 2010).

1.8.4 Molting

Cuticles are rigid exoskeletons and must be shed and replaced through sequential events to allow the insect grow at each stage. This process, known as molting, includes apolysis and ecdysis. During apolysis, the old cuticle separates from the epidermal cell layer, and inactive molting fluid containing an enzyme is secreted from the epidermis into the gap between the old cuticle and the epidermal cells (Hackman, 1982). Molting fluid remains inactive until the new procuticle forms. The epicuticle is synthesized first, followed by the procuticle. The new cuticle expands as the old cuticle is digested and then the old cuticle is shed (ecdysis). The cement layer

is secreted from dermal glands and covers the outermost part of the cuticle (Hackman, 1982).

The newly synthesized cuticle is softer, more flexible, and lighter in color. These mechanical properties aid in shedding their old cuticle or exuvium and in the expansion of the new cuticle in their next instar (S.O. Andersen, Peter, and Roepstorff 1996). After ecdysis, the new cuticle becomes harder and darker owing to the crosslinking of cuticle proteins and dehydration. This process, known as sclerotization, happens concurrently with or soon after molting (Svend Olav Andersen 2010). This complex process involves the oxidation of dopamine derivatives NADA and NBAD (N-acetyldopamine and N-alanyldopamine) to the corresponding o-quinones and their inclusion in cuticular matrix protein by interacting with nucleophilic sites that mediate crosslinking (Andersen, 2010). Cuticles of arthropods differ in rigidity, elasticity, thickness, strength, and color owing to changes in protein composition, chitin-protein ratios, and probable degree of sclerotization, which dictates the functional properties (Andersen, 2010). Alongside sclerotization, melanization occurs in almost all insects investigated, where NADA and NBAD are transformed to DOPA melanin of black pigment, dopamine melanin of brown pigment, NBAD melanin of yellow color, or NADA melanin colorless with the aid of phenoloxidases (PPO) (Sterkel et al., 2019). However, PPO is not identified in ticks, and thus melanizations event may not occur.

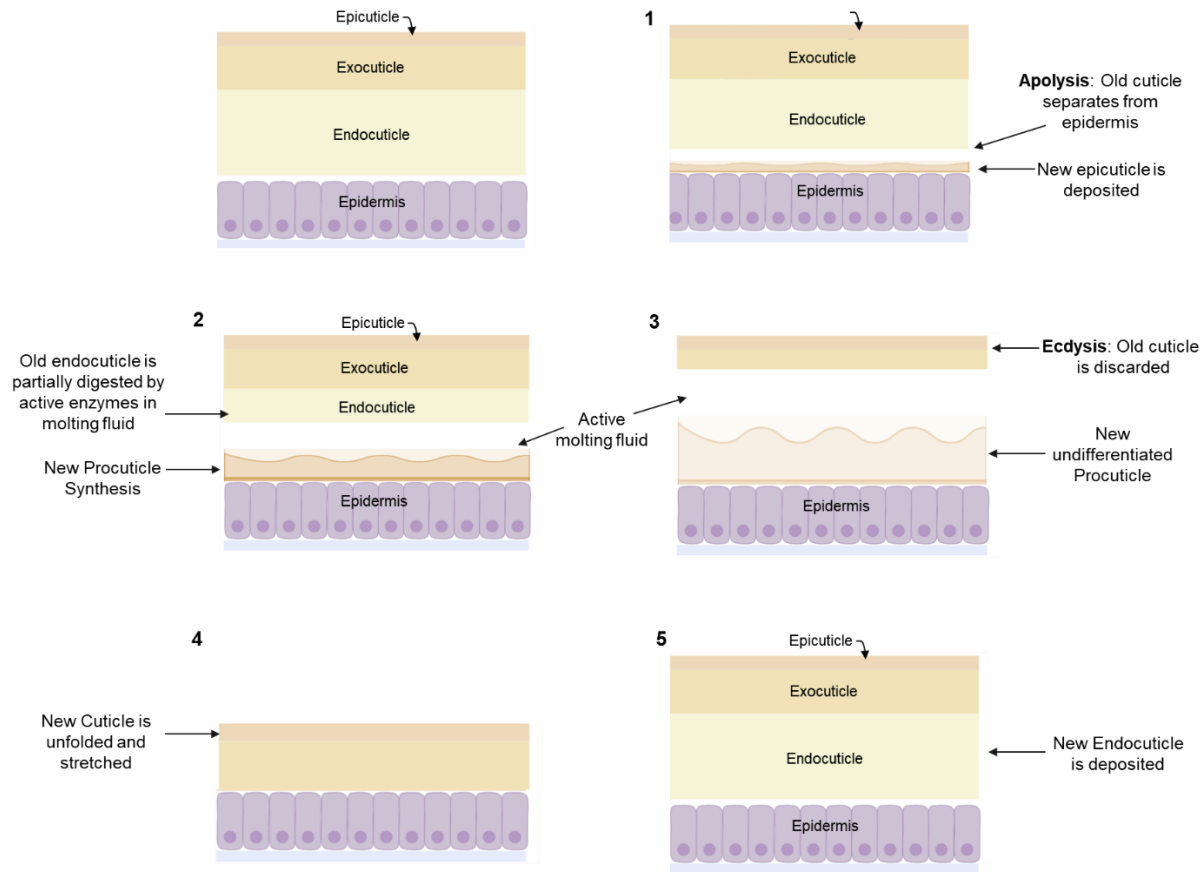


Figure 1.5. Schematic representation of arthropod molting process (Apolysis and Ecdysis). Created with BioRender.com. The molting process is shown from 1 to 5. During apolysis, the old cuticle separates from the epidermis and the new epicuticle is synthesized (1). Active molting fluid-containing enzymes start to digest old endocuticle whereas new procuticle is secreted by the epidermis (2). In ecdysis, the old cuticle is discarded (3). Next, the new cuticle is stretched, and unfolded (4) and the new endocuticle is deposited (5).

1.8.5 Hormones involved in the development and molting processes

Developmental pathways in insects are primarily controlled by neuropeptides, sesquiterpenoids, and ecdysteroids. Neuropeptide hormones, such as Ecdysone-triggering hormone (ETH) and Prothoracicotropic hormone (PTTH), are generated by neurosecretory cells, as their names imply. They control the production and release of

ecdysteroids (Cheong et al. 2015; Yamanaka, Rewitz, and O'Connor 2013). Sesquiterpenoids, including juvenile hormone (JH), methyl farnesoate (MF), and farnesic acid (FA), are produced in the insect corpora alata and regulate molting and metamorphosis (Cheong et al., 2015). JH in arthropods and cholesterol in vertebrates are synthesized primarily from acetyl-coA. JH is exclusive to arthropods and is absent from vertebrates (Cheong et al., 2015).

Insect growth and development, including molting and reproduction, are primarily controlled by two major hormones: steroid 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH). 20E is responsible for all significant developmental transitions, while the JH signal converts the 20E pulse into stage-specific responses (Dubrovsky 2005). Nevertheless, research indicates that ticks and mites lack JH and its precursor methyl farnesoate (MF) (Ogihara et al., 2019) (Neese et al., 2000). Genomic and transcriptome evidence also indicate that acari lack the enzymes necessary to generate JH (J. Zhu et al., 2016). Ecdysteroids, namely Ecdysone (E) and 20E are the primary hormones involved in the development and molting processes of Acari (ticks and mites) and insects and were first recognized as molting hormones (Ogihara et al., 2019) (Niwa and Niwa 2014). Ecdysone is synthesized in the prothoracic glands of insects and then released in the hemolymphs. Once released, it is transported to target cells such as the gut and fat bodies, where it is converted to 20E (Dubrovsky, 2005). 20E interacts with heterodimeric receptors consisting of ecdysone receptor (EcR) and ultraspiracle (USP) and modulates the expression of ecdysone-responsive genes (Uryu, Ameku, and Niwa 2015). Interaction

with 20E with its receptors immediately activates early genes, followed by activation of a larger set of late genes involved in development and reproduction which includes cell growth and differentiation, cell death of obsolete larval tissues, and cuticle production (Dubrovsky 2005). High concentrations of 20E are released before each developmental phase in insects, including before larval molt, pupation, and adult structural differentiation (Kannangara, Mirth, and Warr, n.d.; Dubrovsky 2005).

In ticks, ecdysteroid titers rise after bloodmeal and prior to molting, and administration of ecdysone may trigger the molting of immature ticks and expedite molting cycles, indicating that ecdysteroid also plays significant roles in tick development and molting (Rees 2004; Ogihara, Taylor, and Kataoka 2019). Interestingly, ecdysteroid titers are dependent on the critical weight of the ixodid tick during blood feeding, and they only increase if the hard ticks consume a full bloodmeal (Weiss and Reuben Kaufman 2001; Ogihara, Taylor, and Kataoka 2019). Alternatively, a short pulse of ecdysteroid may trigger ticks to fall off the host (Mao and Kaufman 1999). Studies on the soft tick *Ornithodoros moubata* indicate that ecdysteroid titers begin to rise during the deposition of the cuticle (Germond, Diehl, and Morici 1982). Ecdysteroid levels increase drastically during apolysis (the separating of the old cuticle from the new cuticle) and peak during the deposition of the new epicuticle. When ecdysteroid titers decline, a procuticle is produced and the digestion of the old cuticle begins (Germond, Diehl, and Morici 1982). Similar alterations have also been detected in species of the hard tick *Amblyomma* (Ogihara et al., 2019; Diehl et al., 1982).

Ecdysteroids are generated from cholesterol, similar to vertebrate steroids. However, arthropods cannot synthesize cholesterol, therefore they get it from their hosts (if they are hematophagous) or from plant sterols for ecdysteroid production (Ogihara, Taylor, and Kataoka 2019); (Niwa and Niwa 2014). Cholesterol is transformed to ecdysone in the prothoracic gland, and subsequently to 20E through C-20 hydroxylation in peripheral tissues such as fat bodies and the midgut (Ogihara, Taylor, and Kataoka 2019; Niwa and Niwa 2014). Numerous ecdysteroidogenic enzymes are CYTP450 monooxygenases, such as CYP307A1/spook, CYP307A2/spookier, CYP314A1/shade, CYP315A1/shadow, CYP302A1/disembodied, and CYP306A1/phantom (Niwa & Niwa, 2014). These genes are collectively referred to as the “Halloween genes” because mutations in them result in eerie appearances in *Drosophila* embryos due to faulty cuticle development (Ogihara et al., 2019). Many of the genes involved in this process are unknown and are referred to as "Black Box" with the exception of "spook," "spookier," and "shroud" (Ogihara et al., 2019). The first studied Halloween gene is *disembodied (dib)*; a mutation in disembodied causes aberrant cuticle growth during embryonic development in *Drosophila*, resulting in a cuticle that looks polished and bare (Chavez et al. 2000) (Niwa & Niwa, 2014). In addition, embryonic mortality is caused by the decreased expression of ecdysone-inducible genes and ecdysone titers that result from the complete loss of *dib* function (Niwa & Niwa, 2014). Similarly, mutations in *phantom, shroud, shadow,* and *shade* in *Drosophila* result in lower ecdysone titers, abnormal cuticle formation, and midgut development. (Chávez, n.d.;

Niwa & Niwa, 2014). Except for phantom, all steroidogenic CYTP450s were discovered in the genome of *I. scapularis*, indicating that ecdysteroid biosynthetic pathways are conserved/active (Gulia-Nuss et al., 2016). In fact, the *I. scapularis* genome has a large number of CYTP450s (206) that may be also involved in the detoxification of toxic compounds during bloodmeal and acaricide exposure (Gulia-Nuss et al., 2016). Nevertheless, functions of Halloween enzymes in ticks have not been thoroughly studied, with the exception of the RNA interference-mediated silencing of spook and shadow in the soft tick *O. moubata* interrupted development and resulted in abnormal ecdysis, respectively (Ogihara et al., 2019, 2015).

1.9 Anti-tick vaccine targets

Ticks and TBDs are a growing concern that affects both human and animal health. There are ~900 species of ticks worldwide and they transmit a wide variety of pathogens to humans and animals. Moreover, tick bite itself causes anaphylaxis reactions and potential paralysis. In addition, tick infestations (for example, *Boophilus sp.*) impact the economical production of cattle by affecting cattle health and milk production (J. D. L. Fuente and Kocan 2006).

Controlling tick infestations is highly challenging, as ticks have very few natural enemies. Traditionally, acaricides have been used to control ticks, but that also comes with some problems such as the selection of acaricide-resistant ticks and contamination of milk and meat products. An alternative approach is to development of anti-tick vaccines that will reduce tick infestations and TBD transmissions as well.

The first anti-tick vaccine is developed against one of the economically important parasites of cattle. Willadsen et al. identified an antigen (Bm86) from *Boophilus micropus* (now, *Riphicephalus microplus*) which elicited immunological responses upon vaccinating cattle hosts with Bm86 and is highly protective against tick infestations (Rand et al. 1989a; P Willadsen et al., n.d.). Bm86 provides cross-protection against other tick species as well and is the basis for two commercially marketed vaccinations, TickGARD^{PLUS} in Australia (now discontinued) and GAVAC™ in Cuba (Tabor and Tabor 2018).

Several other tick proteins were identified and studied as possible anti-tick vaccination candidates that target crucial biological processes, such as tick attachment to hosts, tick-host interactions, blood feeding and digestion, vitellogenesis and fertility among others (J. de la Fuente et al. 2016). Multiple cement proteins (aids in attachment) have been identified and evaluated as possible vaccine targets. 64P, a cement protein from *R. appendiculatus*, facilitates tick attachment and feeding and by vaccinating guinea pigs with recombinant 64P protein, nymphal and adult infestation rates were reduced by 48% and 56 to 70%, respectively (Trimnell, Hails, and Nuttall 2002). Several additional cement proteins (P29, HL 34, RIM 36) have also been identified as potential vaccine targets (J. de la Fuente et al. 2016). Numerous proteases and protease inhibitors (HLMP1, serpins, cystatins) involved in tick-host interactions demonstrate promising immunization responses (de la Fuente et al., 2016). In addition, many other potential anti-vaccine targets have been identified,

including Bm91, aquaporin, Ferritin 2, and GST, but none of them are commercially viable (Guerrero et al. 2014; Peter Willadsen et al. 1996; Hajdusek et al. 2010).

A highly conserved protein named subolesin from *I. scapularis* has been found as protective against ticks at all stages of development (Almazán et al. 2005). Moreover, vaccinating mice with 64P and subolesin inhibited TBE viral transmission from infected ticks to mice and *A. phagocytophilum* acquisition from infected mice to ticks respectively (J. D. L. Fuente and Kocan 2006). In the most recent genome analysis study of *I. scapularis*, several candidate vaccine antigens have been annotated with new or updated information which will help us target the best candidates and advance the development of an anti-tick vaccine (De et al. 2023).

Chapter 2: Structural and functional characterization of a novel *I. scapularis* Protein

2.1 Introduction

I. scapularis ticks or blacklegged ticks are obligate blood-feeders that target vertebrate hosts such as small rodents with humans being an accidental host. This tick species is the main carrier of many serious human pathogens, including *Borrelia burgdorferi*, the primary causative agent of Lyme disease. Along with *B. burgdorferi*, blacklegged ticks are also vector for *Anaplasma Phagocytophilum*, *Ehrlichia muris*, protozoa *Babesia microti*, and the Powassan virus (Eisen and Eisen 2018a). Incidence rates for tick-borne diseases have been steadily rising with approximately 476,000 estimated new cases of Lyme disease per year in the United States alone (CDC 2022b). As there is no vaccine available to prevent the majority of tick-borne infections in humans, one of our primary research objectives is to understand tick biology, particularly the structural and functional characterization of a novel *I. scapularis* proteins that constitute preventive targets as anti-tick vaccines.

I. scapularis stays attached to the host for several days, and their feeding success depends on various tick proteins that are associated with successful hematophagy, such as ones expressed in the gut, salivary glands, and associated organs. Nonetheless, the

first step before feeding is to locate hosts for their bloodmeal. Ticks and mosquitoes are ectoparasites that demonstrate host-seeking behavior. Many of these parasites use sophisticated chemosensation methods to detect CO2, a byproduct of vertebrate respiration process, together with host-specific compounds to identify their hosts. To recognize chemical signals, they rely on soluble proteins that are concentrated in the olfactory organs. These proteins activate chemoreceptors by binding to semiochemicals. Two soluble protein classes that serve as chemosensory proteins in insects are OBPs and CSPs (Paolo Pelosi et al. 2014). Pelosi et al. reported that whereas few CSPs are present in other arthropods, OBPs are unique to insects (Paolo Pelosi et al. 2014). Although it is reported that *I. scapularis* genome does not encode any OBP, OBP-like proteins have been identified in the organism's forelegs and hindlegs (Josek et al. 2018a; Gulia-Nuss et al. 2016a; De et al. 2023). Additionally, OBPs have functions outside of chemosensations (Paolo Pelosi et al. 2018).

Attachment of ticks to their hosts begins with the scissor-like chelicerae piercing the host's epidermis, causing damage to the blood vessels and dermal tissues, followed by the secretion of glycine-rich proteinaceous cement compounds (Kemp, Stone, and Binnington 1982; Neelakanta and Sultana 2022). This enables the hard ticks to attach tightly to their hosts for several days. Tick saliva contains proteins and peptides that modulate host responses to tick bites. They weaken their host's hemostasis, dilate blood vessels, impede wound healing, and suppress pain and itch sensations (Kotál et al. 2015; Kazimírová and Štibrániová 2013; Kitsou, Fikrig, and Pal 2021). Once firmly attached to the host skin, they start imbibing blood slowly for the

first few days, and then rapidly during the final 24-48 hours (Franta et al. 2010a). Adult females consume 100 times more blood than their body weight, requiring an enormous expansion of their exoskeleton (Franta et al. 2010a). Studies suggest that hard ticks synthesize cuticles during the slow feeding phase, which appears as folds (Lees 1952a; Svend Olav Andersen and Roepstorff 2005a). During the rapid phase of bloodmeal consumption, the cuticle expands by unwrapping the folds (Dillinger and Kesel 2002). Changes in cuticle structure occur not only during feeding but also during molting, as they molt after each bloodmeal and become larger at each stage, requiring changes in cuticle structures and the subsequent growth of new cuticles to accommodate their increasing size (Dotson, Connat, and Diehl 1995).

To identify tick proteins that are produced during the early stages of the tick engorgement process, we sought to identify gut proteins that are important in tick hematophagy and then assess if their suppression would impair tick feeding and their subsequent development. A mass-spectrometric analysis of approximately 36 hours fed tick gut tissues from our laboratory identified ~5000 proteins, only ~5% of which are unique to ticks (unpublished data). One of the novel genes encodes a secretory protein with structural homology with other known odorant binding proteins named herein as *Ixodes* gut odorant binding protein (IGOBP). Since this protein is produced during the initial stages of tick feeding and is considered to be secreted (due to the presence of a signal peptide sequence), we chose to characterize it further. Orthologs of IGOBP are found in other species from Chelicerata as well. Using in silico tools, we find that IGOBP has substantial structural similarities with mite OBPs. Notably, comparable di-

sulfide bridges are formed by IGOBP and mite OBPs, and cysteine sites are conserved across homologs. In addition, soluble IGOBP proteins exist as higher order complexes. Our data also suggest that *IGOBP* expression in ticks increases following tick attachment and feeding, indicating its importance in tick physiology. We assessed the significance of IGOBP in the tick life cycle by silencing the gene using RNA interferences (RNAi). The impaired feeding, and molting of larvae to nymphs, and nymphs to adults in the absence of IGOBP implies that the protein is essential in the tick life cycle. Additionally, RNAi of IGOBP causes defects in the integrity of the cuticle and affects mainly adult females compared to males. Therefore, this study characterized a novel *I. scapularis* protein and the first tick OBP that is critical for tick hematophagy and development.

2.2 Materials and Methods

2.2.1 Mice, ticks, bacteria

A low passage *B. burgdorferi* clone B31-A3 has been used in this study. Female C3H/HeN mice aged four to six weeks were purchased from Charles River Laboratories or Jackson Laboratories. The nymphal and adult *Ixodes scapularis* ticks that were used in this study originated from a colony that is maintained in our laboratory at 23° C and 85% - 99 % relative humidity as described earlier (Rana et al. 2023). All animal studies were conducted, in compliance with the guidelines and

approval of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Maryland, College Park.

2.2.2 Protein expression and polyclonal antibody preparation

Recombinant IGOBP was purified using a mammalian (CHO cell line) and bacterial (*E. Coli*) expression systems as described (Rana et al. 2023; De et al. 2023). Using gene-specific primers and Platinum SuperFi DNA Polymerase, IGOBP gene was amplified to express in the mammalian expression system (Thermo Life Technologies) using primers listed in **Table 1**. His tag was added to the C-terminal, whereas secretory signal peptides and a Flag tag were attached to the N-terminal. IGOBP was ligated to the pCDNA 3.0 vector using Gibson Assembly Master Mix (New England Biolabs) and CHO cells were transfected with the construct using PEI reagent (Polysciences, USA). G418-resistant clones were selected, and the secretion of recombinant IGOBP protein was examined by anti-Flag antibodies using SDS-PAGE and western blotting. CHO cell supernatants containing the IGOBP proteins were further purified by affinity chromatography using Ni-NTA beads. To generate recombinant IGOBP in bacterial system, gene was amplified removing the signal peptide regions (1-19 amino acid) and cloned into pET28a and pET26b vectors. Vectors containing IGOBP was transformed into *E. coli* BL21 cells and single colonies were selected to check protein induction. IGOBP expression was induced at 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 15-16 hours at room temperature and at 0.5 mM for 5-6 hours at 37c for pET28a and pET26b,

respectively. Protein was isolated using denaturing conditions and native conditions as detailed in the Probond Thermo Protocol using Nickel NTA resin. Polyclonal antibodies against recombinant IGOBP were produced in mice as previously reported (Kariu et al. 2013). Recombinant IGOBP protein (10 ug/mouse) was combined with Complete Freund's Adjuvants (CFA) and administered subcutaneously to mice. Two further boosts (with Incomplete Freund's Adjuvant IFA) were administered with a 10 – 12 days interval between each boost and 1 week after the second boost, the sera were collected. ELISA and immunoblot blot were used to assess the antibody titers and specificity, as previously reported (De et al. 2023).

2.2.3 Isolation of different tick organs

Ixodes nymphs were let to feed on C3H/HeN mice. Ticks were removed using forceps at 48 hours post placement of ticks. Organs were isolated from ticks after being dissected in a drop of sterile distilled water using an Olympus zoom stereomicroscope with a 2X auxiliary objective lens (total magnification, 90x).

2.2.4 RNA Isolation and PCR

Total RNA isolation was performed as stated previously (Rana et al. 2023). The oligonucleotide primer sequences for the quantitative RT-PCR analysis of tick gene expression are indicated in supplemental **Table 1**. For analysis of *IGOBP* expression, ticks were collected at various time points of feeding or as post fed nymphs. Individual ticks were processed separately. Total RNA was extracted from ticks using TRIzol (Invitrogen), treated with RNase-free DNaseI (Qiagen), and then reverse

transcribed to cDNA. Individual gene transcript levels were determined normalized to tick *β-actin* transcripts (Yang et al. 2014). RNA isolation and qRT-PCR analysis of the *IGOBP* gene are performed on intermolt larvae (day 7 to day 37), male and female (unfed ticks), and tick organs (ticks were collected and dissected 48 hours of feeding) as mentioned above.

2.2.5 Western Blot

Western blotting was performed as detailed (Yang et al., n.d.; De et al. 2023). Purified proteins or tick lysate were heated at 95° C in Laemmli buffer containing 5% β-mercaptoethanol. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to nitrocellulose membranes. 5% milk was used for blocking. Membranes were incubated with primary antibodies 1 hour at room temperature or overnight at 4°C with 2% milk in 1X PBS and 0.05% Tween-20. After 3-4 times of washing, HRP conjugated goat anti mouse secondary antibodies were incubated, and image was developed using chemiluminescence substrate.

2.2.6 Immunostaining and microscopy

The samples were prepared for confocal microscopy according to established protocols (Rana et al. 2023). To localize IGOBP in tick different tick organs, ticks were placed on mice. Major tick organs like the gut, salivary glands, fat bodies were extracted from 48 hour-fed ticks. We choose this timepoint, as tick imbibe major bloodmeal after 48 hours of host attachment. Organs were fixed with 4%

paraformaldehyde and permeabilized with acetone. 5% goat serum was used for blocking and 1:200 dilutions of primary antibodies were used. Alexa 488- labeled anti mouse IgG secondary antibodies are used. F-actin and cell nuclei were stained with rhodamine phalloidin and DAPI. Samples were imaged by a LSM 800 confocal microscope (Zeiss). Images of whole ticks were taken using a Canon SX720HS digital camera and an Olympus SZ61 microscope to record any morphological abnormalities during intermolt develop and metamorphosis to the next developmental stage. Scanning electron microscopic (SEM) observation is performed in the XEIA3 microscope (Tescan, Chez Republic) with a high voltage of 10 kV. The samples are placed on the carbon tape and then coated with about 100 nm thick Au-Pd film on the surface to solve the charging problem during SEM observation. For histology, whole ticks were cross-sectioned and stained with hematoxylin and eosin (H&E) as previously described (Rana et al. 2023). The stained slides were scanned with an anatomic pathology scanner from Leica Biosystems and recorded with ImageScope software. Using a Leica Biosystems anatomic pathology scanner and ImageScope software, the stained slides were analyzed and captured.

2.2.7 Sequences, alignment, and analysis

To find OBP homologs in other species, *I. scapularis* IGOBP protein sequences were analyzed by NCBI Blast search. Multiple sequence alignments (MSA) are conducted by Clustal Omega. MEGA 11 is used to construct a phylogenetic tree by aligning IGOBP homolog sequences and using the neighbor-joining method with

1,000 bootstrap replicates. The sequence information used for MSA, and phylogenetic trees are as follows: *I. scapularis,* >XP_002399632. ; *Rhipicephalus microplus,* >XP_037269043.1; *Rhipicephalus sanguineus,* >XP_037514317.1; *Hyalomma asiaticum,* >KAH6944417.1; *Dermacentor silvarum,* >XP_037560530.1; *Dermacentor andersoni,* >XP_050022590.1; *Haemaphysalis flava,* >AYH64978.1; *Haemaphysalis longicornis,* >KAH9360268.1; *Varroa destructor,* >XP_022645714.1; *Galendromus occidentalis,* >XP_003741537.1; *Tropilaelaps mercedesae,* >OQR73552.1; *Tetranychus urticae,* >XP_015786166.1; *Oedothorax gibbosus,* >KAG8183921.1; *Caerostris darwini,* >GIY18324.1; *Caerostris extrusa,* >GIY21294.1, *Pardosa pseudoannulata,* >AYJ71602.1; *Parasteatoda tepidariorum,* >XP_015918592.1; *Centruroides sculpturatus,* >XP_023236094.1; *Isometrus maculatus,* >ACD11898.1; *Limulus polyphemus,* >XP_013788954.1.

2.2.8 Protein Crosslinking via Bis(sulfosuccinimidyl) suberate (BS3)

Crosslinking study is performed as suggested (Thermo Fisher Scientific). Soluble IGOBP protein (0.013 mM) is treated with increasing concentrations of BS3 (0.13 mM, 0.26 mM, 0.4 mM, 0.53 mM, 0.65 mM, 1.32 mM) and the mixtures were allowed to crosslink in room temperature for 30 minutes. The reaction was then terminated with 1 M Tris (pH 7.4). The samples were heated in sample loading buffer with β-Mercaptoethanol (BME) at 95°C for 5 minutes before running in the SDS-PAGE. Control samples were either not treated with both BS3 and BME or were treated with BS3 alone.

2.2.9 RNA interference (RNAi) and tick manipulations

RNAi mediated gene silencing was performed as previously described (Rana et al. 2023) using primers listed in **Table 1**. Briefly, cDNA was generated and used as a template to amplify portions of the open reading frame of *IGOBP* gene. Sets of primers (Table 1) were used for amplification that contain the T7 promoter sequence. A part of the *GFP* gene was also amplified as a negative control. Using a commercial kit (MEGAscript RNAi kit, Invitrogen), dsRNA for *IGOBP* and *GFP* were produced and purified. 4 µl of the dsRNA (3.5 mg/ml) was loaded into a capillary tube and was microinjected into the ticks through their anal pores (Kariu et al. 2011). The injected ticks were placed on mice following day. Some of the ticks were collected at 48 hours and were individually processed and assessed for gene silencing using qRT-PCR analysis. Primers unique to the target dsRNA sequence's upstream or downstream regions were used for the qRT-PCR (**Table1**). For systemic RNAi silencing, unfed larvae or unfed nymphs were placed on mice and collected when fully repleted. dsRNA (3.5 mg/ml) for *IGOBP* and *GFP* were microinjected in the repleted larvae or nymphs using capillary tubes and kept them in the incubator until they molt into nymphs and adults, respectively.

2.2.10 In silico structural analysis

IGOBP structure was predicted by AlphaFold monomer v2.0 program (Jumper et al. 2021; Varadi et al. 2022). The Uniport ID of IGOBP was entered into the AlphaFold protein structure database to retrieve the IGOBP structure. The structure

was accessible in PDB format and was studied using the 3D structure visualization program PyMOL. IGOBP's AlphaFold structure was uploaded in DALI server (http://ekhidna2.biocenter.helsinki.fi/dali/) to identify proteins with similar structures. IGOBP and VdesOBP1 structures were superimposed using PyMOL.

2.2.11 Circular Dichroism

Circular dichroism studies were performed as described (Thakur et al., n.d.). IGOBP were buffer exchanged into 20 mM Sodium Phosphate, 20 mM KCl. Circular dichroism (CD) spectra were obtained using Chirascan CD spectrometer (Applied Photophysics LTD) using a 0.1 mm demountable quartz cell spanning the wavelength range of 180-280 nm. Representative data is shown after repeated scans results in similar data. Data were converted to mean residue molar ellipticity (deg cm2/dmol) after baseline subtraction of the buffer. Secondary structure deconvolution of the CD data was estimated using CDNN deconvolution program (CDNN courtesy of Gerald Bohn, 1997, at the Institut Für Biotechnologie, Martin-Luther Univserität Halle-Wittenberg, through distribution with Applied Photophysics software).

2.2.12 Size exclusion chromatography

Size exclusion chromatography was performed as detailed (Thakur et al. 2022). A Cytiva Superose 12 10/300 GL column was calibrated with gel filtration standards (Bio-Rad) and equilibrated at 0.5 ml/min with PBS. For each run, 100 µl of a protein sample containing 1.0 mg/ml was loaded onto the column, and the sample was eluted for 1.5 column volumes at 0.5 ml/min. Using known proteins and plotting their

apparent molecular masses standard curves were generated. The standard curve was then utilized to approximate IGOBP's apparent molecular mass.

2.2.13 SDS-PAGE mobility shift assay

1 ug of protein in 50 uL of buffer was added to Low-Bind Eppendorf tubes (Eppendorf # 0030108434) and incubated with the indicated amount of DTT for 10 minutes at room temperature. Sample tubes were then heated at the indicated temperatures for 10 minutes at room temperature before adding 4X denaturing SDS-PAGE sample buffer and visualizing using SDS-PAGE and Imperial Blue Coomassie (Thermofisher # 24617).

2.2.14 Nano-Differential Scanning Fluorescence

Nano-Differential Scanning Fluorescence (Nano DSF) studies were performed as detailed (Ronzetti et al. 2022). Briefly, recombinant IGOBP protein was added to Low-Bind Eppendorf tubes at a final concentration of 0.1 mg/mL and incubated with the indicated concentrations of DTT for 10 minutes at room temperature. Samples were then loaded into NanoTemper standard capillaries, placed into the Prometheus NT.48 instrument, and run at 50% excitation power with a thermal ramp of 2.5 °C/minute.

2.2.15 Choice chamber experiments

Choice chamber assays are adopted from (Carr et al. 2017b) and (Sun et al. 2018a). Briefly, store bought plastic choice chambers (two chambers connected with a middle

narrow portions) were used to study ticks' taxis behavior in response to humidity and repellent (DEET). Unfed nymphs were microinjected with *GFP* and *IGOBP* dsRNA and were kept in the incubator overnight (23° C and 85% - 99 % relative humidity). For systemic knockdown ticks, fully fed nymphs were microinjected with control and *IGOBP* dsRNA and were kept in incubator until they molt to adults. Unfed females were used for the tests. For the humidity sensing, one side of the choice chambers was prepared with a ddH2O containing smaller petri dish, and other side was with CaCl2 (desiccant). Before placing the ticks, choice chambers were allowed to equilibrate in the room temperature at 25°C and humidity (RH) was measured after two hours. For DEET sensing, 100% ethanol (ETOH) treated paper tape were placed on one side of the chambers and DEET treated paper tape were placed on the other sides. Next, ticks were placed in the middle of the chambers and taxis behavior of two groups of ticks (control and *IGOBP* knockdown) were tested in parallel in two chambers.

2.2.16 Statistical Analysis

Quantitative results are expressed as means ± standard errors of the means (SEM). Using GraphPad Prism v9 (GraphPad Software Inc.), two-tailed Mann–Whitney U or unpaired t test were conducted to examine the statistical significance of mean values between different groups.

Table 1: Oligonucleotide primers used in chapter 2

Primer Name/Purpose	**Sequence 5'-3'**
IGOBP protein expression (pET28a) forward primer NdeI	GGAATTCCATATGATCCCAGCCGGCGGCAAGGACCT
IGOBP protein expression (pET28a) reverse primer BamHI	CGCGGATCCTTAACCCTGCTGGGCTCCCTGTGGG
IGOBP protein expression (pET26b) forward primer BamHI	TAGGATCCGATCCCAGCCGGCGGCAAGGACCTC
IGOBP protein expression (pET26b) reverse primer XhoI	TAGCTCGAGACCCTGCTGGGCTCCCTGTGGG
IGOBP protein expression (pCDNA 3.0) forward primer	GACGATGACGACAAGATCCCAGCCGGCGGCAAGGAC
IGOBP protein expression (pCDNA 3.0) reverse primer	ATGATGATGATGATGACCCTGCTGGGCTCCCTGTGGG
Forward primer for quantitative PCR of IGOBP	AGCACGACGAGTGCAAGA
Reverse primer for quantitative PCR of IGOBP	CTGGGGATGTGGTAGTCCA
Forward primer for quantitative PCR of Tick *β-actin*	GGTATCGTGCTCGACTC

Forward primer for quantitative PCR of Tick *β-actin*	ATCAGGTAGTCGGTCAGG
Forward primer for RNAi of IGOBP	TAATACGACTCACTATAGGGATGGTGGCATTCAAGGTGTC
Reverse primer for RNAi of IGOBP	TAATACGACTCACTATAGGGGTTCAGGATCTCGGTCCTGG
Forward primer for RNAi of GFP	TAATACGACTCACTATAGGGAGAGCGACGTAAACGGCCACAAGTT
Reverse primer for RNAi of GFP	TAATACGACTCACTATAGGGAGACGGGTCTTGTAGTTGCCGTC

2.3 Results

2.3.1 Characterizations of a novel *I. scapularis* protein, IGOBP

To identify proteins that are important in the tick feeding process, our lab previously processed approximately 36 hours fed tick gut and determined the protein expression profiles by mass spectrometry analysis and compared them to the unfed tick gut proteome. Approximately, 5000 proteins were identified and only 5% of them were unique to ticks (unpublished). One of the proteins (IGOBP) that was significantly upregulated in tick guts after 36 hours of feeding was selected for further studies to determine its roles in tick feeding. BLAST searches identify IGOBP homologs with ~40-80% sequence identities in different tick species, mites, spiders, scorpions, and horseshoe crabs (data not shown). Phylogenetic analysis by MEGA11 shows that all the IGOBP homologs from tick species are clustered together and

closer to the cluster of mite species, while those of other species fall under different clusters **(Fig 2.1 A)**. Interestingly, homologs of IGOBP are not found in any of the arthropod subphylum other than the Chelicerata **(Fig 2.1 B)**. The common features of all chelicerates are the absence of antenna and the presence of sharp anterior chelicerae, which are used for capturing prey (spiders) and piercing the host skin (ticks). Thus, presence of this novel protein only in the Chelicerata suggests the importance of IGOBP in predatory and/or parasitic behavior in this group of arthropods.

IGOBP sequence encodes 187 amino acids where the first 19 amino acid residues are predicted to be the signal peptide. The calculated molecular weight and isoelectric point of IGOBP without signal peptide are ~19 kDa and 5.81, respectively. The presence of N terminal signal peptide sequences suggests that IGOBP is a secreted protein. A Clustal Omega multiple sequence alignment (MSA) revealed the presence of conserved regions in the IGOBP protein across all the organisms in the Chelicerata phylum **(Fig. 2.1 C)**. Moreover, mature IGOBP protein (without the signal peptide) contains 6 conserved cysteines, and their locations are conserved among homologs **(Fig. 2.1 C)**. Interestingly, protein blast analysis of the IGOBP sequence against the NCBI database revealed no additional *I. scapularis* sequences. The expression of two OBP-like proteins, OBPL1 and OBPL2 (IscaXP 002433530 and IscaXP 002405987) in *I. scapularis* forelegs and hindlegs was reported by Josek et al (Josek et al. 2018a), but they only share 25% and 19% sequence identities with IGOBP sequence, respectively. However, their prediction was based on the

resemblance with insect OBP with the presence of N-terminus signal peptide, 4-6 conserved cysteines, and divergence in sequences.

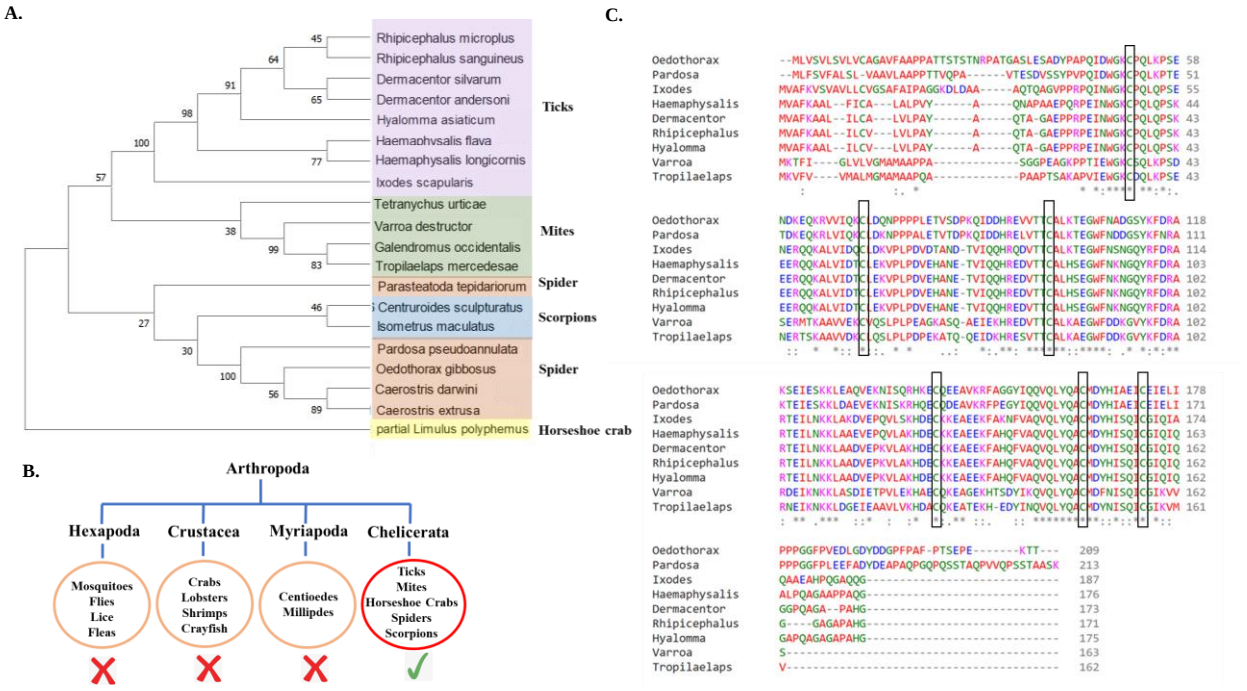


Figure 2.1. IGOBP is conserved across the Chelicerata Phylum. (A) A maximum likelihood phylogenetic tree was constructed using IGOBP homolog sequences from several tick, mite, spider, horseshoe crab, and scorpion species. IGOBP amino acid sequence was utilized as the query sequence in BLAST to retrieve arthropod sequences. The values for branch support are represented by numbers. **(B)** A schematic illustration is demonstrating that IGOBP is exclusive to the subphylum Chelicerata of arthropods. **(C)** Amino acid sequence alignment of IGOBP homologs from representative ticks and other species of Chelicerata. Amino acids with similar properties are labeled with identical colors. The cysteine sites are conserved among homologs, as shown in Box. "*" indicates the identical residues, ":" indicates the residues with strongly similar properties, "." Indicates the residues with weakly similar properties. Accession number for the sequences are mentioned in the method sections.

2.3.2 Structural prediction of IGOBP

In general, functional, and structural characteristics of IGOBP and its homolog proteins are not well characterized in the literature. In silico analysis of IGOBP shows

structural similarities with odorant binding proteins (OBPs). Classical insect OBPs contain 6 alpha helices, 3 disulfide bridges, and a binding cavity that interacts with hydrophobic ligand molecules (P. Pelosi et al. 2006). Vertebrate OBPs are structurally very different from insect OBPs. They belong to the superfamily of lipocalin carrier protein and consist of mainly β-sheets. However, non-insect OBPs are not well-studied. IGOBP's predicted structure using the AlphaFold database has characteristics similar to OBPs **(Fig. 2.2)**. Predicted IGOBP structure consists of 5 alpha helices (H1 to H5) in contrast to other classical insect OBPs, which consist of 6 alpha helices. Disulfide bridges are predicted between Cysteine (C) C1-C6 (at positions 48-169 aa), C2-C3 (at positions 68-95 aa), and C4-C5 (at positions 138-160 aa) instead of the alternative patterns (C1-C3, C2-C5, C4-C6) that are often seen in insect OBPs. The central part of the protein has a relatively ordered structure. Per-residue confidence score (pLDDT) for most of the alpha helix regions is >70 indicating confidence in the predicted model. Whereas the N-terminal and C-terminal regions (1-36 aa and 175-187 aa respectively) are disordered (low pLLDT and high PAE) (data not shown).

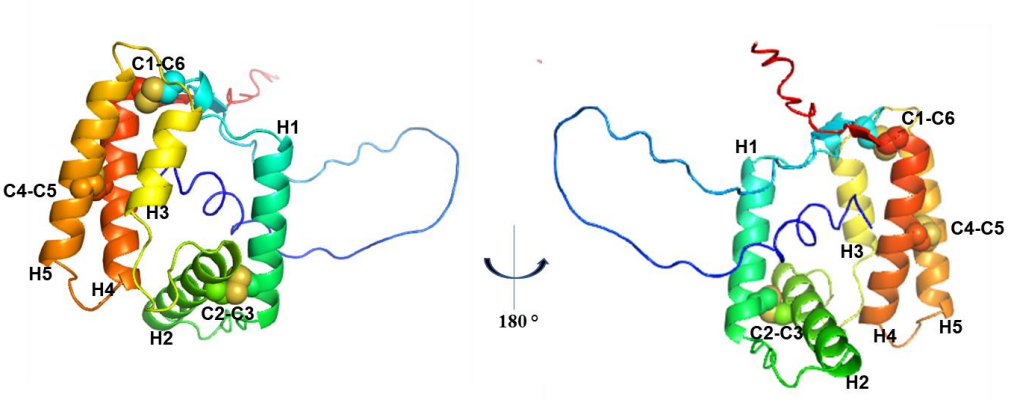


Figure 2.2 IGOBP structure prediction by AlphaFold. Ribbon image of a monomer displaying helices H1 to H5 and disulfide bridges at C1-C6, C2-C3, and C4-C5 positions in PyMOL viewer. The polypeptide chain is colored in rainbow order, from blue (N-terminus) to red (C-terminus). The monomer is depicted from two different angles after being rotated 180 degrees.

2.3.3 Comparison of IGOBP with other OBP structures

To get more insights about IGOBP protein structure, a DALI server (http://ekhidna2.biocenter.helsinki.fi/dali/) search of the Protein Data Bank (PDB) was performed that returned results with a notable hit with one of the OBPs from *Varroa destructor* (mite) (Holm 2022; Amigues et al. 2021a). Crystal structure (PDB ID: 7NZA) of the mite OBP, VdesOBP1, has a Z score of 18.2 and r.m.s.d of 1.3 Å on 141/141 residues (not shown). The structure contains two molecules of VdesOBP1 forming a crystallographic dimer, however biologically relevant form is likely to be the monomer (Amigues et al. 2021a). Structural alignment of IGOBP (s001A) and 7NZA in DALI shows the position of the helices are very similar to each other with 100% match in the position of helices H2, H3, and H5 **(Fig. 2.3 A)**. Superimposition of 7NZA with IGOBP structure in PyMOL shows all the helices superimpose nearly identically with an r.m.s.d value of 0.558 Å **(Fig. 2.3 B)**. Like IGOBP, VdesOBP1 crystal structure consists of 5 helices and 3 disulfide bridges at cysteine positions of C1-C6, C2-C3, and C4-C5. VdesOBP1 and IGOBP sequences shared around 56% of the same amino acids. Furthermore, the amino acid residues predicted to form the binding wall for VdesOBP1 are conserved in the IGOBP **(Fig. 2.3 C)**. Comparing IGOBP and VdesOBP1 to traditional OBPs indicates that it lacks the sixth helix

(Amigues et al. 2021a). Predicted structure of another OBP, TurtOBP1 from spider mite *Tetranychus urticae* has a very similar structure as in IGOBP and VdesOBP1 (J. Zhu et al. 2021). Therefore, OBPs from Chelicerata and those from insects vary primarily in their cysteine pairing and overall three-dimensional folding. Thus, although we may speculate that OBPs from insects and Chelicerata originated from a common predecessor, their significant sequence divergence suggests evolution of their potential adaptive functions in specific group of arthropods.

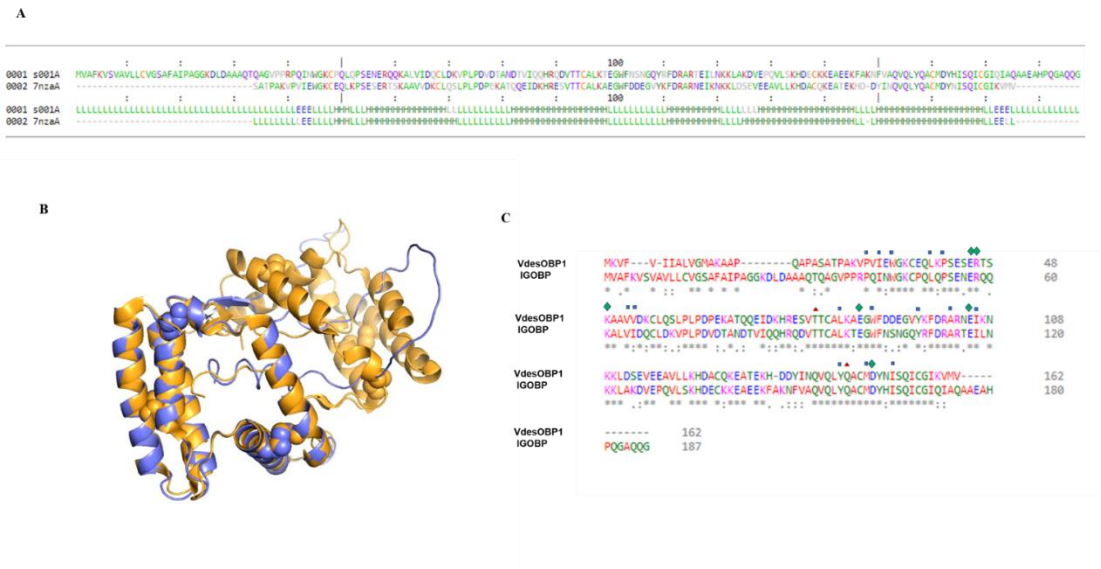


Fig 2.3. Structural homology of IGOBP and mite OBP. (A) Stacked pairwise alignment of IGOBP and VdesOBP1 (PDB ID 7NZA) by DALI server shows conservation of secondary structures. Secondary structures are denoted as H (helix), E (strand), and L (loop). **(B)** The predicted structure of IGOBP monomer is superimposed with the crystal structures of *V. destructor* OBP dimer (PDB ID 7NZA; root-mean-square deviation of 0.558 Å). IGOBP is colored in violet and VdesOBP1 is in yellow. **(C)** Alignment of VdesOBP1 and IGOBP amino acid sequences shows conserved amino acids. Square boxes denote hydrophobic residues, triangles denote polar non-charged, and green diamonds show charged residues in the binding cavities of VdesOBP1.

2.3.4 IGOBP Protein purification and structural characterizations

In order to further characterize IGOBP, the protein was expressed and purified. The cDNA sequence excluding the N-terminal signal sequence was cloned in pET28a and expressed as recombinant poly histamine (6X-His)-tagged IGOBP in bacterial systems. IGOBP was induced at room temperature with 0.3 mM IPTG. However, the recombinant IGOBP was accumulating in the inclusion bodies and thus we purified the protein using Ni- nitrilotriacetic (Ni-NTA) column under denaturing conditions followed by buffer exchange in 1X PBS **(Fig. 2.4 A)**. Next, we wanted to confirm the presence of disulfide bridges in IGOBP in solution. The addition of reducing agents decreased the temperature of aggregation (Tagg), as shown in nanoDSF assays with increasing concentrations of DTT and TCEP **(Fig 2.4 B)**. Additionally, we detected IGOBP mobility shift using SDS-PAGE after exposure to reducing agents at increasing concentrations **(Fig. 2.4 C)**. Both our nanoDSF and SDS-PAGE results indicate the presence of the disulfide bridges in IGOBP. Circular Dichroism (CD) was performed to determine the secondary structure of the protein purified in denaturing conditions and was buffer exchanged in 20 mM Sodium Phosphate, 20 mM KCl. CD analysis shows the presence of 24 % helical, 28.2 % antiparallel, 9.4 % parallel, 20.4% beta-turn and 30.2% random coil **(Fig. 2.4 D)**. However, the structural prediction states that IGOBP should primarily consist of alpha helices if it is an odorant binding protein. Thus, it is possible that the IGOBP does not retain its original structure after being purified under denaturing conditions. We have generated polyclonal antibodies in mice using the purified proteins.

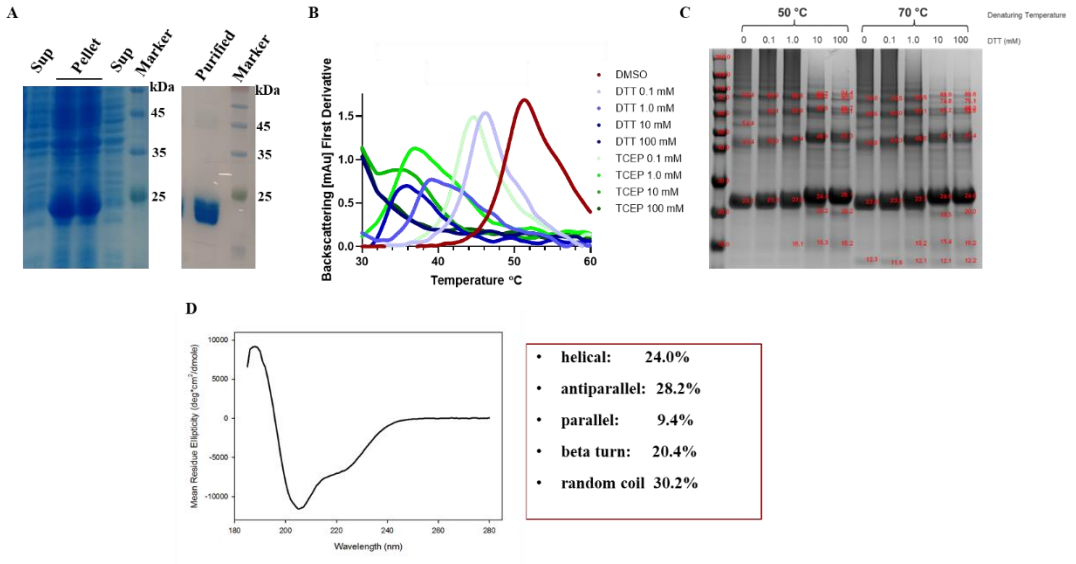


Figure 2.4. Expression, purification, and characterizations of IGOBP protein. (A) Left panel; protein was expressed in a bacterial system using pET28a, and it is present in the pellet (P) but not in the supernatant (S). Right panel; separation of purified IGOBP by SDS-PAGE. **(B)** Effects of reducing agents on IGOBP protein. NanoDSF measurements of the temperature of aggregation (Tagg) demonstrating the addition of reducing agents to 0.1 mg/ml protein stocks significantly reduced the Tagg with increasing concentrations of DTT, and TCEP. **(C)** Comparison of protein mobility in SDS-PAGE under reducing and non-reducing conditions. Bands were visualized with Imperial Blue Coomassie. **(D)** The recombinant IGOBP protein isolated under denaturing conditions was analyzed by circular-dichroism (CD) spectroscopy. Spectra of IGOBP from 200 to 260 nm are presented as mean residue ellipticity (deg-cm2/dmol) versus wavelength after baseline subtraction of the buffer. Spectra indicated the presence of alpha helices, beta sheets, beta-turns, and random coils.

2.3.5 Purification and Characterizations of soluble IGOBP protein

To determine protein structure and functions, we sought to purify IGOBP in native conditions. IGOBP was expressed in Chinese hamster ovary (CHO) cell lines using pcDNA3.0 vector **(Fig. 2.5 A, upper panel)**. However, this technique did not yield adequate IGOBP concentrations required for subsequent characterization assays

(Fig. 2.5 A, lower panel). Thus, we produced the protein in a bacterial system using the pET26b cloning vector. pET26b contains pelB, which guides recombinant protein into the periplasm. Periplasm possesses an oxidizing environment and enzymes that assist generate disulfide bonds. We were able to express IGOBP in the soluble fraction (in supernatant fractions) and purify it under native conditions in adequate amounts **(Fig. 2.5 B)**.

We further analyzed the oligomeric states of IGOBP in solution using bis (sulfosuccinimidyl) suberate (BS3) crosslinking agent. IGOBP proteins were incubated with increasing concentrations of BS3 for 30 minutes, then treated with Tris, β-mercaptoethanol (βME), and heated before performing SDS-PAGE. The results show that IGOBP is present in monomers, dimers, trimers, and higher order complexes **(Fig. 2.5 C)**. Although the precise oligomeric states of protein could not be confirmed, size exclusion chromatography (SEC) indicates that IGOBP exists in multiple oligomeric states as well **(Fig. 2.5 D)**. Next, we compared the size of the recombinant protein (expressed in the bacterial system) to the size of the native IGOBP from tick lysates. Immunoblot analysis with IGOBP antisera reveals that both recombinant protein and the native form of protein from whole ticks have similar molecular weight, which are detectable at around 19 kDa, suggesting that the mature IGOBP may lack significant post-translation modifications like glycosylation **(Fig. 2.5 E).**

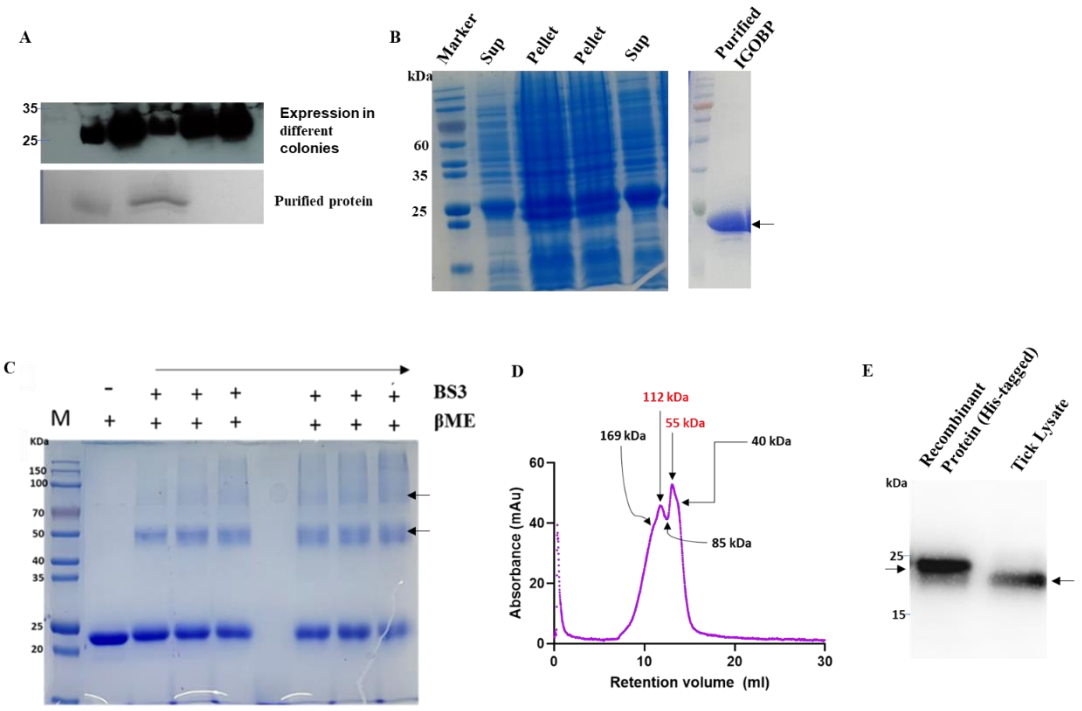


Figure 2.5. Expression, purification, and characterization of soluble IGOBP protein (A) Expression and purification of proteins in a mammalian (CHO) system. Upper panel: IGOBP-expressing CHO clones were identified by evaluating IGOBP expression with anti-Flag antibodies in western blot. Lower panel: gel electrophoresis of IGOBP, isolated from CHO cells. **(B)** Gel electrophoresis of purified protein in soluble conditions. Protein was expressed in bacterial system (using pET26b vector) and was present in both supernatant (S) and pellet (P) fractions (left panel). Gel electrophoresis of purified IGOBP under native conditions is shown in the right panel with an arrow. **(C)** Analysis of the oligomeric states of IGOBP. (Upper panel) Crosslinking study of recombinant IGOBP in solution using bis (sulfosuccinimidyl) suberate (BS3). IGOBP were incubated with increasing concentrations of BS3 for 30 minutes at room temperature, as shown by arrow, before being assessed by SDS-PAGE. + denotes the presence of the BS3 or βME and – the absence of the BS3 or βME. Arrows indicate the crosslinked protein. **(D)** Oligomeric states of recombinant IGOBP protein were examined via size exclusion chromatography on Superose 12 10/300 GL column. Molecular mass for main peaks is highlighted with red and shoulder peaks are denoted with black. **(E)** Recombinant protein and native protein from tick lysates were immunoblotted using IGOBP antisera and detected at around 19 kDa. Black arrows indicate the IGOBP protein.

2.3.6 IGOBP expression in vivo

From our earlier mass spectrometry data, we detected IGOBP protein in the tick gut at 36 hours of tick placement (unpublished data). Here, we studied the spatial and temporal expression of IGOBP. Gene expression was investigated at various stages of life cycle, organs, and feeding times of nymphs. *IGOBP* expression is highest at 12-24 hours of feeding nymphs **(Fig. 2.6 A)**. *IGOBP* is most abundant in hypostomes, and legs followed by salivary glands and carcasses with the gut having the lowest expressions in the 48 hours fed nymphs **(Fig. 2.6 B)**. *IGOBP* expression increases at the end of the larval development in intermolt larvae **(Fig. 2.6 C)**. Additionally, *IGOBP* has relatively higher expression in adult female ticks compared to adult male ticks **(Fig. 2.6 D)**. Confocal immunofluorescence analysis using IGOBP antibodies indicated that the protein is detected in all the organs tested, including the gut, salivary glands, and fat bodies **(Fig. 2.6 E)**. We also examined protein expression levels in organs by immunoblotting from 48-hour-fed nymphs and found that IGOBP protein was more abundant in the salivary glands than in the tick gut, supporting the RNA expression results **(Fig. 2.6 F)**.

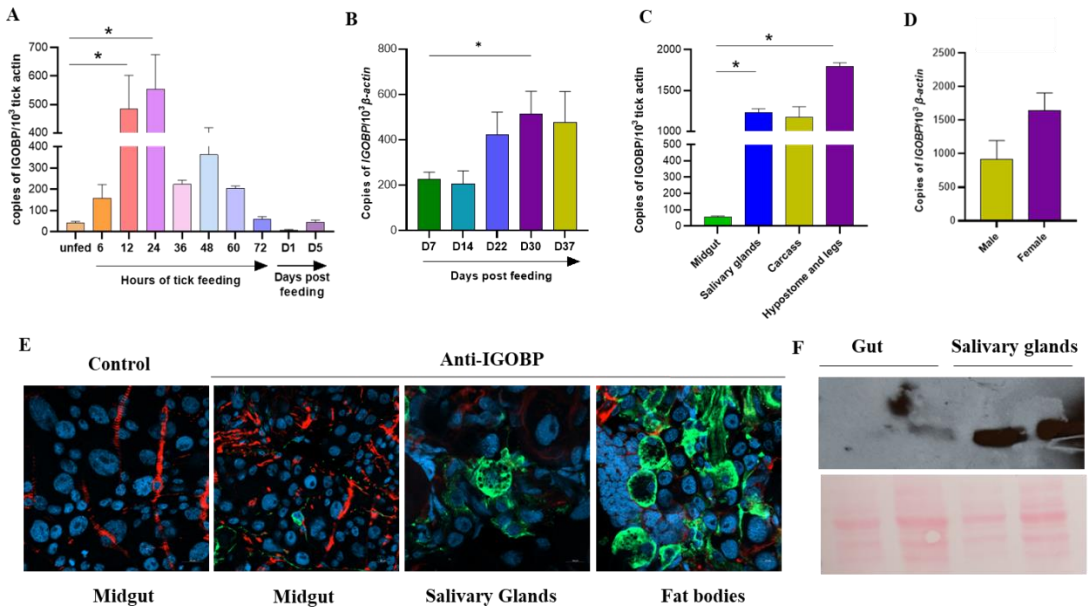


Figure 2.6. Temporal and spatial expression of IGOBP in naïve ticks. (A) Expression of *IGOBP* at various time points. Nymphs were placed on mice and collected (5 ticks/group) at different time points after placement of ticks; individual ticks were processed for measurements of *IGOBP* transcripts. **(B)** The expression of *IGOBP* was examined in several tick organs. Ticks were collected 48 hours after being placed on mice. Organs were separated and combined from 10-12 ticks for each pool (2 pools/ group). **(C)** Expression analysis of *IGOBP* in intermolt larvae. Fully repleted larvae were maintained in an incubator, and 7-day, 14-day, 22-day, 30-day, and 37-day post-feeding ticks were separately processed for qRT-PCR analysis to evaluate IGOBP expression; 5 ticks/group were used. **(D)** *IGOBP* expression in male and female ticks. Unfed male and female ticks (5 ticks/group) were individually processed, and qRT-PCR analysis was done. **(E)** Localization of IGOBP in tick. Isolated organs (gut, fat bodies) from 48 hours fed nymphs were processed for confocal immunofluorescence staining using IGOBP antisera and FITC-labeled anti-mouse IgG (labeled green). The nuclei in tick tissues were labeled with DAPI (blue), and actin filaments were stained with Phalloidin (red). **(F)** IGOBP protein expression was compared in tick organs (gut and salivary glands) by western blot using IGOBP antisera. The total protein amount in the tick organs is shown by Ponceau S staining. *p< .05.

2.3.7 IGOBP supports tick hematophagy

To further understand the significance of IGOBP in the tick life cycle, we sought to knockdown its expression via RNA interference. To achieve this, we

synthesized *IGOBP* sequence specific double-stranded RNA (dsRNA) and used an unrelated gene (*GFP*) to synthesize control dsRNA. The dsRNA (3.5 mg/ml) for *IGOBP* and *GFP* (control) were microinjected into the ticks before placing them on mice **(Fig. 2.7 A)**. Individual qRT-PCR analysis of 6-8 ticks from each group at 48 hours after tick placement indicates considerably lower expression ($p < 0.001$) of *IGBOP* transcript levels in *IGOBP* dsRNA treated (designated as ds*IGOBP*) ticks compared to nymphs injected with *GFP* dsRNA (designated as ds*GFP*), indicating effective knockdown of the *IGOBP* **(Fig. 2.7 B)**. The remaining ticks were fed until repletion and completely repleted ticks were recovered from the water. The number of ticks collected was significantly lower ($p < .01$) in the ds*IGOBP* group compared to the ds*GFP* group **(Fig. 2.7 C)**. We also assessed their weights and discovered that *IGOBP* knockdown ticks had considerably lower tick weights ($p < .0001$) than the control group **(Fig. 2.7 D)**. Thus, our data suggest that IGOBP is essential for the tick hematophagy process.

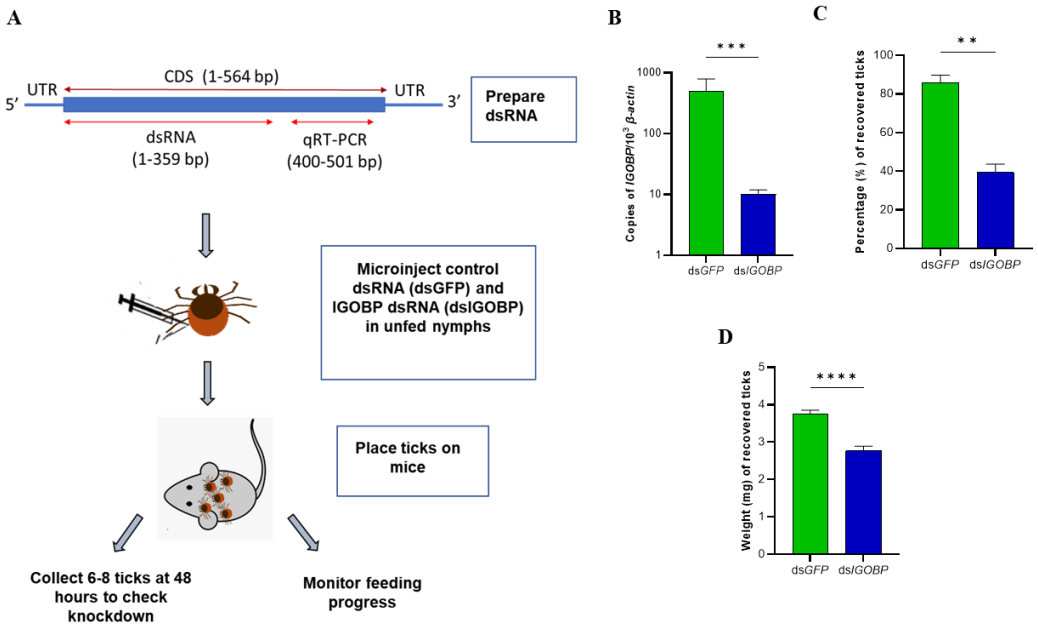


Figure 2.7. Knockdown of *IGOBP* impairs tick feeding and reduces tick weights significantly. (A) Illustration of dsRNA mediated silencing experiment. 1-359 bp regions were targeted for RNA interference and 400-501 bp regions were chosen for gene expression analysis. **(B)** Knockdown of *IGOBP* transcripts is induced by RNA interference. Nymphal ticks were injected with *IGOBP* dsRNA or control *GFP* dsRNA, fed on mice and ticks were collected (6-8 ticks/group) at 48 hours after placing them on mice. Individual ticks were processed for measurements of *IGOBP* transcripts. **(C)** *IGOBP* knockdown impairs tick feeding. The remaining ticks were allowed to continue their feeding and collected after their repletion (completion of bloodmeal). **(D)** The weights of repleted ticks from the IGOBP knockdown group are significantly lower compared to the control group. Experiment was performed at least 4 times. **p < .01, *** p < .001, **** p< .0001.

2.3.8 Knockdown of IGOBP transcripts

We injected dsRNA into ticks via their anal pores, which are part of the hindgut and are connected directly to the midgut. Additionally, we observe that *IGOBP* expression is lowest in the midgut. Therefore, we examined whether dsRNA injections are effective locally in the midgut or whether they may effectively silence *IGOBP* expression in other organs, including salivary glands, hypostome, legs, and

carcasses, where *IGOBP* expression is higher. After microinjecting, ds*IGOBP* and ds*GFP*, ticks were placed on mice. Ticks were collected after 48 hours, and the organs were separated under a light microscope. Each pool of organs was collected from 10-12 ticks and transcript levels were evaluated using qRT-PCR. We found that IGOBP silencing is effective in all tick organs tested, including the midgut, salivary glands, carcass, hypostome, and legs **(Fig. 2.8 A)**.

Subsequently, we investigated whether RNAi-mediated silencing affects IGOBP protein levels in ds*IGOBP* ticks. We collected ticks after 48 hours and used immunoblotting with anti-IGOBP antibodies to assess IGOBP protein levels in both control and knockdown ticks. We found lower IGOBP levels in the whole tick lysates and carcasses of knockdown ticks as compared to control ticks, demonstrating that *IGOBP* knockdown reduces protein levels effectively **(Fig. 2.8 B)**. Ponceau staining pictures are shown to compare the total protein in the tick lysates **(Fig. 2.8 B, lower panel)**.

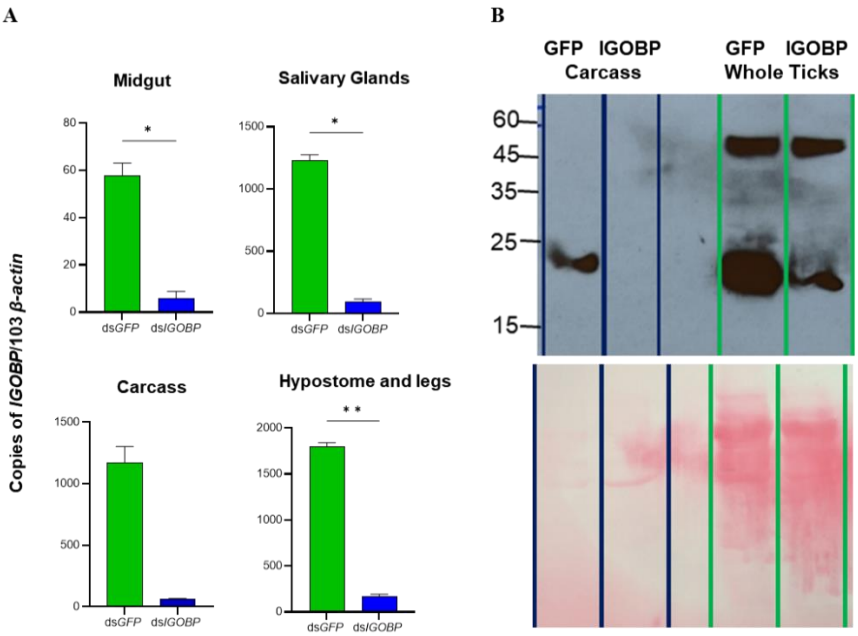
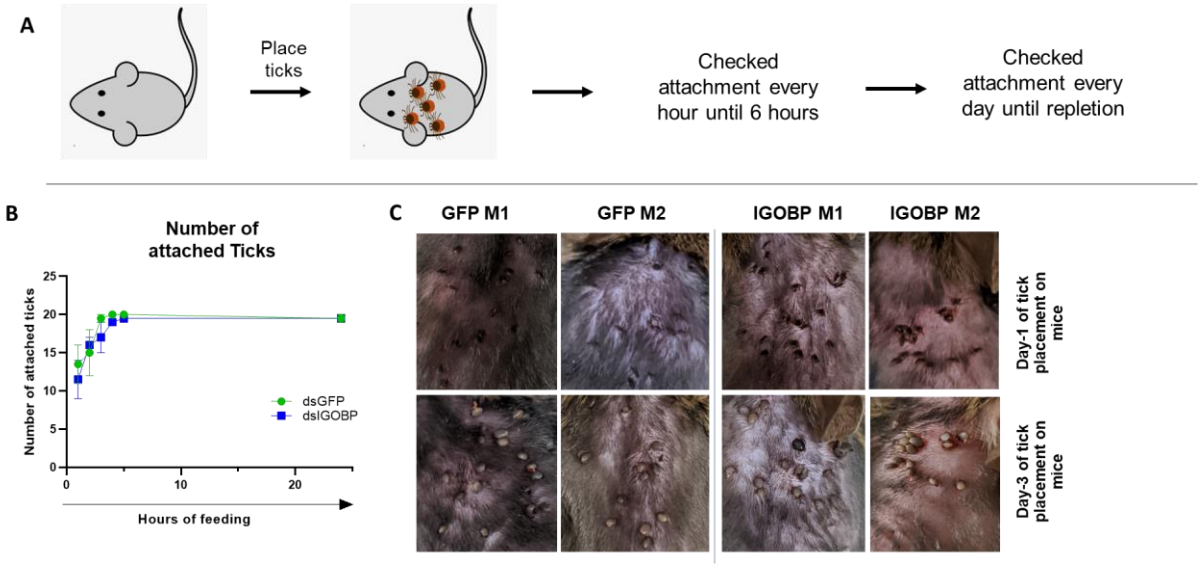


Figure 2.8. dsRNA treatment reduces *IGOBP* RNA and protein levels in tick organs. (A) Expression of *IGOBP* was reduced in various tick organs in the *IGOBP* dsRNA treated ticks compared to the control ticks. **(B)** Western blot analysis of knockdown ticks with IGOBP antisera reveals reduced protein expression in the knockdown ticks (upper panel). Whole protein in tick lysates is shown by Ponceau staining (lower panel). Experiment was performed at least two times. *p < 0.05, **p < .01

2.3.9 Silencing of *IGOBP* impacts tick engorgement process

Given that IGOBP deficiency impacts tick feeding, we inquired whether the lower feeding efficiency was related to ticks' inability to attach to the host skin. Therefore, we microinjected dsRNA for *GFP* and *IGOBP* in unfed nymphs, placed them on mice, and carefully observed them for 6 hours (at 30-minute intervals) after placement, as well as once a day until repletion **(Fig. 2.9 A)**. Comparable numbers of ticks are found attached to both groups until 6 hours to 24 hours suggesting that attachment is unaffected by the silencing of *IGOBP* **(Fig. 2.9 B)**. We continued

monitoring for the next few days until their repletion and we observe that ticks are still attached and that their sizes are comparable up to day 3 following tick placements **(Fig. 2.9 C).** Interestingly, ticks from ds*IGOBP* groups (right panel) are unable to complete their engorgement process, as illustrated in **Fig. 2.9 D-F**. We noticed that, in the absence of IGOBP, ticks have severe impacts from day-4 (upper panel) and day-5 (bottom panel) during the rapid feeding phase **(Fig. 2.9 D)**. The ticks in the IGOBP dsRNA treated group were in the midst of complete engorgement on day 4, but they were dead on day 5 (blue and yellow arrows) **(Fig. 2.9 D).** In the control group, we are able to recover 75–90% of fully repleted ticks, while in the *IGOBP* dsRNA treated group, only 5–10% of fully repleted ticks are recovered **(Fig. 2.9 E-F).** Our findings indicate that the lack of IGOBP did not affect tick attachment to hosts, but rather the engorgement process, particularly during the rapid phase feeding that occurs during the final 24-48 hours.



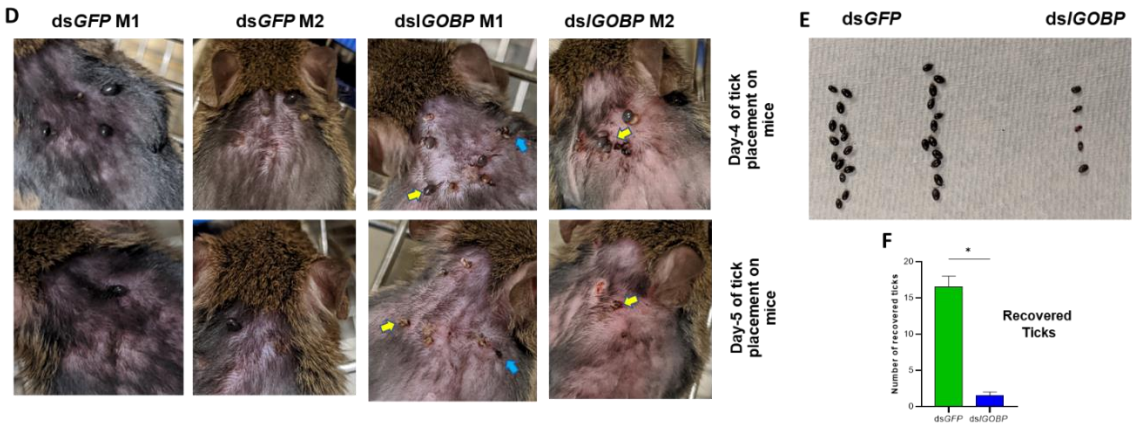


Figure 2.9 *IGOBP* knockdown affects the tick engorgement process but not tick attachment. (A) Schematic representations of the attachment experiment. dsRNA treated nymphs were placed on mice and attachment and feeding progress were monitored until repletion. **(B)** A comparable number of ds*GFP* and ds*IGOBP* ticks were attached to mice. For the first 6 hours, the number of attached ticks was counted every 30 minutes for both groups, and then every day until repletion. The graph shows the time till 24 hours. **(C)** The number of attached ticks, their size, and their feeding progress are comparable, as shown in the representative images (control group mice; ds*GFP* M1 and ds*GFP* M2, experimental group mice; ds*IGOBP* M1 and ds*IGOBP* M2) at day -1 (upper panel) and day-3 (bottom panel). **(D)** There were differences in tick repletion on days 4 and 5 following tick placement. The upper panel depicts a few dead ticks on day 4 in the ds*IGOBP* group (ds*IGOBP* M1 and ds*IGOBP* M2). When the same mice are compared on day 5 (bottom panel), the ds*IGOBP* group had more deceased ticks. The yellow and blue arrows represent the identical ticks in the same mouse on day 4 and day 5. **(E-F)** Ticks recovered from the control and *IGOBP* knockdown groups are shown in panels E and F. In the ds*IGOBP* group, fewer ticks were repleted and recovered ticks were dead. Experiment was performed at least three times. *p< 0.05

2.3.10 Systemic knockdown of IGOBP impacts tick molting and engorgement

Since the effects of gene silencing are evident between days 4 and 5, we speculated that the presence of premade IGOBP protein (presence of previously expressed protein before silencing of *IGOBP*) might explain the lack of impacts at earlier time points. To better understand the role of IGOBP, we employed systemic

RNAi silencing, in which the interference effect persists and is maintained even after the molting of the organism into the next stage of their life cycle and herein referred to as systemic RNAi (Fire et al. 1998; Grishok, Tabara, and Mello 2000). Therefore, we microinjected dsRNA into newly engorged larvae and allowed them to molt in an incubator **(Fig 2.10 A)**. dsRNA treatment reduced *IGOBP* levels in freshly molted nymphs, as assessed by qRT-PCR analysis, indicating systemic knockdown of the *IGOBP* gene **(Fig 2.10 B)**. Tick molting efficiency is decreased by IGOBP deficiency, as seen in **Fig 2.10 C**. Despite the fact that we conducted the experiment three times independently, in one of the repeat experiments, the control tick groups exhibited poorer molting efficiency owing to fungal contamination in our incubator, which may have influenced the statistical outcomes of the entire study. The ability of systemic knockdown ticks to attach and feed on mice was then investigated. Similarly, to our prior findings, both the *IGOBP* dsRNA treated, and control groups are effectively attached to hosts. However, as we observed in our last experiment, they are unable to complete the feeding process **(Fig 2.10 D)**. This confirms that the IGOBP affects engorgement rather than attachment.

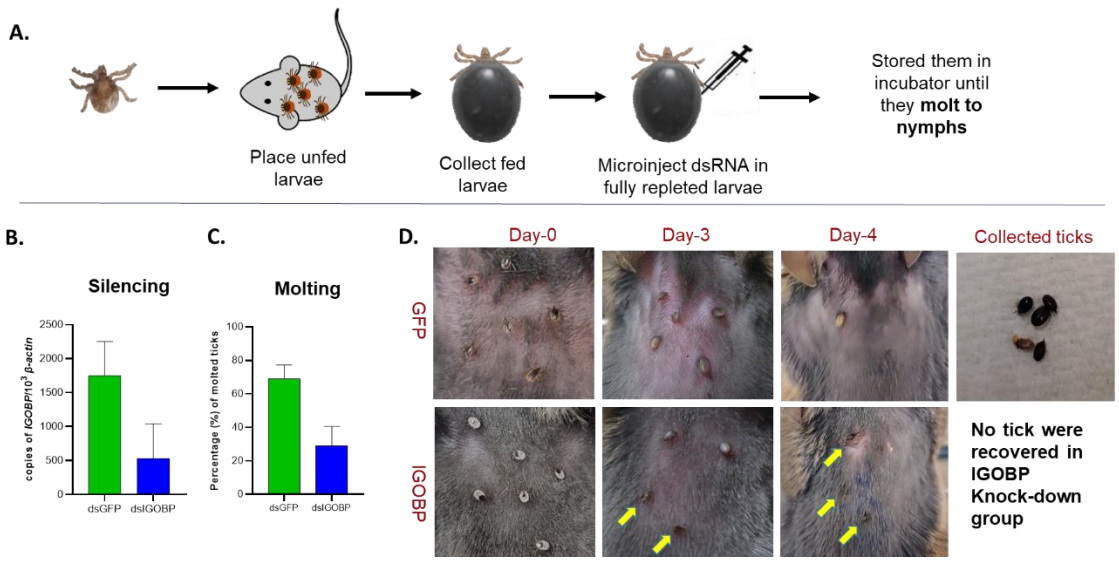


Figure 2.10. Systemic knockdown of *IGOBP* impacts larval molting. (A) The generation of systemic nymphs from fed larvae is shown schematically. Unfed larvae were placed on mice and collected when completely repleted. *GFP* and *IGOBP* dsRNA were microinjected into fully fed larvae and incubated until they were molted to nymphs. **(B)** The impact of RNAi-mediated *IGOBP* knockdown is systemic. The level of *IGOBP* transcripts in molted ticks was lower in the ds*IGOBP* group than in the control group. **(C)** The molting percentage is lower in the *dsIGOBP* treated larvae compared to *dsGFP* treated larvae. **(D)** The knockdown of *IGOBP* in systemic nymphs has no impact on the attachment but does impair the engorgement process. Systemic nymphs were put on mice and their attachment and feeding progress were monitored. Despite successfully attaching ticks in both groups, ds*IGOBP* ticks were unable to complete their bloodmeal, and none of the ticks were repleted. Experiment was repeated at least two times, representative pictures from one of the replicates are shown here.

2.3.11 Feeding impairment of *IGOBP* knockdown ticks is not attributed to itch

As seen in our previous trials, ticks feed normally until the third or fourth day, after which they were unable to continue feeding and were discovered dead. In the absence of IGOBP, it is possible that tick bites cause discomfort or itching in mice, allowing them to detect the ticks and remove them from the skin. To test our

hypothesis, we opted to execute a tick feeding experiment where ticks can engorge on murine skin contained within a protective chamber implanted on the skin, as detailed earlier (Poché et al. 2020)**.** We glued plastic caps (as a tick chamber) to the mice's skin and placed control and *IGOBP* systemic knockdown ticks under the caps to protect them from the mice's access **(Fig 2.11 A)**. We euthanized the animals five days after tick placement, and the ticks were retrieved from the caps. There are fewer ticks discovered alive in the knockdown group than in the control group **(Fig 2.11 B-C)**. Interestingly, the external phenotypes of the few ticks that survived in the knockdown group differ markedly from those of the control ticks **(Fig 2.11 C, bottom panels)**. Full-fed control ticks have a shinier, darker, and dent-free exoskeleton compared to full-fed knockdown ticks. Some of the ticks in the control group started feeding late probably due to accumulated condensations in the capsules. Therefore, taken together, we concluded that despite the presence of caps, ticks are unable to feed, indicating that the itching responses of the hosts are not the major reason of the observed impeded feedings.

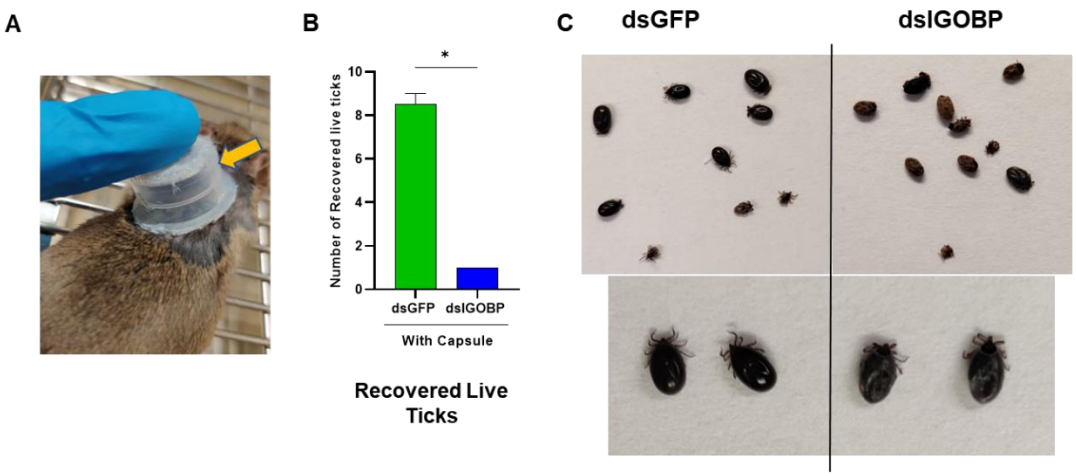


Figure 2.11 Impaired feeding in *IGOBP* knockdown ticks is not due to host itch responses. (A) To protect ticks from mice, plastic caps were adhered to the mice's skin, and ticks were placed under the caps as shown by a yellow arrow. **(B)** Even in the presence of caps, the number of live repleted ticks is lower in the *IGOBP* knockdown group. On day 6 after tick placements, mice were euthanized, and ticks were collected from the capsules. **(C)** A collection of ticks is exhibited. The majority of the ds*IGOBP* ticks looked to be deceased (upper panel). Two live ticks are depicted in the bottom panel, where the appearance of ds*IGOBP* ticks is unusual.

2.2.12 Nymph to Adult molting is impacted by systemic *IGOBP* knockdown

Since IGOBP is implicated in the molting of larvae to nymphs, we sought to determine if it is involved in nymph to adult molting. In the freshly repleted nymphs, we microinjected *IGOBP* and *GFP* dsRNA, and we maintained them in the incubator until they were molted into adult females or males. When compared to the control group, we observe fewer female ticks molt in the ds*IGOBP* group. Thus, we ran the experiment again but with a few minor changes. At the nymphal stage, the sex of ticks are not determined. Therefore, we collected fully repleted nymphs from mice cages and separated them into two groups based on their weight. Since female adult

ticks are larger than male adult ticks, we hypothesized that heavier nymphs molt into females and lighter nymphal ticks metamorphose into males. Therefore, individual ticks were weighed, and ticks weighing less than 3.5 mg were included in the lighter group, whereas heavier groups contained nymphal ticks weighing more than 3.7 mg. Ticks were maintained in the incubator until they completed their molting into adults.

We observed that ticks in the lighter group molted into males and ticks in the heavier group molted into females, as predicted by our hypothesis **(Fig. 2.12 A)**. Therefore, we further proceed to determine the impact of IGOBP knockdown on lighter (male) and heavier (female) ticks' molting. For both groups of ticks, we microinjected control and *IGOBP* dsRNA in the fully repleted nymphal ticks and kept them in an incubator until they were molted into adults **(Fig. 2.12 B)**. This experiment was repeated at least 4 times. Ticks in the control group of males and females and *IGOBP* dsRNA injected males have molted between 71-100%, compared to only 46-56% of the ticks in the *IGOBP* dsRNA injected female group **(Fig. 2.12 C)**. In addition, 100 % of female ticks treated with *IGOBP* dsRNA have aberrant appearances, and a significant number of female ticks die after molting **(Fig 2.12 D-E)**, demonstrating that IGOBP is essential to the physiology of adult female ticks. However, at day 30 post feeding, *IGOBP* knockdown ticks did not exhibit any such changes in body weight **(Fig 2.12 F)**.

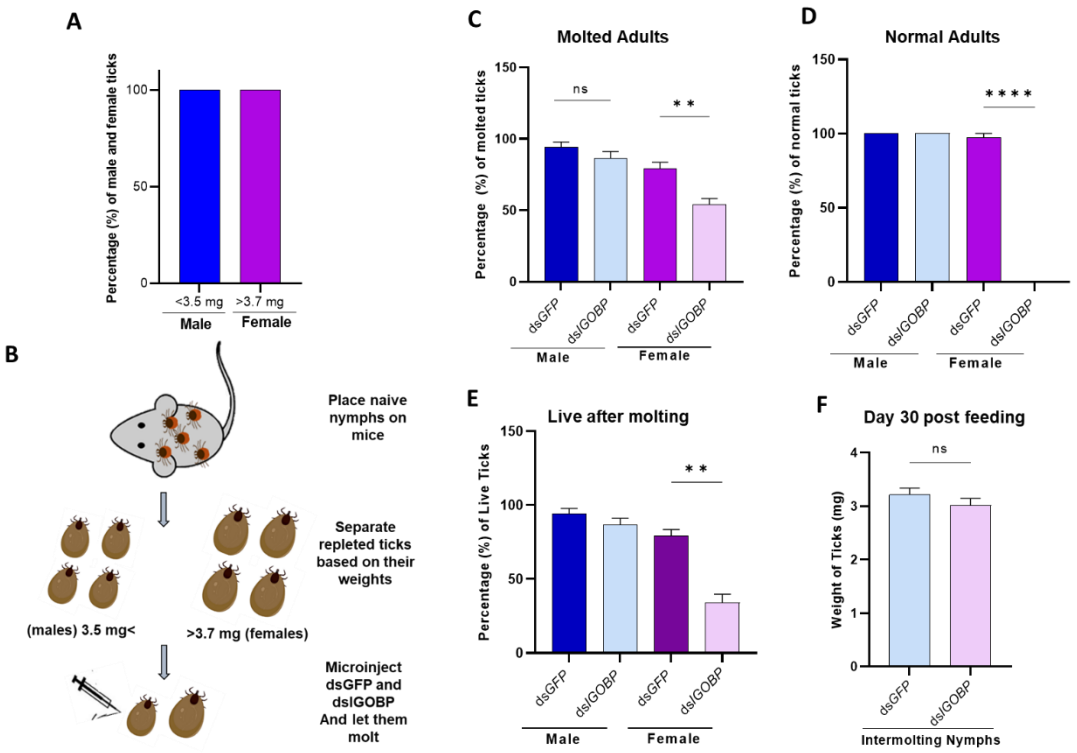


Figure 2.12. *IGOBP* knockdown affects nymph to adult molting. (A) Weight of the fully repleted nymphal ticks suggests whether they will molt to male or female adults. Lighter weighted ticks (< 3.5 mg) molt to males and heavier ticks (> 3.7 mg) molt to females. **(B)** Illustrations depicting the systemic RNAi-mediated experiment in fed nymphs. Nymphs were placed on mice and collected after their bloodmeal was complete. Fully fed nymphs were weighed and divided into two groups (<3.5 mg and > 3.7 mg). dsRNA for *GFP* and *IGOBP* were microinjected into them and maintained in the incubator until they molted into adults. **(C)** Silencing of *IGOBP* influences adult female molting efficiency. After 45-50 days of dsRNA administration in fed nymphs, the number of molted adult male and female ticks was counted. Although males in both the ds*GFP* and ds*IGOBP* groups molted in comparable numbers, female molting efficiency in the ds*IGOBP* group is significantly lower. **(D)**, **(E)** The absence of IGOBP has an effect on female tick fitness. In the ds*IGOBP* group, almost all of the females exhibit aberrant looks, and a significant number of females died. **(F)** The weights of the ticks at the 30-day intermolt period were comparable. Experiment was performed at least 4 times. **p< .01, ****p< .0001. The absence of error bars in diagrams (**A)** and (**D)** is due to the fact that the same proportion (100%) of ticks molted or have normal or atypical phenotypes in each replicate experiment.

2.3.13 *IGOBP*-knockdown introduce anatomical defects

To further understand how IGOBP is involved in tick physiology, we carefully examined the phenotypic abnormalities in the newly molted nymphs and adults. *IGOBP* dsRNA treated systemic nymphs exhibit an opaque appearance, as illustrated in **Fig. 2.13 A**, while the control group appears transparent, and the gut is visible through the cuticles. Furthermore, *IGOBP* knockdown ticks' dorsal abdomen is flat and dented, unlike the control ticks,' as shown in **Fig. 2.13 A** with the yellow arrow. In order to identify what caused these phenotypes, we dissected the ticks, separated the cuticles, and looked at them under a light microscope where we observe that the control tick cuticles could transmit light, but the IGOBP tick cuticles could not, making them darker in appearance **(Fig. 2.13 B)**.

Systemic *IGOBP* silencing in fully repleted nymphs resulted in phenotypic abnormalities, particularly in newly molted females. In comparison to control ticks, the whole female exterior structure appears irregular and is lighter in color **(Fig. 2.13 B, left panels)**. Notably, the female alloscutum appears different while the hard scutum portion does not display any abnormalities. The majority of a male tick's body is covered in hard scutum, which covers any variations, however very little of it is abnormal, such as white or lighter in color, as indicated by the red arrows **(Fig. 2.13 B, right panels)**. We therefore concluded that *IGOBP* knockdown affects the alloscutum but not the scutum, resulting in morphological and likely to be structural abnormalities in the cuticle. Female ticks, which have alloscutum covering the majority of their body parts, grow 100 times their body weight after feeding because

the alloscutum expands rather than the hard sclerotized scutum, whereas male ticks do not feed as much and have a hard skeleton (scutum) covering their bodies that can prevent their major expansion. We infer that IGOBP may be involved, directly or indirectly in cuticle synthesis or expansion during feeding, impacting ecdysis and molting.

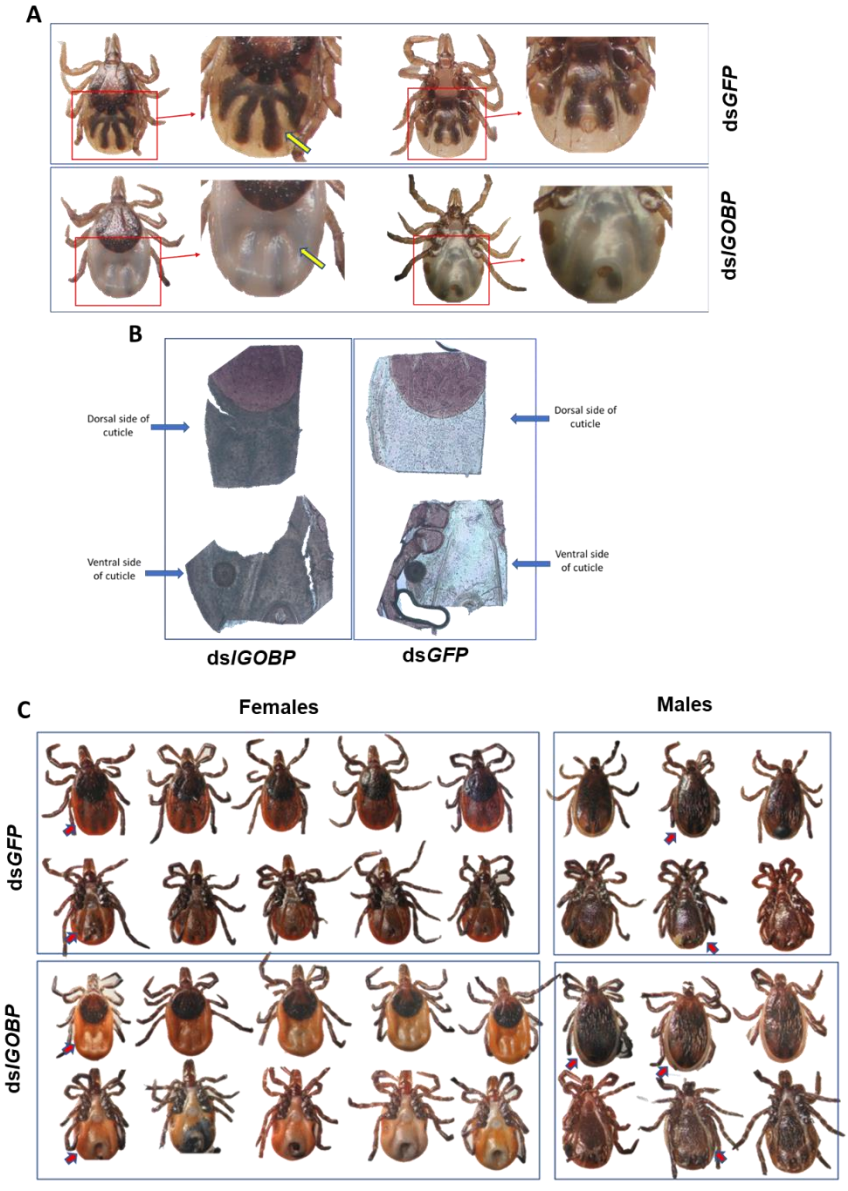


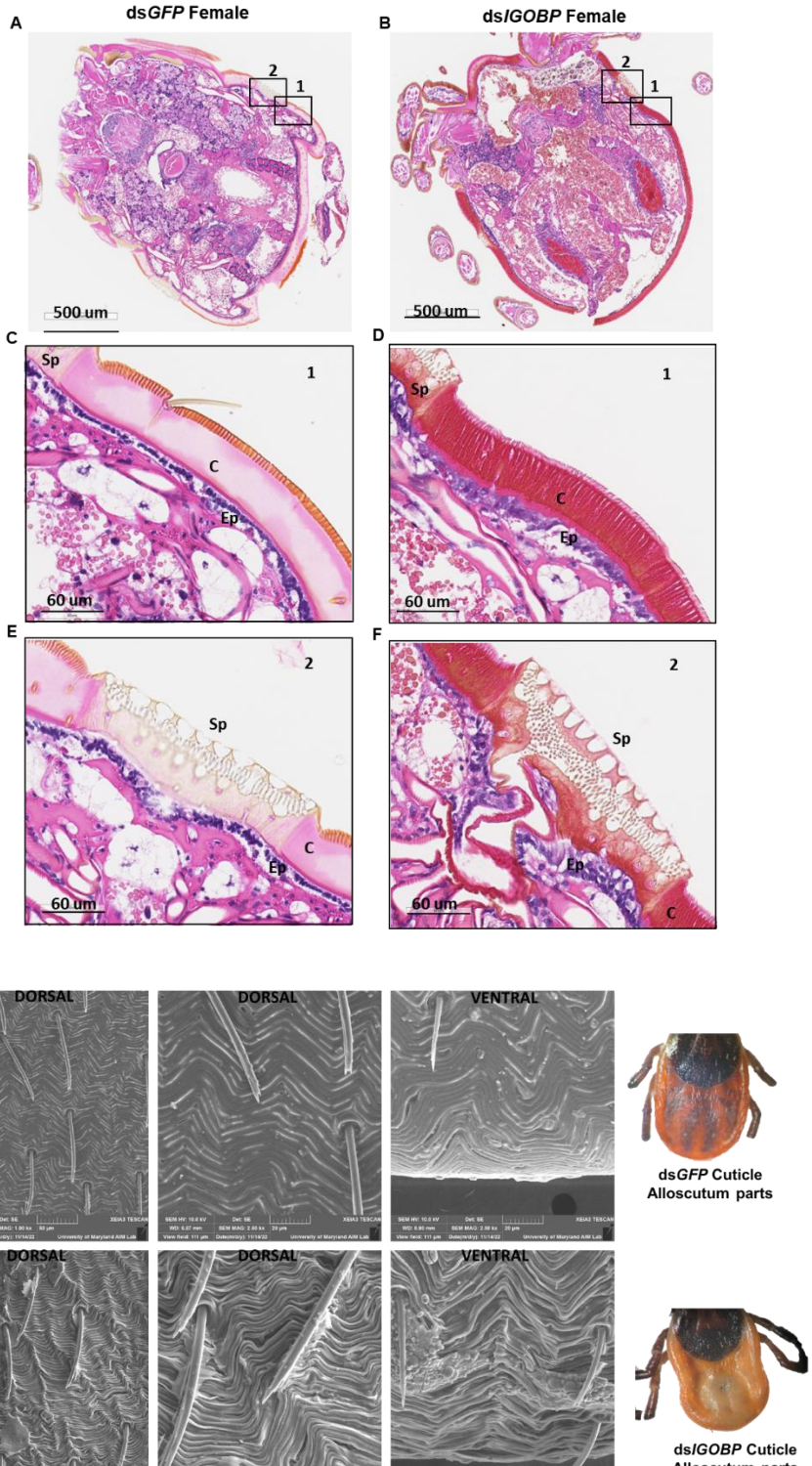
Figure 2.13. Systemic knockdown of *IGOBP* results in morphological abnormalities in molted ticks. (A-C) *IGOBP*-knockdown ticks with severe anatomical abnormalities. Representative images of ticks are shown here. The morphological differences in freshly molted nymphs and adults were investigated. While ds*GFP* ticks are normal (top panels; A and C), ds*IGOBP* nymphs and females have abnormal colors and structures. In panel A, the red squares have been enlarged. The differences between nymphal ticks (A) and adult ticks (B) are represented by yellow and red arrows, respectively. **(B)** The cuticles of ds*IGOBP* nymphs are opaque. Cuticles are isolated and examined using light microscopes. While the cuticle of a control tick could transmit light and so seem transparent, the cuticle of an *IGOBP* knockdown tick could not.

2.2.14 RNAi of *IGOBP* impacts cuticle integrity

We next performed histological (H&E) staining of ds*GFP* and ds*IGOBP* treated female ticks to determine any structural differences between control and *IGOBP* knockdown ticks. Whole tick cross-sections are depicted in **Fig. 2.14 (A-B)** and boxes 1 and 2 indicate regions that are enlarged in **Fig. 2.14 (C-F)**. In the ds*IGOBP* group, the cuticle (C) possesses villi-like structures that may be porous, thereby holding the stain and making the cuticle appear redder **(Fig. 2.14 D)**. In contrast, the control ticks' cuticle has a more solid appearance and retains less stain **(Fig. 2.14 C)**. We also noticed differences in spiracle (Sp) Structures, which refer to the respiratory apertures in the exoskeleton of insects that are connected to the trachea and aid in oxygen intake **(Fig. 2.14 E-F).** However, we could not confirm if there are any such differences in the other organ structures.

In our previous findings, the cuticle exhibits irregularities in the alloscutum, but not in body sections covered with the hard sclerotized scutum, such as mouthparts, top dorsal regions, and legs. Moreover, *IGOBP* dsRNA treated systemic ticks' cuticles are readily breakable (data not shown). Therefore, the effects of RNAi

mediated silencing of *IGOBP* in female cuticles were examined by scanning electron microscopy (SEM) since they had the greatest phenotypic impacts. Images of the exoskeleton surface from dorsal and ventral alloscutum of both nymphs (data not shown) and adult females demonstrate an even structure in the control ticks **(Fig. 2.14 A-B).** In contrast, the alloscutum surface of *IGOBP* knockdown ticks is unevenly pleated. Interestingly, the surface structure in other sclerotized body parts has no differences between *GFP* and *IGOBP* dsRNA treated tick cuticles **(Fig. 2.14 C)**. We also studied cross-sections of systemic nymphs' cuticles to analyze the structure underneath. Control tick cuticle contains well-organized layers as can be seen in **Fig. 2.14 D** whereas, ds*IGOBP* treated ticks lack organized structure and in fact appear porous and hollow. These findings suggest that IGOBP is important for maintaining cuticular integrity.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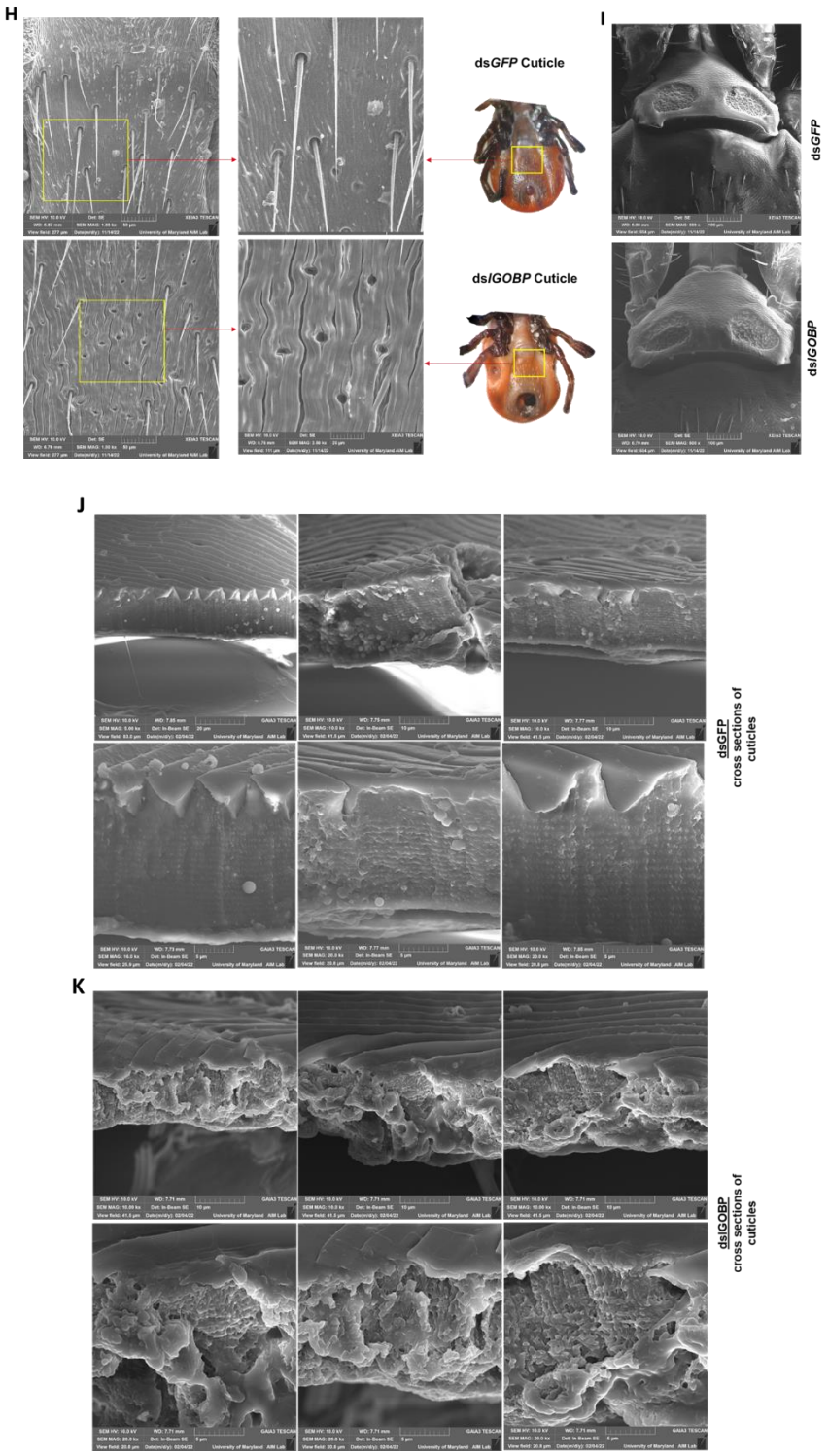
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K
dsGOBP cross sections of cuticles

Figure 2.14. *IGOBP* knockdown affects cuticle integrity. (A-F) H&E staining of cross sections of female ticks. Systemic knockdown female ticks were sectioned and processed for H&E staining. A and B show whole tick cross sections. C and D depict enlarged areas in box 1 from A and B, respectively. E and F show enlarged areas from box 2 in A and B, respectively. C, cuticle; Ep, epidermis; Sp, spiracle. **(G-I)** SEM pictures of the ventral and dorsal sides of female tick. Fully repleted nymphs were microinjected with ds*GFP* or ds*IGOBP* and evaluated for phenotypic differences after molting. *IGOBP* knockdown ticks exhibit altered exoskeleton structures on both the dorsal and ventral sides of the alloscutum (G-H), but not in the areas that are covered with scutum (I) The yellow box areas are enlarged in (H). **(J-K)** SEM images of nymphal cuticle cross sections. *GFP* and *IGOBP* dsRNA were microinjected into fully fed larvae. Cuticles from recently molted nymphs were isolated, cut using sharp blades, and imaged using a scanning electron microscope. ds*GFP* ticks have orderly structures (J), whereas ds*IGOBP* ticks have unorganized hollow structures (K). A-B pictures have been taken with 40X magnifications. Scale bars in A-B is 500 μm; C-F, 60 μm; G, 50 and 20 μm; H, 50 and 20 μm; I, 100 μm, J-K, 20, 10, 5 μm.

2.2.15 IGOBP does not impact ticks' ability to detect humidity and DEET

IGOBP has structural similarities with insect OBPs, thus our next goal is to understand the role of IGOBP in ticks' response to different odorants, repellents, and humidity. To test the attraction of ticks to high humidity, we performed choice chamber experiments which were adapted and modified from earlier studies (Sun et al. 2018b). One side of the choice chamber was prepared with a ddH2O containing a smaller petri dish, and another side was with CaCl2 (desiccant). Before putting the ticks, choice chambers were allowed to equilibrate in room temperature at 25°C, and relative humidity (RH) was measured after two hours. Unfed nymphal ticks were microinjected with *IGOBP* or, *GFP* dsRNA (12 ticks/ group), and each group of ticks was placed in the middle of chambers. Behaviors of two groups of ticks in response to humidity were tested in parallel in two chambers. The humidity choice chamber experiment reveals no behavioral difference between *IGOBP* knockdown ticks and

the control group, indicating that IGOBP may not affect ticks' sensitivity to humidity **(Fig. 2.15 A)**.

RNAi knockdown while reducing transcript copies, does not influence already translated or premade proteins. We anticipated that the presence of previously expressed IGOBP protein (before dsRNA treatment) might obscure the results when *IGOBP* was knocked down in unfed nymphs and used for the choice-chamber experiments the next day. Therefore, we conducted the experiment using systemic female ticks (*GFP* and *IGOBP* dsRNA were microinjected in fully fed nymphs and kept in an incubator until they molt to adults) in order to evaluate humidity sensing. Female ticks were kept in a 50 ml centrifuge tube containing CaCl2 for approximately twenty-four hours before being placed in the choice chambers. Female ticks were used because, in the absence of IGOBP, they have greater observed effects (such as cuticle aberrations, reduced molting efficiency) than those of male ticks. We observed that six out of seven ticks were in the water chamber for both control and knockdown groups, indicating no behavioral differences between ds*GFP* and ds*IGOBP* systemic female ticks **(Fig. 2.15 B)**. Keeping ticks in low-humidity environments may cause dehydration, which may obscure the knockdown impact during behavioral tests. Therefore, further tests must be conducted to determine if IGOBP plays a role in tick humidity sensation in which ticks are placed directly in the choice chambers, without prior incubation in decreased humidity conditions.

Additionally, we observed that even when ticks were kept in reduced humidity settings inside the centrifuge tube containing CaCl2 salt, both groups remained alive

for 24 hours, demonstrating that *IGOBP* knockdown ticks may survive dehydration despite having compromised cuticle integrity (data not shown). However, we have only tested for a duration of 24 hours, future experiments over longer time periods will be needed to determine whether IGOBP is involved in reducing dehydration. We also compared the expression of *IGOBP* transcripts using qRT-PCR in response to dehydration between the control nymphal ticks (were kept in an incubator at 85-99% humidity settings) and desiccated ticks (were kept in low humidity settings for 2 hours and 24 hours) and found comparable expression in all the groups **(**data not shown).

Using a similar choice chamber setup, we tested the response of *IGOBP* knockdown nymphal ticks for relevant odorants (deer hair extracts, not conclusive, data not shown) and repellents (DEET) and found no significant differences in ticks' behavior as well in the presence or absence of DEET **(Fig. 2.15 C)**. Although ticks in both the control and experimental groups were not drawn away from the repellent chambers, they were more likely to be drawn to the ethanol chamber rather than the DEET chambers.

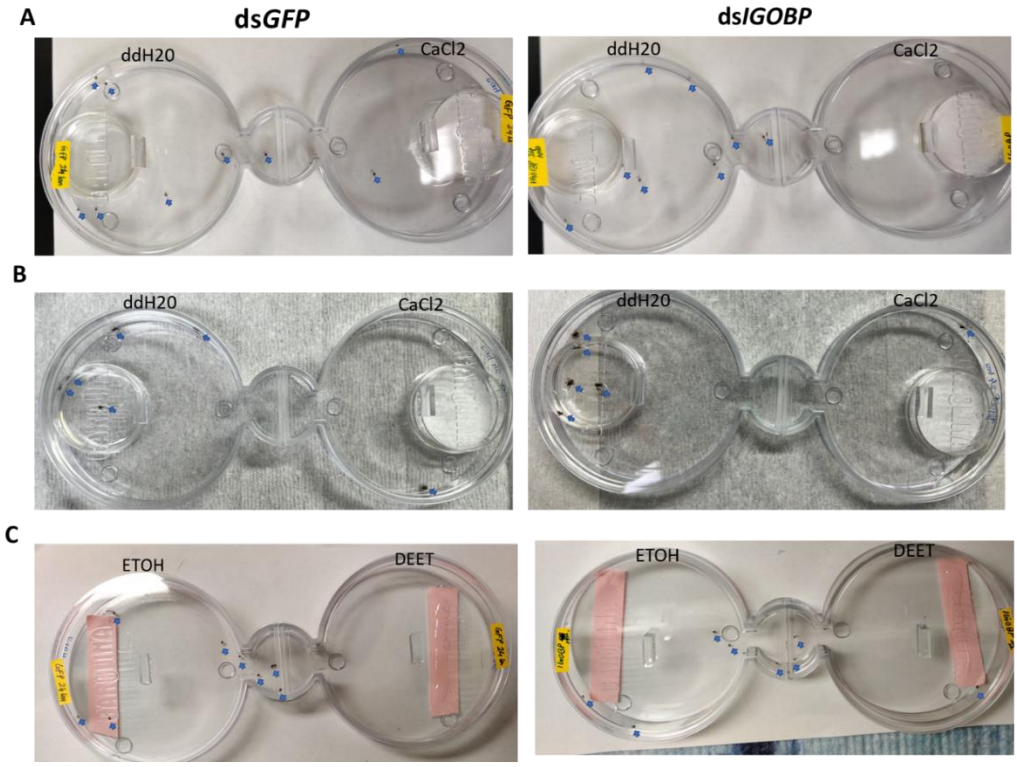

Figure 2.15 Knockdown of *IGOBP* does not impact humidity and DEET sensing. (A-B) Humidity responses are determined using choice chamber experimental setup. ds*IGOBP* and ds*GFP* ticks were placed in the center of two separate choice chambers. Each chamber had a relative humidity (RH) of 18-25% on one side ($CaCl_2$) and 88-90% (ddH2O) on the other. Ticks' responses to humidity were observed by their movement towards the test chambers from the center. Blue arrows indicate individual ticks. *GFP* and *IGOBP* dsRNA treated unfed nymphs were used in panel A. Systemic knockdown unfed females were used in panel B. **(C)** Choice chamber experiments to determine DEET sensing. Each chamber had ethanol (100 %) coated paper tape on one side and DEET coated paper tape on the other side. dsRNA treated unfed nymphal ticks was placed in the center and their movements were observed.

2.4 Discussion

Our study characterizes a novel *I. scapularis* protein, termed as IGOBP. To the best of our knowledge, this is the first tick OBP that is structurally and functionally characterized in such detail. Although we are unable to determine its functions in olfaction, we termed the protein OBP since it closely resembles the insect

OBP in many structural details, including the N terminal signal sequence and 4-6 cysteine residues. Our findings are consistent with prior research on mite OBPs (*V. destructor* and *T. urticae* OBP) demonstrating that these Chelicerata OBPs have comparable structural characteristics (Amigues et al. 2021b) (J. Zhu et al. 2021). Chelicerata OBPs vary from insect OBPs in terms of cysteine pairing (alternative pattern), resulting in distinct three-dimensional folding. Intriguingly, BLAST searches using IGOBP amino acid sequences revealed no other arthropod proteins except Chelicerata. Despite the fact that OBPs are a set of closely related proteins, they appear diverse among insect orders and even within the same species (P. Pelosi et al. 2006). For example, *Drosophila* OBPs have 15% sequence identity with the OBPs of moths and butterflies, and 10-15% with their own OBPs (P. Pelosi et al. 2006). In fact, we noted that BLAST searches did not identify any more *I. Scapularis* homologs. However, two OBP-like proteins, IscaXP 002433530 and IscaXP 002405987, were identified in the forelegs and hindlegs of *I. Scapularis* (Josek et al. 2018b) and have only 25% and 19% sequence identities with IGOBP sequence, respectively. Therefore, we cannot confirm that IGOBP homologs are restricted to Chelicerata on the basis of sequence identities as they might possess structural and functional homology rather than sequence similarities.

OBPs are typically small, water-soluble proteins with hydrophobic ligand-binding sites. The negative Grand average of hydropathicity index (GRAVY) indicates that the 19 kDa IGOBP protein is soluble. IGOBP and VdesOBP1 share 56% of their sequences and superimpose almost similarly with ~0.5 Å r.m.s.d value.

In fact, all the amino acids (hydrophobic, polar non-charged, and charged residues) in the wall of VdesOBP1's binding pocket are conserved in IGOBP, indicating that IGOBP may also contain similar binding cavities. In fact, the majority of the amino acid residues in the walls of the ligand-binding pockets of VdesOBP1 are hydrophobic (Amigues et al. 2021a).. However, the N terminal portions of VdesOBP1 are not ordered in the crystal structure, and the elongated N and C terminal regions form two-stranded pseudo beta sheets. The N and C terminals of the projected AlphaFold IGOBP structure exhibit extremely low pLLDT scores and are disordered as well. In several insect OBPs, the expanded C terminus functions as an additional alpha helix or folds back into the binding pocket in response to a pH shift, acting as a mechanism for ligand removal. However, we do not know whether the C terminal sections of IGOBP and VdesOBP1 have a comparable function. Moreover, it has been observed that OBPs often form dimers (Amigues et al. 2021a), and our BS3 crosslinking, and size exclusion chromatography confirms that IGOBP occurs as multiple oligomeric forms. However, it is uncertain whether this reflects the functional form in vivo.

As obligate hematophagous organisms, ticks are exclusively reliant on blood as energy source which they require to store for a long time. In addition, they lack antennae and proboscis to locate hosts. Tick forelegs include Haller's organs, which resemble insect antennae. Ticks raise their forelegs to sense the presence of hosts and latch to them when they are close range – a behavior well known as tick questing. Haller’s organs have the capacity to detect pheromones, carbon dioxide (CO2) and

humidity, which are crucial for ticks to find their hosts and survive in dry weather. However, without Haller's organs, they still uptake their bloodmeal if they are placed on the hosts (Josek et al. 2018b). Our qRT-PCR data indicate that the expression of the IGOBP gene is highest in the hypostome and legs, which may imply potential involvement in chemosensation. However, the presence of IGOBP in other organs leaves open the possibility that it plays additional roles. Moreover, transcriptomic study of the forelegs and hindlegs of *I. scapularis* by Josek et.al reveals that IGOBP is not unique to forelegs, even though forelegs express *IGOBP* at a greater level than hindlegs (Josek et al. 2018b). In fact, our data show that the expression of this gene increases during feeding and at the end phase of larval molting suggest that it may have roles in tick feeding and development. It is common for OBPs to have roles outside of chemosensation, given that they perform non-traditional tasks such as development, immune responses, and nutrient solubilizations (Paolo Pelosi et al. 2018).

RNAi mediated silencing indicates that IGOBP plays crucial roles in tick physiology and life cycle. The protein is required during feeding and molting. In addition, the knockdown impairs the fitness of adult female ticks. Gene silencing significantly decreased the percentage of survival engorged nymphs and their weights. We confirmed that RNAi is effective and gene expression is lower in all the organs tested in ds*IGOBP* treated groups. Consequently, protein levels are reduced in knockdown ticks, indicating targeted and efficient gene knockdown.

Although IGOBP has structural similarities with OBPs, our studies suggest that this protein may not play roles in many aspects of olfaction, such as the detection of humidity and repellant (such as DEET detection). However, our findings do not confirm conclusively that the IGOBP is not implicated in chemosensation or humidity sensation. Although we employed systemic knockdown (ds*GFP* and ds*IGOBP* were microinjected in fully fed nymphs and let them molt to adult) female ticks for humidity sensing, we exposed them to a low humidity environment for 24 hours before testing their behavior. This may result in extreme thirst, and when placed in the middle of the chambers, both groups of ticks move toward the water. This hypothesis can be supported by two independent studies of *Drosophila* humidity sensations where flies with mutant hygrosensory genes initially exhibited impaired humidity sensing but gravitated toward the humid environment by the end of the test. The authors suggested the behavior may be due to extreme thirst (Sun et al. 2018b) (Liu et al. 2007). Sun et al. also observed that flies with the OBP59a (one of the Drosophila odorant binding proteins) deletion are more resistant to desiccation and survive longer under desiccating conditions (0% RH) than control flies (Sun et al. 2018b). Therefore, additional testing is required in which *IGOBP* knockdown ticks should be examined for humidity sensing without prior exposure to lower humid conditions, as well as assessing the ticks' ability to withstand desiccation for longer durations (more than 24 hours). Although we examined whether *IGOBP* silencing exists in systemic nymphal ticks after molting, but we did not investigate adult ticks. It is reasonable to presume that the *IGOBP* knockdown is still present because of the

aberrant phenotypes and compromised cuticle integrity in females, but we must confirm this.

OBP59a expression is confined to the fly antennae, specifically in hygrosensitive sensilla, in contrast to IGOBP, which is abundantly expressed in different tick tissues. Similarly, the removal of the initial pair of tick legs rendered them incapable of recognizing DEET indicating that the Haller's organ is especially involved in repellent sensing (Carr et al., 2017b). Even though IGOBP expression is highest in the hypostome and legs, it is also present in other organs and saliva. It is possible that IGOBP is involved in these chemo or hygro-sensory pathways alongside other proteins. Consequently, it is highly unlikely that IGOBP has direct effects on hygroreceptions or DEET sensing. However, we have not tested any other odorants or repellents other than DEET. We cannot therefore generalize that IGOBP does not participate in chemosensation, but we can confirm that (despite its structural similarity to OBPs) IGOBP certainly participates in non-conventional pathways other than chemosensation.

IGOBP knockdown ticks are still able to attach to hosts, indicating that they are able to locate adequate attachment sites. In fact, ds*IGOBP* treated nymphs do not have any impairment during the first few days, and they appear and consume blood similarly to the control ticks. The dramatic effects over the last 24-48 hours of feeding suggests a few possibilities; 1) hosts remove ticks as soon as they detect them and, 2) the attached tick’s growth is inhibited during rapid feeding, 3) Or it is possible

that our RNAi approach is unable to completely deplete the IGOBP and a basal level of the protein can fulfill the function.

The saliva of ticks contains a range of proteins that enable them to continue feeding on blood. These include anesthetic substances (such as histamine-binding proteins) that eliminate pain and itch feelings in their hosts (Šimo et al. 2017d; Krause et al. 2009a). Even after recurrent tick infections, laboratory mice do not develop acquired tick resistance as guinea pigs and rabbits do (Schoeler, Manweiler, and Wikel 1999; Krause et al. 2009a). Studies explain that laboratory mice similar to natural reservoir white footed mice generate limited inflammation in response to repeated tick bites, which does not hamper tick feeding (Anderson et al. 2017b). However, in the case of guinea pigs, repeated tick feeding causes strong inflammatory responses and highly elevated pain, and itching responses cause the host to expel or kill the ticks (Narasimhan et al. 2007; Krause et al. 2009a; Anderson et al. 2017a). In fact, tick saliva contains members of the lipocalins (binds and transports small hydrophobic molecules) protein family, histamine-binding proteins that sequester histamine, lipids, serotonin, and prostanoids as a means of evading the host immune system by decreasing inflammation at tick bite sites (Kim et al. 2016a) (Kitsou, Fikrig, and Pal 2021). Being structurally similar to odorant binding protein (OBPs typically bind and transport hydrophobic ligands), it is possible that IGOBP could trap host immune factors to impede host immune responses. To evaluate if mice scratching dislodges attached ticks in our case, we protected the ticks from the mice using plastic capsules. However, ticks were still found to be dead, showing that mice

itching was not the primary reason. In addition, we did not see any delayed feeding, which we would have anticipated if their blood intake were reduced or slowed.

Unlike other hematophagous insects, such as mosquitoes, the tick's digestive process begins intracellularly while feeding. Images of ticks' midgut epithelial cells reveal that they undergo a series of alterations during feeding (Franta et al. 2010d). In fact, blood digestion accelerates rapidly near the end of slow feeding phase and reaches its peak in fully fed ticks (Franta et al. 2010d) (Sojka et al. 2013b). Although we have not extensively studied the gut structures and the PM structures in knockdown ticks, H&E staining does not show any striking differences in the gut structures between control and experimental groups.

Male ticks do not consume bloodmeal as much as female ticks (100 times their body weight); hence their bodies do not grow and are mostly coated with a rigid scutum that does not expand significantly in volume. In contrast, two-thirds of the female tick's body is coated in soft, flexible alloscutum, which expands greatly while feeding. Interestingly, we discovered that the cuticles of *IGOBP* knockdown ticks are malformed or aberrant and adult females with defective alloscutum exhibit impaired fitness. Our observations suggest that IGOBP might be involved in alloscutum formation or expansion during molting or feeding. Thus, we processed adult female ticks for H&E staining, where we observed that the cuticle and spiracle structures of knockdown ticks are porous and compromised. Spiracles are the openings for the trachea through which air exchanges occur. Studies indicate that during the feeding process, the trachea stretches to accommodate the massive size of feeding ticks

(Starck et al. 2018b). Therefore, it is possible that tracheal expansion does not occur during feeding in *IGOBP* knockdown ticks, impairing the process of engorgement. However, we did not directly test this occurrence, which will be essential in the future to validate.

Scanning electron microscopy images reveal that the hard scutum sections, such as the mouthparts and legs, are comparable between the two groups, whereas the alloscutum reveals substantial changes in the *IGOBP* dsRNA treated ticks, further supporting our hypothesis that IGOBP may play a role in alloscutum expansion during feeding. Cuticle hardness depends on many factors, for example, types of proteins, chitin and protein ratios, amount of sclerotization. The structural disorganizations in the cuticle elicited by *IGOBP* dsRNA treatment may cause the cuticle brittle, fragile, or incapable of expanding or holding considerable amounts of bloodmeal. Additionally, significant proportions of *IGOBP* dsRNA treated adult ticks do not survive even after molting, probably due to a deformed cuticle, as was also found in *Anthonomus grandis*, the cotton boll weevil (Firmino et al. 2020). Cuticle protects insects against physical injury, chemical damage, infections, and water loss (K. Y. Zhu et al., 2016) (Pesch et al. 2016). Therefore, a greater percentage of dying females could also be the result of water loss or bacterial or fungal infections.

In summary, to the best of our knowledge, this study is the first study to define any tick protein structurally and functionally with homology to insect OBP. In addition, our work investigates a novel *I. scapularis* protein and sheds light on hematophagy and developmental processes, expanding our understanding of tick biology. Our

findings demonstrate that silencing IGOBP results in lethality at all stages of the tick life cycle examined. Thus, IGOBP may serve as a target for the development of innovative tick control methods, such as an anti-tick vaccine.

Chapter 3: Understanding Molecular Mechanism Underlying IGOBP's functions

3.1 Introduction

The blacklegged tick *I. scapularis* is a three-host tick and they vector diverse human pathogens including the agent of Lyme disease, *B. burgdorferi*. During a single bloodmeal, ticks may consume up to one hundred times their initial weight (Perner et al. 2016). This enormous growth is aided by the rapid extension of their exoskeleton, expansion of the digestive system, including enlargement of the gut lumen, and dramatic proliferation of gut cells (Agyei and Runham 1995). In contrast to other model organisms such as *Drosophila*, little is known about molecular mechanism of tick physiology and development. Although gene functions in ticks are studied using RNA interference, gene editing approaches such as CRISPR-CAS9 remained technically challenging in ticks owing to a number of inherent constraints, including a long (two to three year) life cycle, intrauterine egg development, injection difficulties with eggs, and multistage cells (Sharma et al. 2020). Nonetheless, the *I. scapularis* tick genome was one of the first to be sequenced among tick species which has been re-sequenced and re-assembled in recent years (Hill and Wikel 2005b; Gulia-Nuss et al. 2016a; J. R. Miller et al. 2018; De et al. 2023). This resulted in the discovery of several genes, which, in conjunction with RNAi and omics tools, made it feasible to comprehend the functions of numerous genes and proteins in tick biology.

IGOBP, a 19 kDa protein has structural similarities with insect and Chelicerata OBPs. This protein is upregulated during feeding and first identified in tick gut suggesting roles during bloodmeal uptake. However, it is present in other extra-gut organs in ticks and in fact more highly detectable in the hypostome, legs, salivary glands, and carcasses. IGOBP is also found in saliva proteome (Kim et al. 2016b) at different time points during the feeding suggesting that the protein is secreted into the host bite sites. Although it is unclear if IGOBP has roles in odorant sensations, silencing of *IGOBP* does have dramatic effects resulting in impaired tick feeding and molting, abnormal cuticle formation and female ticks' fitness probably due to impaired cuticular structures.

The tick genome features redundancy in gene counts and repetitive sequences (De et al. 2023) (Jia et al. 2020). The fact that silencing a single gene, which is expressed in multiple organs, has profound impacts on complex biological processes, such as tick feeding and development, suggests that IGOBP may play a role in multiple cellular pathways. Consequently, deciphering the molecular mechanism of IGOBP will enhance our knowledge of the biology of tick feeding, tick development, and the tick-host interface as well as the possible discovery of anti-tick vaccine targets. In this study, we used RNA sequencing (RNA-seq) to identify molecular components and their functions that regulate *IGOBP* gene expression, or vice versa. Several downregulated and upregulated genes involved in diverse processes or functions, such as ecdysone metabolism, development, heme binding activity, and transporter activity, are identified. IGOBP was identified as the most downregulated

gene in the ds*IGOBP* ticks compared to the ticks in the control group, indicating effective knockdown.

To gain cues and a better understanding of IGOBP function, we chose a few downregulated genes and attempted to determine their roles via silencing their expression using the RNA interference (RNAi) mechanism. Although we were unable to confirm their roles in the tick life cycle, our study identifies a number of potential genes involved in the IGOBP functions governing tick hematophagy, development, and cuticle integrity, and serve as potential vaccine targets to prevent tick infestation.

3.2 Materials and Methods

3.2.1 Mice, ticks, bacteria

A low passage *B. burgdorferi* isolate B31-A3 has been used in this study (Yang et al. 2014). Female C3H/HeN mice (four to six weeks) were bought from Charles River Laboratories or Jackson Laboratories. The nymphal and adult *I. scapularis* ticks that were used in this research work originated from a colony that is maintained in our laboratory. All animal studies were conducted, in compliance with the guidelines and approval of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Maryland, College Park.

3.2.2 RNA interference (RNAi) and tick manipulations

RNAi mediated gene silencing was performed as previously described (Rana et al. 2023). Briefly, the targeted sequences were amplified with sets of primers that

contain the T7 promoter sequence (primer list in **Table 2).** dsRNA for each gene were prepared and purified using MEGAscript RNAi kit, Invitrogen. About 4 µl of the dsRNA (3-4 mg/ml) was loaded into a capillary tube and was microinjected into the ticks through their anal pores, as detailed (Kariu et al. 2011). Ticks were placed on mice the after overnight incubation. Systemic RNAi silencing was performed as detailed in chapter 2. dsRNA (3-4 mg/ml) were microinjected for each individual gene in the repleted larvae or nymphs using capillary tubes and kept them in the incubator until they molt into nymphs and adults, respectively.

3.2.3 RNA Isolation and PCR

Total RNA was extracted from ticks using TRIzol (Invitrogen) and reverse transcribed to cDNA as described previously (De et al. 2023) and in chapter 2. 5-10 ticks were collected at 48 hours and were individually processed and assessed for gene silencing using qRT-PCR analysis and normalized to tick *β-actin* transcripts. Primers unique to the target dsRNA sequence's upstream or downstream regions were used for the qRT-PCR (**Table 2**).

3.2.4 RNA-Seq and analyses

dsRNA (3.5 mg/ml) for *GFP* and *IGOBP* were microinjected in unfed nymphal ticks and placed on naïve mice. At 48 hours ticks were collected and RNA was isolated from each tick separately followed by checking knockdown by qRT-PCR. 3 pooled samples were prepared by mixing RNA samples from 4 ticks (4 ticks/ pool; 3 pools/ group) and the samples were then submitted for Illumina polyA

enriched mRNA-Seq library preparation and sequenced using Illumina NovaSeq sequencing at the Institute for Genome Sciences, University of Maryland, School, of Medicine. The *I. scapularis* transcript data were downloaded from VectorBase database and utilized as the reference sequence (https://vectorbase.org/vectorbase/app/) (Giraldo-Calderón et al. 2022). Standard software was used to match the sequencer's reads with the reference transcriptome. The counts produced by three biological replicates of each treatment were analyzed using DESeq2 and the log fold differences were calculated, and the most differentially expressed genes were identified using the p-value and the padj (adjusted p-value) of p 0.05. Gene Ontology (GO) enrichment analysis were performed by DAVID and QuickGO.

3.2.6 Statistical Analysis

The statistical significance of the difference between the mean values of groups was analyzed using the two-tailed Mann–Whitney U test. Results were analyzed using GraphPad Prism v9 (GraphPad Software Inc.); $P < 0.05$ was considered significant.

Table 2. Oligonucleotide primers for chapter 3

Primer Name/Purpose	**Sequence 5′-3′**
Forward primer for RNAi of *IGOBP*	TAATACGACTCACTATAGGGATGGTGGCATTCAAGGTGTC

Reverse primer for RNAi of *IGOBP*	TAATACGACTCACTATAGGGGTTCAGGATCTCGGTCCTGG
Forward Primer for RNAi of *GPX*	TAATACGACTCACTATAGGGCATCTCCAAGAAGCTCTCCA
Reverse Primer for RNAi of *GPX*	TAATACGACTCACTATAGGGCTGCTTCTCCTTCAGGTACT
Forward Primer for RNAi of *LDLR*	TAATACGACTCACTATAGGGTCGACTGAAGTTCCTTTGAC
Reverse Primer for RNAi of *LDLR*	TAATACGACTCACTATAGGGGAGCCTCTGGCGATGTTT
Forward primer for RNAi of *3A14*	TAATACGACTCACTATAGGGGCGAACAGACGGCGAAGGTGATT
Reverse primer for RNAi of *3A14*	TAATACGACTCACTATAGGGGAAAGCCAACTTCGGGATACT
Forward primer for RNAi of *3A24*	TAATACGACTCACTATAGGGCCTTTCCGAAGCTCCCTTATAGGAC
Reverse primer for RNAi of *3A24*	TAATACGACTCACTATAGGGGCGAAGGTCAATGACCTTGCTTAAG
Forward primer for quantitative PCR of *IGOBP*	AGCACGACGAGTGCAAGA
Reverse primer for quantitative PCR of *IGOBP*	CTGGGGATGTGGTAGTCCA
Forward primer for quantitative PCR of Tick *β-actin*	GGTATCGTGCTCGACTC
Forward primer for quantitative PCR of Tick *β-actin*	ATCAGGTAGTCGGTCAGG
Forward primer for quantitative PCR of *GPX*	CTTCCTCTTCAACGCCATCA
Reverse primer for quantitative PCR of *GPX*	GAGAAGGTCTGCTTCGATTGT
Forward primer for RNAi of *GFP*	TAATACGACTCACTATAGGGAGAGCGACGTAAACGGCCACAAGTT
Reverse primer for RNAi of *GFP*	TAATACGACTCACTATAGGGAGACGGGTCTTGTAGTTGCCGTC

Forward primer for quantitative PCR of *LDLR*	CCTCATCTTGGTCGGTGTT
Reverse primer for quantitative PCR of *LDLR*	CGACGGATTGTTTAGGATCAAAG
Forward primer for quantitative PCR of *ACSF2*	AGATCGTCGATTCGCAAGG
Reverse primer for quantitative PCR of *ACSF2*	CTTGTCGTCCTGCTTCAGAT
Forward primer for quantitative PCR of *3A14*	CAGGCTTGCTTATCTCGATCTT
Reverse primer for quantitative PCR of *3A14*	CGTACTGTCCAAGAGGCATTT
Forward primer for quantitative PCR of *3A24*	GTACAGGGTCACCACATCTAAC
Reverse primer for quantitative PCR of *3A24*	GAGCTCTAGGTTGACTTCAATACA
Forward primer for quantitative PCR of *SAM*	CGAGGCGGAGAGTCTCATAA
Reverse primer for quantitative PCR of *SAM*	GGGCCATAAGCAGGAAGAAT

3.3 Results

3.3.1 *IGOBP* regulated genes

In our previous studies, we find out that IGOBP has roles in tick hematophagy, development and maintaining cuticle integrity**.** To further understand the mechanism

behind IGOBP's functions in tick biology, we compared the transcriptomic profiles of ds*GFP* (control) and ds*IGOBP*-treated ticks to identify additional genes whose expression was linked with *IGOBP*. RNA was isolated from the 48 hours fed dsRNA treated ticks and was assessed for effective knockdown by qRT-PCR before submitting for RNA-sequencing **(Fig. 3.1 A)**. To map the sequences, a VectorBase reference genome for *I. scapularis* was used (https://vectorbase.org/vectorbase/app/) (Giraldo-Calderón et al. 2022). To determine the genes regulated by IGOBP, mapped reads from control and *IGOBP* knockdown samples were analyzed by DGE using the standard tool. The MA plot displays the differentially expressed genes in *IGOBP* dsRNA injected tick samples in comparison to *GFP* dsRNA treated samples. Red dots represent the genes with varying degrees of expression levels, with *IGOBP* being the most downregulated gene **(Fig. 3.1 B).** We narrowed the list of the genes that were up- and down-regulated based on the adjusted p-value with less than 0.05 and log fold differences with equal or greater than 0.6.

In accordance with the aforementioned criteria, we selected 104 up-regulated genes and 73 down-regulated genes and categorized with Gene ontology (GO) keywords onto cellular component, molecular function and biological Process using the DAVID tool (https://david.ncifcrf.gov/) (Huang et al. 2007) (Tang et al. 2021). Downregulated genes have cellular components enrichment in the membrane location. Enrichment for biological process is observed in the biosynthetic and metabolic processes (including the metabolism and biosynthesis of steroids, ecdysone, and cellular hormone), developmental processes including larval or pupal

development, post-embryonic development, and cell cycle. Upregulated genes are involved in the carnitine biosynthetic process, extracellular matrix organizations, fatty acid biosynthetic process, production of siRNA involved in RNA interference. Extracellular regions (12), comprising extracellular matrix (3) and extracellular space (5), are the predominant areas of GO enrichment in cellular compartment for the upregulated genes. Molecular function includes dioxygenase activity and transporter activity.

To further evaluate, we used the VectorBase database to manually extract each gene's Uniprot ID and used QuickGO program (https://www.ebi.ac.uk/QuickGO/annotations) to examine GO keywords (Binns et al. 2009). Twenty of the 31 gene products for downregulated genes are membrane-associated, whereas four are located in the nucleus and four in the cytoplasm **(Fig. 3.2 A)**. Out of the 73 genes that were downregulated, 38 were annotated for molecular function. Of these, 13 genes are involved in oxidoreductase activity, 8 genes in heme binding, iron binding, and monooxygenase activity, 6 genes in transferase activity, and 3 genes in transmembrane transporter activity **(Fig. 3.2 B)**. Additionally, there is RNA binding, structural constituent of cuticle, methyltransferase activity and hydrolase activity, among others. A set of 22 genes are involved in a variety of biological processes, including 4 genes involved in transmembrane transport, 2 genes involved in protein transport, a single gene involved in cellular oxidant detoxification, 2 genes involved in chromatin organizations **(Fig. 3.2 C)**.

A set of 49 genes are annotated for cellular components in the GO analysis of upregulated genes, of which 17 genes are in the membrane, 7 genes are in the nucleus, 10 genes are in the extracellular region, and 6 genes are in the cytoplasm **(Fig. 3.2 D)**. Among the 55 gene products, 11 genes are involved in the molecular function of oxidoreductase activity, 12 genes in hydrolase activity, 9 genes in transferase activity, 10 genes in metal ion binding, 3 genes in serine-type endopeptidase activity, and 4 genes in DNA-binding transcription factor activity **(Fig. 3.2 E)**. Other molecular function activities include binding of iron and zinc ions, glucose-6-phosphatase, metalloendopeptidase activity, fatty acid synthase activity. 44 genes are designated for biological processes, with 4 involved in transmembrane transport, 4 in transcriptional control, 3 in proteolysis, 2 in lipid metabolism and 2 in siRNA processing among others **(Fig. 3.2 F)**.

Next, we wanted to validate the differential gene expression pattern by qRT-PCR. Five genes were selected based on their differential expression levels. *LDLR, GPX, SAM, and 3A14* have log fold changes greater than one and *ACSF2* has a log fold change less than 0.5 in the RNA-Seq results. cDNA samples were first prepared from the 48-hour-fed control ticks and *IGOBP* knockdown ticks, and *IGOBP* silencing was confirmed before analyzing expression of the other genes. *IGOBP* has been efficiently silenced each time as shown by one of the representative figures (**Fig 3.3**). Using the same cDNA samples, we next evaluated the expression of the selected genes. Expression of all five genes is lower in the *IGOBP* knockdown ticks than in the control group, with *LDLR* transcript levels being significantly reduced and *ACSF2*

having the least amount of change, corroborating the RNA-sequencing data on differential expression levels **(Fig. 3.3)**.

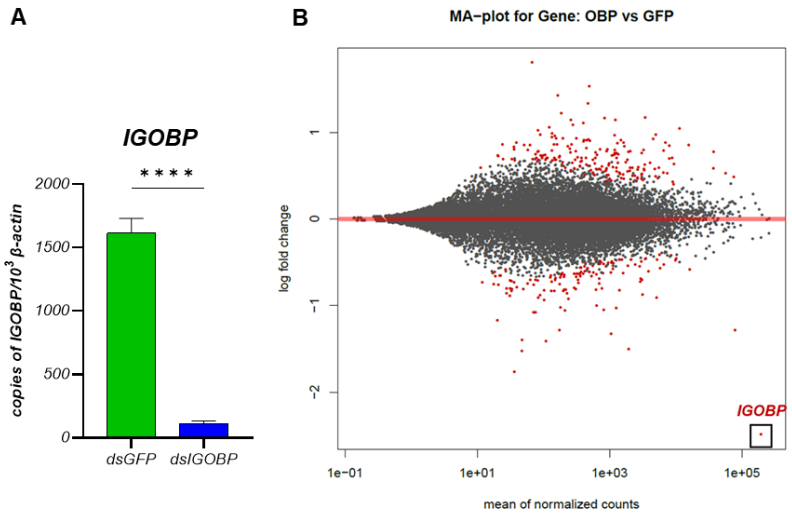

Figure. 3.1. Differentially expressed genes. (A) *IGOBP* is effectively downregulated by RNAi. Ticks were treated with dsRNA for *IGOBP* and *GFP* and analyzed for knockdown by qRT-PCR before submitting the samples for RNA-Seq. ****p<0.0001 **(B)** MA plot showing differentially expressed genes by RNA-Seq. The log 2-fold changes show the mean expression level (y-axis) normalized to the read counts (x-axis) for each gene. Red dots indicate up or down-regulated genes in the IGOBP group in comparison to GFP group. The dot inside the square box on the right corner of the MA plot represents *IGOBP*.

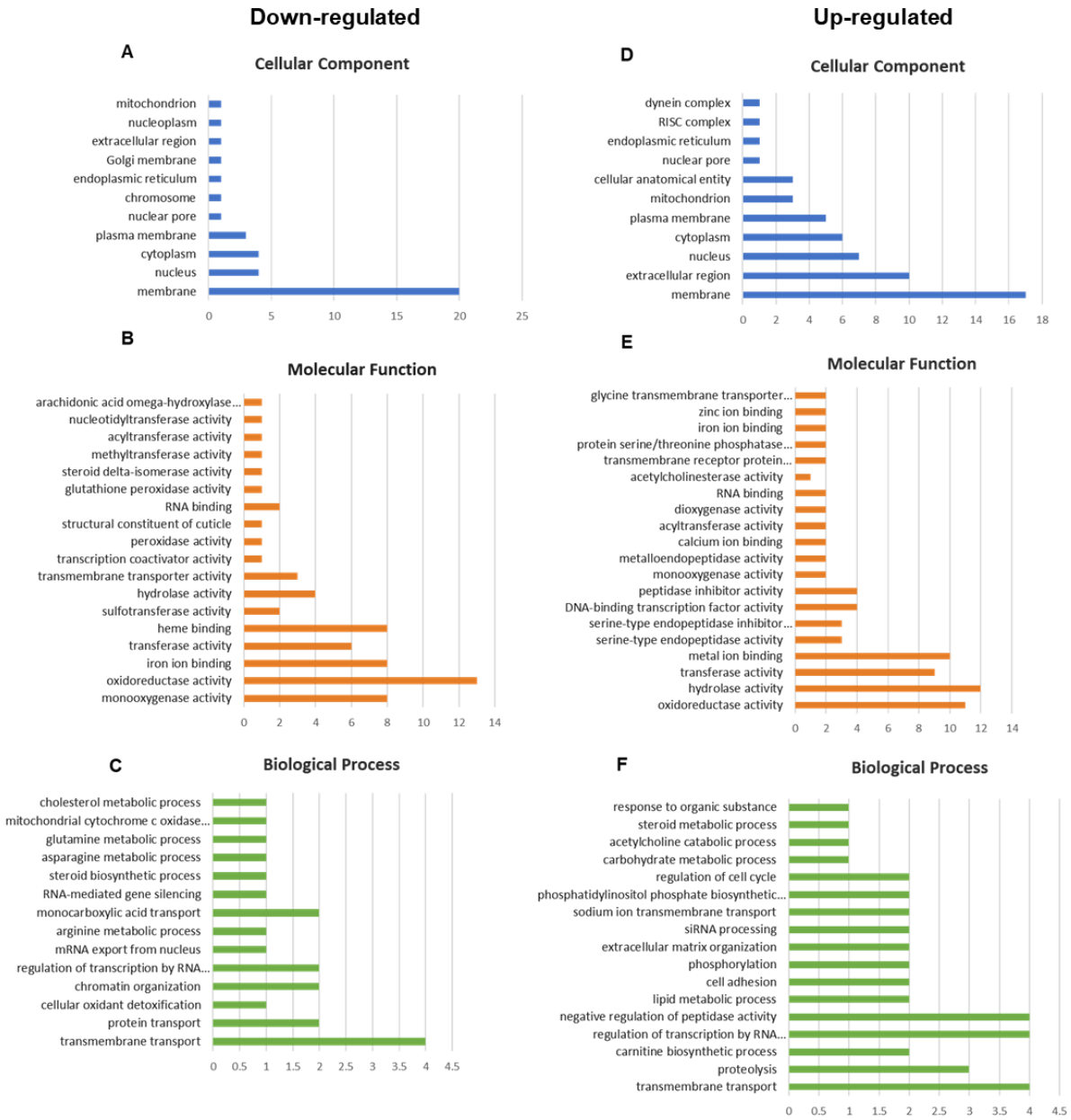

Figure. 3.2. Bar chart representation of gene ontology (GO) for differentially expressed genes in RNA-Seq. When compared to control 48-hour-fed nymphal ticks, gene expressions for *IGOBP* knockdown ticks were shown to be up- or down-regulated. The parameters for the genes considered for GO analysis are ≥ 0.6-fold differences and an adjusted p-value of < 0.05. **A-C** represents cellular component, molecular function and biological process for down-regulated genes and **D-F** shows the same for up-regulated genes.

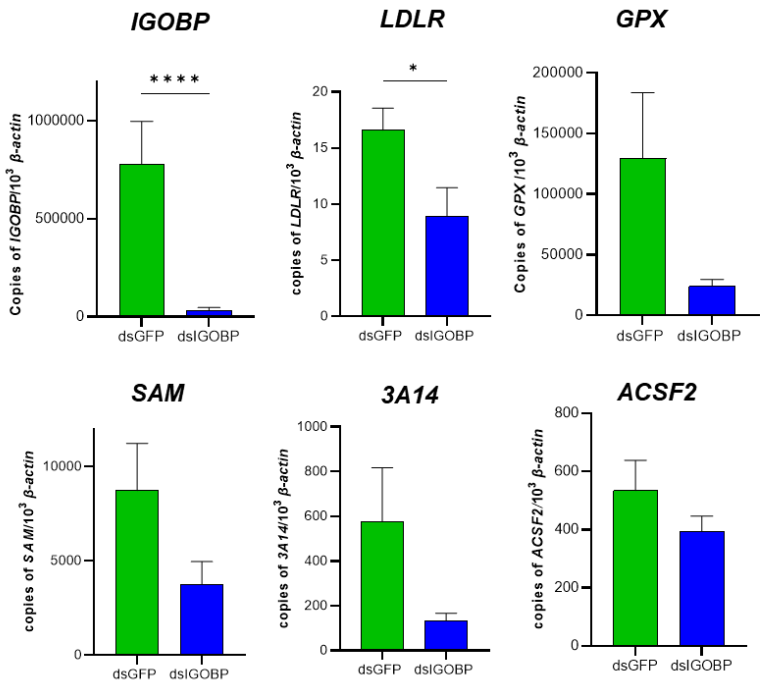

Figure 3.3. qRT-PCR validation of selected genes identified by RNA-Seq analysis. Analysis of *IGOBP* silencing in tick cDNA samples revealed that *IGOBP* expression is significantly lower in the knockdown group. The expression of other genes was investigated using the same cDNA samples. For each group, 7–10 ticks were examined, and each gene's expression was tested twice. "****" and "*" denotes significance with $p < 0.0001$ and $p < 0.05$, respectively.

3.3.2 Roles of IGOBP regulated genes in tick physiology

To further determine the underlying mechanism of IGOBP's roles, four genes are chosen based on their predicted roles and most dramatic expression differences between *IGOBP* dsRNA-treated and control ticks. Two of these are recognized in the VectorBase database as cytochrome P450s (CYTP450), while the other two are recognized as phospholipid-hydroperoxide glutathione peroxidase (PHGPx) and low-density lipoprotein receptor apical endosomal glycoprotein (LDLR). Blast searches

reveal that all four of them have homologs in other hard ticks and are identified as CYTP450 3A14 like protein, CYTP450 3A24 like protein, glutathione peroxide (GPX), and MAM and LDL-receptor class A domain-containing protein (LDLR) respectively. CYTP450s are monooxygenase and heme binding proteins that are involved in a wide range of reactions, including the ecdysone biosynthetic pathways, development, cuticle formation, and detoxifications of toxic compound (Davies et al. 2006; Sztal et al. 2012a; Rewitz et al. 2006). GPXs act as antioxidants by scavenging harmful oxidative radicals in cells and cell membranes and may contribute to acaricide resistance (Cossío-Bayúgar, Miranda, and Holman 2005; Mittapalli, Neal, and Shukle 2007). LDLR is a lipid transporter and helps in lipid or cholesterol trafficking in insect organs (Ravikumar and Vijayaprakash 2013).

RNAi-mediated silencing was next conducted on these selected genes to elucidate their functions in tick physiology. Portions of their coding regions were amplified, and dsRNA was prepared followed by microinjection in the unfed nymphs. dsRNA treated ticks were placed on mice and some of the ticks were removed at 48 hours to analyze knockdown by qRT-PCR **(Fig. 3.4).** *LDLR* expression levels were significantly reduced in the LDLR dsRNA treated ticks, indicating that RNA interference was effective **(Fig. 3.4)**. Although, knockdown of other tested genes is not statistically significant, the knockdown group exhibited relatively reduced gene expression **(Fig. 3.4)**. Number of repleted ticks and their weights were measured at the end of the feeding, where no effects were observed (data not shown). We injected dsRNA into newly repleted larvae and nymphs to conduct systemic RNAi silencing

and there was no effect in development (data not shown). Tick physiology was unaltered for the rest of the gene as well (data not shown). However, silencing was not effective significantly for several genes (*3A14*,*3A24*, *GPX*) and thus cannot conclude the significance of these genes in tick biology **(Fig. 3.4)**.

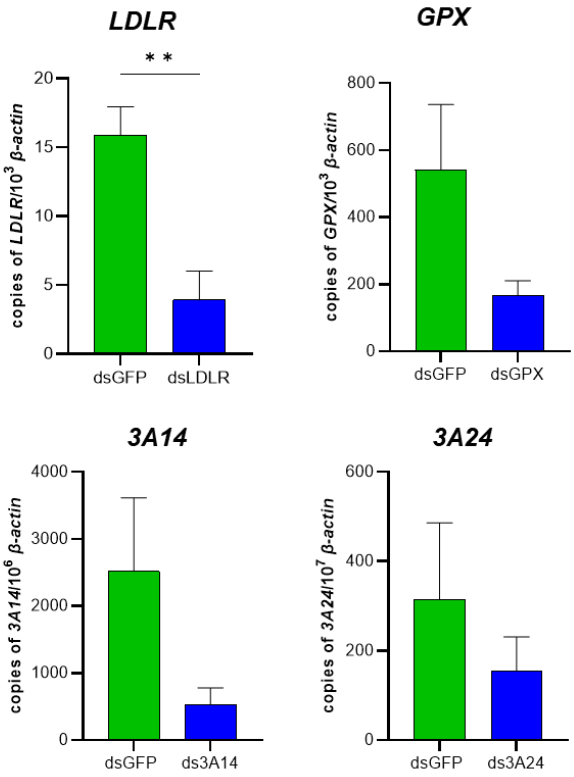

Figure 3.4. Candidate genes are knocked down by RNA interference in nymphs. For each of the selected genes, dsRNA was prepared and microinjected into the nymphs. Ticks that had been fed for 48 hours were collected and their gene expression was examined. Significant downregulation of LDLR (**p< .01) indicated efficient knockdown. Although there was no significant downregulation in the expression of *GPX*, *3A14*, or *3A24*, the knockdown groups had lower expression patterns. 5-7 ticks in each group were used to check knockdown of target genes.

3.4 Discussion

Our previous studies have shown that the IGOBP is essential throughout ticks' life cycle. In this study, experiments were conducted to understand the underlying mechanisms of IGOBP's functions. Using RNA-Seq of control *GFP* and *IGOBP*-knockdown ticks, we discovered that numerous genes express differentially in the absence of *IGOBP*. The DAVID tool's GO analysis indicates that a number of genes that are downregulated in the *IGOBP* knockdown ticks are related to the metabolism of steroid hormones. Although DAVID tool has been used in other studies to analyze *I. scapularis* transcriptome (Tang et al. 2021), very few genes were annotated in our case. Thus, we used QuickGO program to analyze our data in detail. Several of the downregulated genes belong to CYTP450 family members. CYTP450s are heme-containing proteins that belong to one of the most prevalent protein families act as detoxifying agents and plays roles in metabolism of xenobiotic, ecdysteroids and juvenile hormones (Feyereisen 1999) (Sztal et al. 2012b). In insects, CYTP450s catalyze the conversion of 20E from cholesterol through several stages, which controls insect molting and metamorphosis (Iga and Kataoka 2012). Mutations in several of the known CYTP450 genes in *Drosophila* result in malformed development (Warren et al. 2002). Despite the fact that certain homologs (annotated as neverland, shroud, shade, shadow, disembodied) have been found in *I. scapularis*, many others remain unidentified and uncharacterized (O'Neal et al. 2022) (Gulia-Nuss et al. 2016a). Several CYTP450s were downregulated including 3A14 and 3A24 with 1.5 and 1.3 log fold differences respectively in our RNA-Seq data. Therefore, we

selected 3A14 and 3A24 to further investigate their roles in the *I. scapularis* life cycle. However, silencing 3A14 and 3A24 had no significant influence on tick feeding and development, indicating that either the knockdown is ineffective or silencing 3A14 and 3A24 alone may not have a noticeable effect on the tick life cycle.

Glutathione peroxidase (GPX), one of the other genes we examined, exhibits 1.2 log fold lower expression in the *IGOBP* knockdown group in the RNA-Seq data. Similar to CYTP450s, GPX aids in antioxidant defense by reducing the negative effects of reactive oxygen species (ROS) (Mittapalli, Neal, and Shukle 2007). In fact, GPX protects against cellular damage in response to local tissue injury by scavenging ROS produced by leukocytes in the necrotic mouse tissues (Das et al. 2001) (Steiling et al. 1999). GPX also might protect parasitic helminths from the host cell derived ROS (Zelck and Janowsky 2004; Zhang et al. 2015). Tick attachment and feeding result in a wound in the host bite sites. In order to stay attached and feed for several days, they attempt to avoid host immune responses and limit the generation of ROS and thus secreting antioxidants into the bite sites may help them in attaining uninterrupted feeding (Das et al. 2001). Salp25D, an antioxidant protein found in *I. scapularis* saliva, may play a detoxifying role in both host tissues and the salivary glands of the vector (Das et al. 2001). Surprisingly, Salp25D has 75% similarity with GPXs from bovine and *Drosophila* (Das et al. 2001). Moreover, during blood feeding, digestion of bloodmeal gives rise to free heme and generates ROS. Several antioxidant enzymes including GPX, glutathione-S-transferase (GST), catalase (CAT)

are thus involved in maintaining homeostasis in redox balances (Sabadin et al. 2021). Additionally, the blood fluke *Schistosoma japonicum* exhibits malformed egg development when PHGPx (phospholipid hydroperoxide glutathione peroxidase) is knocked down, indicating their functions during vitellogenesis (Zhang et al. 2015). We thus attempted gene silencing to determine whether GPX plays a function in tick hematophagy and the developmental process. Although there is lower expression of GPX in the IGOBP knockdown groups compared to the control ticks, there are no observable effects on tick feeding and development, presumably because the silencing was not significant or there is redundancy or unknown compensatory events in GPX functions; therefore, no conclusions can be drawn regarding its importance.

The fourth gene selected for the further study comprises putative low density lipoprotein receptor (LDLR). In mammals, LDLRs regulate cholesterol homeostasis and serve as signaling molecules, whereas in insects LDLR helps in sequestering lipids and cholesterols, mainly diacylglycerol and phospholipid through receptor-mediated endocytosis in different tissues and organs primarily in fat bodies and ovaries (Ravikumar and Vijayaprakash 2013) (Cheon et al. 2006). Insects derive their energy primarily from lipids, and fat bodies serve as lipid storage, metabolism, and transport sites (Cheon et al. 2006). Moreover, lipids are transported from fat bodies to ovaries and taken up by vitellogenin receptors during embryonic development (Ravikumar and Vijayaprakash 2013). Specific LDLRs for fat bodies have been identified in insects, and they are similar to vertebrate LDLR (Ravikumar and Vijayaprakash 2013). Defective LDLR in *Drosophila* causes sterility in females

suggesting their roles in vitellogenesis (Ravikumar and Vijayaprakash 2013). Insects cannot produce cholesterol on their own, so they acquire it from their hosts and use it to form cell membranes and steroid hormones (such as 20E). In the hematophagous kissing bug, *R. prolixus*, lipoproteins (Lp) and LDLRs absorb cholesterol from the host's bloodmeal and transmit it to other tissues, including fat bodies and ovaries (Entringer, Majerowicz, and Gondim 2021). Furthermore, 20E affects the expression of Lp and LDLRs (Cheon et al. 2006). In our prior studies, we found that silencing of *IGOBP* had an impact on tick feeding and molting. In addition, IGOBP is known to be secreted by tick saliva, and as an OBP, it may bind hydrophobic compounds and play a role in their transport. We, therefore, anticipate that abnormal lipid or cholesterol (source of 20E hormones) trafficking from hosts to ticks or within tick tissues or organs may affect tick hematophagy and development.

Zhao et al. additionally demonstrate that RNAi silencing of the lipid transporter proteins apolipophorin I and II results in molting defects in *Locusta migratoria* and decreased cuticle surface lipids, which compromises the cuticle barrier functions (Zhao et al. 2020). Cuticular lipid or cuticular hydrocarbons (CHC) are transferred to the cuticle during molting and immediately after the formation of the new cuticle (Zhao et al. 2020). In *Drosophila*, the fatty acid synthesis initiating enzymes acetyl-CoA Carboxylase (ACC) and fatty acid synthase 3 (FASN3) and the lipophorin receptors LpR1 (LDLR) and LpR2 (LDLR) are essential for CHC generation, trafficking, and limiting water-loss (Zhao et al. 2020) (Wicker-Thomas et al. 2015). Moreover, in our prior studies, we found that molted nymphs and adults

from *IGOBP* knockdown group have aberrant cuticles and decreased fitness. Hence, we selected LDLR and silenced the gene by RNAi mechanism to understand the roles of this gene. Although gene silencing was statistically significant, it does not have any impact on tick feeding, weight, and molting. Nonetheless, it is plausible that the cuticle defects are caused by the synergistic actions of multiple genes as we have observed in the case of *Drosophila* lipoprotein receptors 1 and 2 LpR1 (LDLR) and LpR2 (LDLR), where CHC levels were unaffected in mutants with deletions of either LpR1 or LpR2, but they were drastically reduced in mutants with deletions of both LpR1 and LpR2 (Wicker-Thomas et al. 2015). However, RNA-seq discovered several additional genes whose expression was considerably upregulated or downregulated in the *IGOBP* silenced ticks. Nevertheless, at least 89% of the 104 upregulated genes and 83% of the 73 downregulated genes have putative roles or are completely uncharacterized. It is therefore evident that additional research is required to comprehend the functions of these genes in relation to the tick life cycle and the molecular mechanisms that underlie IGOBP.

Chapter 4: IGOBP's roles in vectorial competence

4.1 Introduction

Lyme disease, also known as Lyme borreliosis, is the most common vector-borne illness distributed across the United States and Europe caused by the bacterium *B. burgdorferi* sensu lato complex. In the natural infection cycle, *B. burgdorferi* is maintained in two disparate hosts: a vertebrate host, such as *Peromyscus leucopus*, the white-footed mouse in North America, and an arthropod vector, *I. scapularis* or the deer tick. When naïve ticks bite infected hosts and take an infectious bloodmeal, they can acquire *B. burgdorferi*, maintain the pathogen transstadially, and when they seek bloodmeal again, they can transfer the bacteria to naïve hosts, triggering clinical complications in multiple organs, called Lyme disease. The CDC estimates that around 476,000 estimated cases of Lyme disease are diagnosed each year, but only fractions are reported (Kuehn 2013; CDC 2022b). Along with *Borrelia, I. scapularis* transmits many other human pathogens, such as the causative agents for human and animal granulocytic anaplasmosis, ehrlichiosis, babesiosis, encephalitis. Over the past two decades, the geographic ranges have expanded for *I. scapularis* along with Lyme disease and other tick-borne diseases (TBD). Moreover, tick bite itself causes IgE production and allergic reactions in humans which is another growing concern to public health (Crispell et al. 2019b).

Belonging to the hard tick family, *I. scapularis* stay attached to the host and feed for several days and uptake 100 times more blood than their body weight (Daniel

E. Sonenshine and Roe 2013). Tick saliva contains molecular effectors that counteract the host immune responses at the tick-host interface (Šimo et al. 2017b; Kim et al. 2016a; Chmelar et al. 2012). In addition, their feeding success and development depend on various tick proteins which express in the gut, salivary glands, and ovaries (Šimo et al. 2017b; Sojka et al. 2013a; Kemp et al. 1989; Kusakisako, Fujisaki, and Tanaka 2018). Additionally, these factors create a suitable environment for tick-borne pathogens that aid their survival in the vectors and transmission to the hosts (Šimo et al. 2017b). Hence, their capability for harboring multiple disease pathogens and ability to take blood from different hosts make *I. scapularis* a successful vector. Except for tick-borne encephalitis, there is currently no human vaccine available for most of TBDs (Research 2021). Although the use of acaricides as a tick control method has shown efficacy, it is not widely accepted due to a rise in ticks with chemical resistance and concerns about milk and meat product contamination (J. D. L. Fuente and Kocan 2006; José de la Fuente and Contreras 2015).

The alternative strategy for controlling tick infestation and TBD is developing anti-tick vaccines that can prevent tick attachment and blood feeding. However, a lack of knowledge about tick-protective antigens is impeding the development of effective vaccines, despite the fact that robust research activity in the field has led to the first successful and commercially available vaccine to control tick infestation in cattle based on *R. microplus* Bm86 antigen (Alzugaray et al. 2017). Being an important vector, the genome of *I. scapularis* is the first tick genome to be

sequenced, which provides resources for identification of multiple candidate tick antigens (Hill and Wikel 2005b), while more recent efforts yielded an improved genome with the discovery of new protein-coding genes (De et al. 2023). Targeting essential tick proteins required for tick hematophagy or development, either singly or using a combination of different tick or microbe-targeted proteins as vaccine targets may also have greater efficacy in inhibiting tick-borne diseases (Rego et al. 2019).

We identified a novel protein of unknown function named IGOBP from fed nymphs' gut, which was also discovered in other tick organs such as the legs and hypostomes, fat bodies, and salivary glands of nymphs. Silencing of the *IGOBP* gene in ticks had profound effects on tick hematophagy and development, which suggests IGOBP has important functions in tick biology. Since the protein bear tick-specific sequences, antigenic and are extracellular, it constitutes a potential vaccine target to limit tick infestations and related disease transmission, such as *B. burgdorferi*. In this study, the efficiency of IGOBP protein as a potential anti-tick vaccine was evaluated in mice as well as we examined its impact on vectorial competence impairing transmission of *B. burgdorferi*. Mice were immunized with recombinant IGOBP protein, and ELISA was used to measure antibody titers. Although mice generated high-titer antibodies against IGOBP, they were incapable of blocking tick infestations or unable to block *Borrelia* transmission from tick to hosts. We further examined the transmission of *Borrelia* via passive anti-IGOBP antibody transfer in ticks and also found no significant effects. Similarly, *IGOBP* silencing also had no effect on pathogen transmission and acquisition, suggesting that IGOBP function does not

influence pathogen persistence or transitions between vectors and hosts. Intriguingly, we discovered that mice develop antibodies against IGOBP when they are infested with ticks, indicating that IGOBP is an exposed antigen that is recognized by hosts.

4.2 Materials and Methods

4.2.1 Mice, ticks, bacteria

A low passage *B. burgdorferi* clone B31-A3 has been used in this study as detailed (Thakur et al., n.d.). Female C3H/HeN mice of four to six weeks old were purchased from Charles River Laboratories or Jackson Laboratories. As sex does not influence tick feeding or *B. burgdorferi* infection, we mostly use female mouse for the study (as they are easy to group and house). The nymphal and adult *I. scapularis* ticks that were used in this study originated from a colony that is maintained in our laboratory at 23 °C, 16L:8D photoperiod and 85% - 99 % relative humidity, as detailed (De et al. 2023). All animal studies were conducted, in compliance with the guidelines and approval of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Maryland, College Park.

4.2.2 Protein expression and purification

The recombinant IGOBP for immunization was purified using a bacterial (*E. Coli*) expression system, as detailed (De et al. 2023) and in chapter 2. *IGOBP* gene was amplified excluding the signal peptide regions (1-19 amino acid) and cloned into pET28a vector. Vectors containing IGOBP was transformed into *E. coli* BL21 cells

and single colonies were selected to check protein induction. IGOBP expression was induced at 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 15-16 hours at room temperature. Protein was isolated using denaturing conditions as detailed in the Probond Thermo Protocol using Nickel NTA resin.

4.2.3 Active Immunization study

Immunization studies were performed as detailed (Yang et al., n.d.). Recombinant IGOBP protein (10ug/mouse) was combined with Complete Freund's Adjuvants (CFA) and administered subcutaneously to mice (3 mice/group). Two further boosts (with Incomplete Freund's Adjuvant, IFA) were administered with a 10 to 12 days interval between each boost and one weeks after the second boost, the sera were collected. Control mice were immunized with adjuvants and PBS only. ELISA and immunoblot blot were used to assess the antibody titers and specificity, as previously reported, (De et al. 2023). To check the effect of IGOBP immunization in tick feeding, mice were infested with naive ticks (12 ticks/mouse) and their feeding progress were monitored. Fully repleted ticks were collected and their weights were measured. To assess pathogen transmission, group of mice (3 mice/group) were immunized as mentioned above and 10 days after the second boost, *B. burgdorferi* infected ticks were placed on the mice. 12 days after tick feeding, mice were euthanized, and murine tissues were collected and assessed for *Borrelia* burden by quantitative RT-PCR.

4.2.4 Antibody transfer studies

Passive immunization studies were performed as detailed (Yang et al., n.d.). Anti-IGOBP antibodies or normal mouse serum was microinjected into infected nymphs. Next day, the ticks were placed on mice and were allowed to feed until repletion. Twelve days after the ticks completed their bloodmeal, mice were sacrificed, and the organs were collected. *B. burgdorferi* levels were determined by qRT-PCR using primers for borrelial *flaB* and normalized using mouse *β-actin*.

4.2.5 RNA isolation and PCR

Isolation of RNA and PCR analyses were performed as detailed (Rana et al. 2023). Fully engorged or 72 hours fed ticks were collected and individually processed for qRT-PCR analyses to determine pathogen burdens in the vector. Total RNA was extracted using TRIzol (Invitrogen), treated with RNase-free DNaseI (Qiagen), and then reverse transcribed to cDNA. *Borrelia flaB* and *Anaplasma 16S rRNA* transcripts were measured and then normalized to the level of tick *β-actin* transcripts using the primers listed in **Table 2**. For assessment of *B. burgdorferi* levels in murine tissues, Borrelia *flaB* were measured and then normalized to the level of mouse *β-actin* transcripts using the primers listed in the table 3.

4.2.6 RNA interference (RNAi) and tick manipulations

RNAi mediated gene silencing was performed as previously described (Rana et al. 2023), using primers listed in **Table 3**. Briefly, portions of the open reading frame of *IGOBP* and *GFP* were amplified. Using MEGAscript RNAi kit (Invitrogen),

dsRNA for *IGOBP* and *GFP* were produced and purified. 4 µl of the dsRNA (3.5 mg/ml) was loaded into a capillary tube and was microinjected into the ticks through their anal pores as detailed (Kariu et al. 2011). The injected ticks were placed on mice following day. Some of the ticks were collected at 48 hours and were individually processed and assessed for gene silencing using qRT-PCR analysis. Primers unique to the target dsRNA sequence's upstream or downstream regions were used for the qRT-PCR (Table 3).

4.2.7 Western Blotting

Immunoblotting was performed as detailed (Rana et al. 2023). Briefly, purified proteins were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and immunoblotted with either naïve mice sera or IGOBP immunized mice sera.

4.2.8 Statistical Analysis

Quantitative results are expressed as the mean ± S.E.M. The statistical significance of the difference between the mean values of groups was analyzed by two-tailed unpaired *t*-test, or the Mann–Whitney *U*-test using GraphPad Prism v.9. $P < 0.05$ was considered to be significant.

Table 3. Oligonucleotides primers for chapter 4

Primer Name/Purpose	**Sequence 5'-3'**

IGOBP protein expression (pET28a) forward primer NdeI	GGAATTCCATATGATCCCAGCCGGCGGCAAGGACCT
IGOBP protein expression (pET28a) reverse primer BamHI	CGCGGATCCTTAACCCTGCTGGGCTCCCTGTGGG
Forward primer for quantitative PCR of *Borrelia flaB*	TTGCTGATCAAGCTCAATATAACCA
Reverse primer for quantitative PCR of *Borrelia flaB*	TGAGACCCTGAAAGTGATGC
Forward primer for quantitative PCR of *A. phagocytophilum* 16S rRNA	CAGCCACACTGGAACTGAGA
Reverse primer for quantitative PCR of *A. phagocytophilum* 16S rRNA	5CCCTAAGGCCTTCCTCACTC
Forward primer for quantitative PCR of mouse *β-actin*	AGAGGGAAATCGTGCGTGAC
Reverse primer for quantitative PCR of mouse *β-actin*	CAATAGTGATGACCTGGCCGT
Forward primer for quantitative PCR of Tick *β-actin*	GGTATCGTGCTCGACTC
Forward primer for quantitative PCR of Tick *β-actin*	ATCAGGTAGTCGGTCAGG
Forward primer for RNAi of *IGOBP*	TAATACGACTCACTATAGGGATGGTGGCATTCAAGGTGTC
Reverse primer for RNAi of *IGOBP*	TAATACGACTCACTATAGGGGTTCAGGATCTCGGTCCTGG
Forward primer for RNAi of *GFP*	TAATACGACTCACTATAGGGAGAGCGACGTAAACGGCCACAAGTT
Reverse primer for RNAi of *GFP*	TAATACGACTCACTATAGGGAGACGGGTCTTGTAGTTGCCGTC

4.3 Results

4.3.1 Active immunizations against IGOBP neither impact tick feeding nor protect mice from *B. burgdorferi* infections

In our previous studies, we observed that in absence of IGOBP, tick feeding is impaired, therefore we next assessed whether antibodies against IGOBP can also impair tick feeding and pathogen transmission. Groups of mice (3 mice/group) were immunized with recombinant IGOBP (we used PBS for the control group), and the antibody titer was measured by enzyme linked immunosorbent assay (ELISA) before challenging them with naive nymphal ticks. In contrast to the control groups, IGOBP induced high titers of antibodies **(Fig 4.1 A).** The mice were subsequently infested with naïve ticks (12 ticks/mouse) and feeding parameters were monitored. A comparable number of ticks were collected from both groups and the tick weights were unaffected by IGOBP immunization in mice **(Fig 4.1 B-C)** suggesting that immunizations with IGOBP antibodies do not interfere in tick hematophagy. We also assessed the impact of immunizations on *Borrelia* transmission from infected ticks to naïve hosts. Mice were infested with infected ticks (5 ticks/mouse) and the murine tissues were collected 12 days after tick repletion. The spirochete levels were comparable in mice from both groups indicating that IGOBP immunization does not protect mice from pathogen transmissions **(Fig. 4.1 D)**.

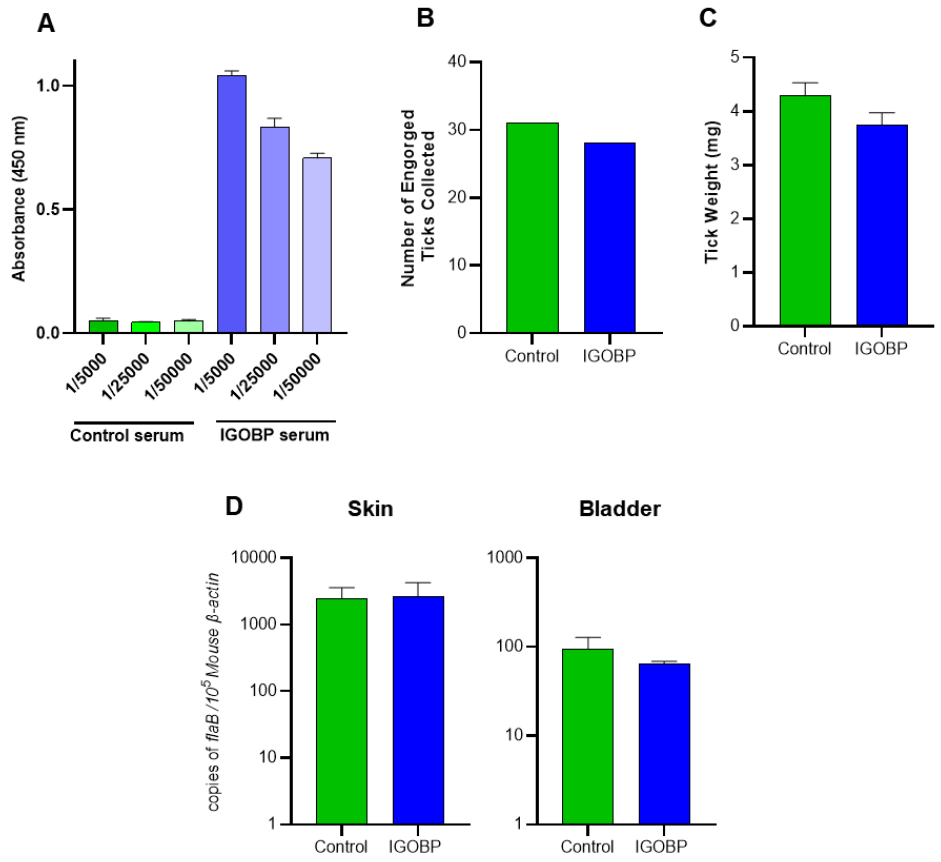

Figure. 4.1. Immunization with IGOBP protein does not affect tick feeding and *B. burgdorferi* transmission. (A-C) Naïve ticks were allowed to feed on mice immunized with IGOBP protein. **(A)** Mice sera were collected, and antibody titers were measured by ELISA. **(B-C)** The number of total repleted ticks are comparable in both groups as well as their weights (mg) and their feeding process were monitored and the weights of fully repleted ticks were measured. **(D)** *B. burgdorferi* burden in mouse skin and bladder were analyzed. Mice were immunized and were infested with *Borrelia* infected ticks. Mice organs were collected after 12 days of tick feeding and spirochetes levels were evaluated by qRT-PCR.

4.3.2 Passive antibody transfer in tick gut does not affect tick feeding and pathogen transmission

As previously described, active vaccination with IGOBP proteins was unsuccessful in preventing tick infestations and disease transmission, presumably due

to the delayed delivery or quantity of ingested antibodies along with the bloodmeal into the tick midgut. Therefore, to avoid that possibility IGOBP antibodies (IGOBP immunized mice serum) or control mice serum were directly introduced into unfed nymphs via microinjection technique (15 ticks/mouse) and subsequently placed on mice (3 mice/group) and feeding progress were monitored. We observed that tick feeding, and tick weights remain similar in both groups **(Fig. 4.2 A-B)**. Passive antibody transfer has a negligible effect on the transmission of pathogens from ticks to hosts as well **(Fig. 4.2 C).**

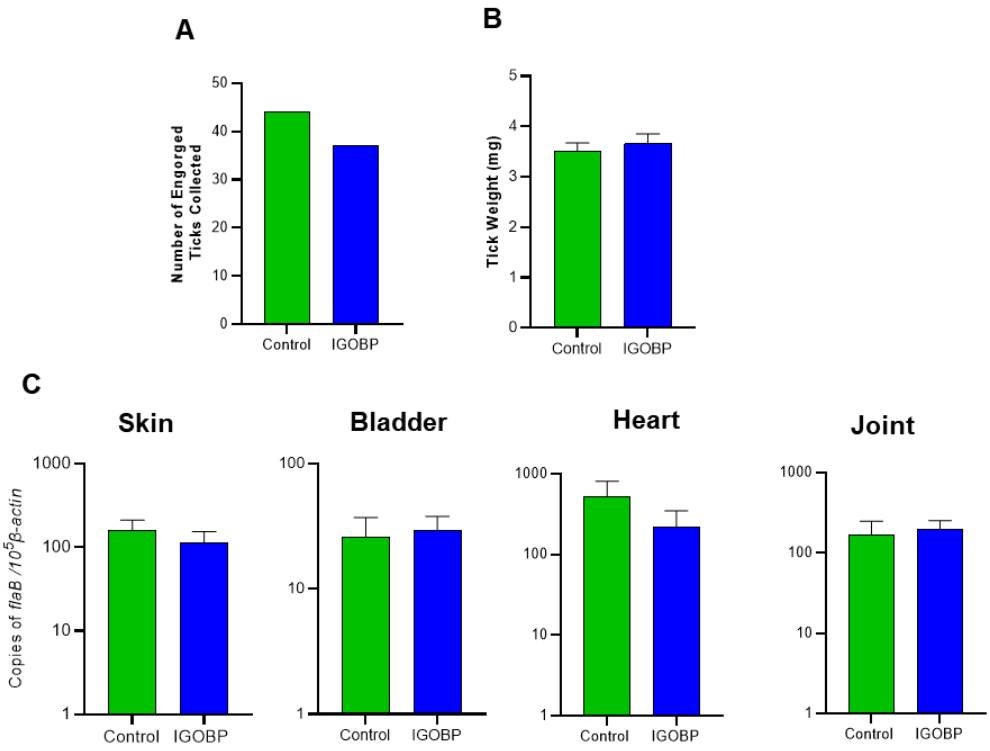

Figure. 4.2. Passive transfer of IGOBP antibodies does not impact tick feeding and transmission of *B. burgdorferi* from infected ticks to mice. **(A-B)** Nymphs were microinjected with IGOBP or control antibodies and placed on mice (3 mice/group). Total

number of repleted ticks and their weights were measured from both groups. **(C)** Passive transfer of IGOBP antibodies does not impact spirochete transmission. Infected nymphs were microinjected with antibodies and placed on mice. Mice were sacrificed and organs were collected 12 days after tick repletion. *B. burgdorferi* burdens in murine skin, bladder, heart, and joints were assessed.

4.3.3 RNAi mediated silencing does not interfere with pathogen persistence and transmission

Next, we aimed to characterize the function of IGOBP in pathogen transmission since RNA interference has been found to efficiently lower *IGOBP* RNA and protein levels while impairing the tick hematophagy process. For transmission studies, dsRNAs were microinjected in infected nymphal ticks and were allowed to feed on naïve mice. Ticks that were fully engorged were collected, and qRT-PCR was used to determine their *Borrelia* load. Twelve days following a tick feeding, tissues from mice were collected. Silencing had a negligible effect on *Borrelia* persistence in mice or ticks **(Fig 4.3 A-B)**. In fact, the knockdown groups show slightly greater burden levels, although these differences are not significant. Next, we examined whether the absence of IGOBP influenced *B. burgdorferi* or *A. phagocytophilum* acquisition in ticks from infected hosts. Naive nymphal ticks were allowed to infest mice after being microinjected with dsRNA and collected as fed ticks. We discovered that the pathogen loads were equivalent in control and knockdown ticks, indicating that IGOBP might have no effect on the persistence of either *Borrelia* or *Anaplasma* in ticks **(Fig 4.3 C).**

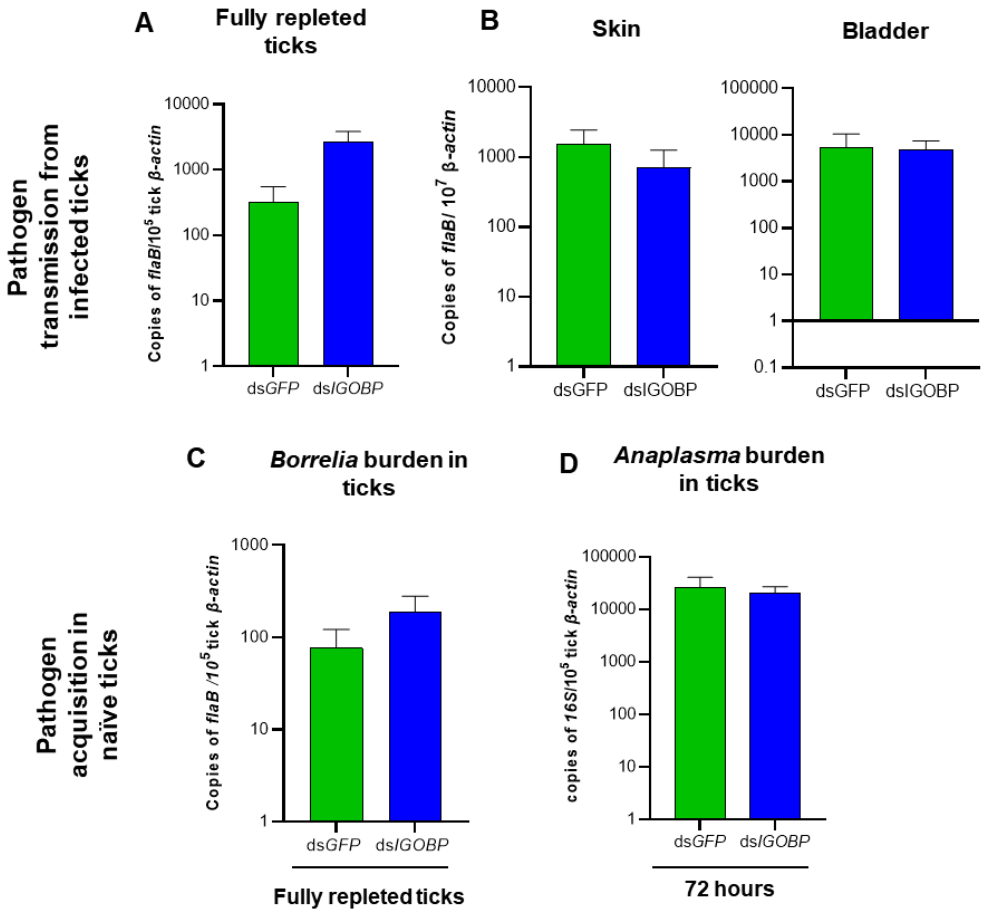

Figure 4.3. RNAi mediated knockdown of IGOBP does not affect *Borrelia* persistence. (A-B) Infected ticks were microinjected with dsRNA for GFP and IGOBP and placed in mice. Ticks were fed until repletion and *Borrelia* burden were assessed in ticks (5 ticks/group) by qRT-PCR. Mice were sacrificed 12 days after tick feeding and pathogen levels were analyzed in the skin and bladder. **(C-D)** Naïve ticks were microinjected with GFP and IGOBP dsRNA and fed on *Borrelia* and *Anaplasma* infected mice. Fully repleted (6 ticks/group) and 72 hours fed ticks (10 ticks/group) were analyzed for *Borrelia* and *Anaplasma* levels, respectively.

4.3.4 Tick infested hosts develop antibodies against IGOBP

Although IGOBP is immunogenic, immunization studies revealed that antibodies had a negligible impact on tick feeding. According to our earlier results

(Fig. 2.6), *IGOBP* is abundantly expressed in the salivary glands. IGOBP is also present in saliva, as shown by its presence in the saliva proteome, indicating that the protein is released into tick bite sites through saliva (Kim et al. 2016b). Therefore, we sought to discover whether mice that are tick exposed, produce antibodies against IGOBP. We immunoblotted recombinant IGOBP with serum from control and tick-infested mice and found that tick-infested mice serum could detect the recombinant protein, indicating that IGOBP is released to the host bite sites and that hosts recognizes and generates antibodies against the protein **(Figure 5.4).**

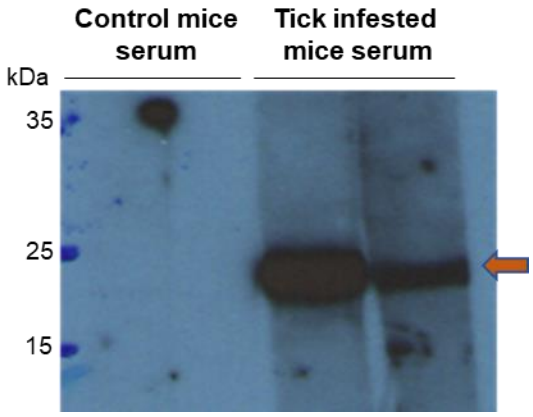

Figure. 4.4. Tick infested mice serum develops antibodies against IGOBP. Recombinant IGOBP proteins were immunoblotted with serum from control mice and serum from tick infested mice. Prominent bands at around 20 kDa are observed, as shown by an arrow.

4. 4 Discussion

Our previous studies show that the IGOBP gene silencing results in reduced tick feeding, tick weights, and molting. However, IGOBP antibodies had negligible

effects on both tick feeding and pathogen transmission. In addition, our western blotting data reveal that IGOBP is highly immunogenic in mice that are exposed to ticks, suggesting that IGOBP is an exposed antigen, which may be one of the reasons why antibodies are ineffective in reducing tick feeding. 'Exposed' antigens are ones that are secreted in the host dermis via tick saliva and are thus routinely exposed to the immune system of the host during tick feeding. As a result, the host recognizes the antigens and produces antibodies against them through tick infestations (J. D. L. Fuente and Kocan 2006). In contrast, 'concealed' antigens are not exposed to the host immune system (such as ones that are expressed selectively in the gut or other internal tick organs), necessitating repeated vaccinations against this protein in order to generate antibodies against buried antigens (J. D. L. Fuente and Kocan 2006). As an exposed antigen, ticks may have developed methods to suppress host immune responses to the exposed IGOBP antigens. This may explain why IGOBP immunization does not have the same effects as RNAi silencing and does not impair tick hematophagy process. The commercially available BM86 is a tick gut protein that belongs to concealed antigens and is highly conserved across tick species, making it effective against tick infestations (J. D. L. Fuente and Kocan 2006; Rand et al. 1989b). Despite the fact that IGOBP is substantially conserved in other hard tick species, it may not be an ideal vaccine target owing to its prior exposure to host immune systems.

Nevertheless, we cannot rule out the possibility that IGOBP is not a promising vaccine target solely by testing on mouse models. Mice used in the laboratory and the

wild white-footed mice (natural reservoirs) do not develop acquired tick resistance (ATR) in response to repeated tick bites. However, recurrent tick infestations cause guinea pigs, rabbits, and cows to spontaneously acquire ATR (Sajid et al. 2021). Humans demonstrate cutaneous hypersensitivities in response to repeated tick infestations, indicating that they develop resistance to repeated *I. scapularis* infestations (Sajid et al. 2021) (Krause et al. 2009b). According to Narasimhan S. et al., *I. scapularis* salivary proteins produced during the first 24 hours are sufficient to generate ATR and inhibit tick feeding (Narasimhan et al. 2007). Our earlier investigations indicate that *IGOBP* expression peaks between 12 and 24 hours. Interestingly, a cocktail of 19 salivary proteins from *I. scapularis* (19ISP) encoding mRNA vaccines induces tick immunity in guinea pigs, resulting in erythema at the bite sites and impaired tick feeding and *Borrelia* transmissions (Sajid et al. 2021). However, when mice were immunized with 19ISP, they did not develop erythema and there was no impact on tick feeding, indicating that tick salivary proteins induce tick resistance in non-natural hosts (Sajid et al. 2021). Therefore, to confirm our hypothesis, we need to additionally test the protein in non-natural reservoirs. As part of their normal life cycle, ticks may have adapted to combat the immunological responses that mice generate. As humans are also non-natural hosts, they may behave similarly to guinea pigs. Therefore, our future objective is to evaluate IGOBP as a potential vaccine target in the guinea pig models.

Despite the fact that RNAi silencing inhibits tick feeding, it did not prevent *Borrelia* transmission and acquisition and *Anaplasma* acquisitions, indicating that it

may not play a role in pathogen persistence. However, RNAi silencing does not eliminate previously synthesized proteins, which may have contributed to pathogen transmission and acquisition. Approaches like systemic RNAi targeting *IGOBP* could be induced in larval ticks where silencing of *IGOBP* could carry over to nymphs with depleted proteins. Thus, our future objectives remain to conduct transmission studies using systemic knockdown ticks for which no premade proteins are available to assess if complete depletion of IGOBP impacts transmission of pathogens from ticks to hosts.

Chapter 5: Conclusion and Future Studies

Ticks parasitize almost any vertebrate class across the globe and are considered to be one of the most widespread and predominant vectors of serious human infectious diseases. In the United States and many other parts of Europe, nearly 95 percent of reported cases of the vector-borne disease were associated with ticks, such as Ixodes ticks, making these the most medically important group of arthropod vectors. While our understanding of arthropod biology comes largely from studies involving model insects, especially the fruit fly and mosquitoes, this knowledge cannot be readily translated to ticks, largely because ticks are phylogenetically very distant from these dipterans, have a unique and relatively long-life cycles, and due to their ancient origin, appearing several hundred million years earlier than modern flies. It is therefore reasonable to assume that ticks have evolved unique biological properties distinct from model insects that can only be discovered by experimental studies using ticks. In this dissertation, we have characterized a unique protein from *I. scapularis* that has structural properties similar to insect Odorant Binding Proteins (OBP), termed *I. scapularis* gut OBP (IGOBP). Despite its homology to conventional OBP, our studies suggest its possible functions seemingly unrelated to tick olfaction, rather supporting tick hematophagy through maintenance of cuticular integrity in sub-adult ticks and females where acquisition of a complete bloodmeal is indispensable to execute subsequent growth, metamorphosis, and development of the ectoparasite. IGOBP function in *I. scapularis* is tied to its

influence on global gene expression, including ones relevant to steroid or hormone metabolism, detoxification, and transport, among others.

I. scapularis, one of the most significant and widespread tick species, transmits numerous human and animal pathogens, such as agents of Lyme disease, anaplasmosis, babesiosis, and Powassan virus disease. In fact, Lyme disease is the most prevalent vector-borne disease in the United States, with 476,000 estimated cases diagnosed annually (CDC 2022b). Existing and most commonly used anti-tick measures, such as the use of acaricides for tick control have drawbacks, such as contamination of milk and meat products and the development of tick populations resistant to acaricides (José de la Fuente and Contreras 2015). In addition, there is currently no anti-tick vaccine available for humans. Therefore, it is evident that we need better targets or preventive approaches in order to combat tick infestations and tick-borne diseases. One of the strategies involves a better understanding of the molecular biology of ticks and the identification of effective vaccine candidates that target crucial tick physiological processes, such as hematophagy and development.

In the current dissertation, we, therefore, characterized a novel *I. scapularis* protein, IGOBP, which was discovered to be upregulated in the tick gut in partially fed nymphs after 36 hours of tick attachment to the host, indicating the protein's potential importance in tick feeding. To the best of our knowledge, this is the first attempt at structural and functional characterization of a tick protein that has similarities to odorant-binding protein (OBP). Unlike model insects, Chelicerata, which includes ticks are relatively understudied, our repetitive BLAST searches over

the past several years do not reveal any additional *I. scapularis* homologs. However, as IGOBP is conserved amongst ticks and in fact does contain homologs from other tick species, it represents an excellent target for a broader multi-species vaccine target.

The predicted structure of IGOBP is most closely similar to a mite OBP from *Varroa destructor* (VdesOBP1). A common presence of three disulfide bridges and five helices in the same arrangement in both proteins suggests their similar gross structural organization. Sequence alignment by Clustal Omega reveals that the amino acid residues of the VdesOBP1 and IGOBP's binding cavities are conserved and contain a majority of hydrophobic residues, indicating that the proteins may bind hydrophobic ligands. To definitively demonstrate whether IGOBP is an odorant binding protein and its potential mechanism of functions, additional structural confirmation, an evaluation of cysteine pairing, validation of its binding cavities, and identification of IGOBP's ligands are needed. Despite our experimental attempts, we were unable to confirm the roles of IGOBP as an odorant-binding protein, especially in the sensing of DEET and humidity. While these efforts cannot rule out a possible function of IGOBP in tick olfaction, further research utilizing additional odorants must be conducted to assess its possible OBP functions.

Even though the IGOBP protein was initially identified in the gut, this gene is highly expressed in the hypostome and legs, salivary glands, and fat bodies, with peak expression occurring between 12- and 24-hours during feeding. This pattern of IGOBP expression during the early feeding period suggests it has a possible role in

hematophagy. In fact, our study revealed that IGOBP plays essential roles during tick hematophagy, and development, as silencing the gene by RNAi significantly impairs tick feeding, and also molting and development. We confirmed that impaired tick feeding is not a result of tick removal by hosts due to host recognition or itching responses, but rather a result of an impaired tick engorgement process. In fact, *IGOBP* knockdown ticks were able to attach to the hosts similarly to the control groups, indicating that IGOBP plays a role in the later stages of feeding when ticks take the so-called "the big sip" ingesting major part of the bloodmeal requiring rapid expansion of the body and the cuticle. We further observed that the knockdown of *IGOBP* influences the molting of larvae to nymphs and nymphs to adults impacting mainly the females, who feature a softer and more expandable cuticle. Consequently, to determine whether IGOBP has roles throughout the ticks' life cycle, we need to ascertain whether IGOBP also participates in female feeding, in addition to egg development and oviposition.

Systemic *IGOBP* knockdown, i.e., suppression of *IGOBP* through larval, nymphal, and adult stages results in abnormal cuticle formations and loss of cuticle integrity. Female *IGOBP* knockdown ticks have abnormal spiracle structures, necessitating further examination of their tracheal structure during feeding and body expansion (trachea extends during feeding which may support the increase in body size and circulate oxygen to the distal organs (Starck et al., 2018). Additional research is required to determine the structural and compositional differences between the control and *IGOBP* knockdown tick cuticles.

NMR studies have been used for decades to detect cuticle components (Christensen et al. 1991; Kramer, Hopkins, and Schaefer 1995; Schaefer et al. 1987; Duplais et al. 2021). Currently, carbon (^{13}C) and proton (^{1}H) nuclear magnetic resonance (NMR) studies are being conducted to identify the components responsible for the abnormal cuticle of female *IGOBP* knockdown ticks that potentially could lead to the identification of the mechanism by which IGOBP affects tick physiology. While in our recent preliminary data, we have detected compositional differences in NMR spectra in *IGOBP* knockdown tick cuticles **(Fig. 5.1)**, the identification of precise molecular makeup and structures that are altered and the mechanism of their alteration remains a subject of future investigation.

Despite the fact that solid-state ^{13}C NMR and water-soluble ^{1}H proton spectra for both control and ds*IGOBP*-treated tick cuticles are very similar, indicating that the chemical compositions are unchanged between the two groups (data not shown), when samples were suspended in DMSO-d6 and analyzed by high-resolution magic angle spinning (HRMAS) proton magnetic resonance spectroscopy (1H MRS) and Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion NMR, they display variations in the relative intensity of peaks in between two groups of cuticle samples (**Fig 5.1 A,B)**. Preliminary interpretation of 2D proton total correlation spectroscopy (TOCSY) data reveals variations in mobile/soluble components at around 5.1 ppm in the experimental (ds*IGOBP*) cuticle sample compared to the control group tick cuticles **(Fig. 5.1 C)**. While we have detected compositional differences in NMR spectra in IGOBP knockdown tick cuticles, these are merely preliminary results, and

we are currently in the process of analyzing the data in depth. As a result, we are unsure of the compositions detected by NMR and the compositional differences that exist. Consequently, further analysis and investigation are necessary.

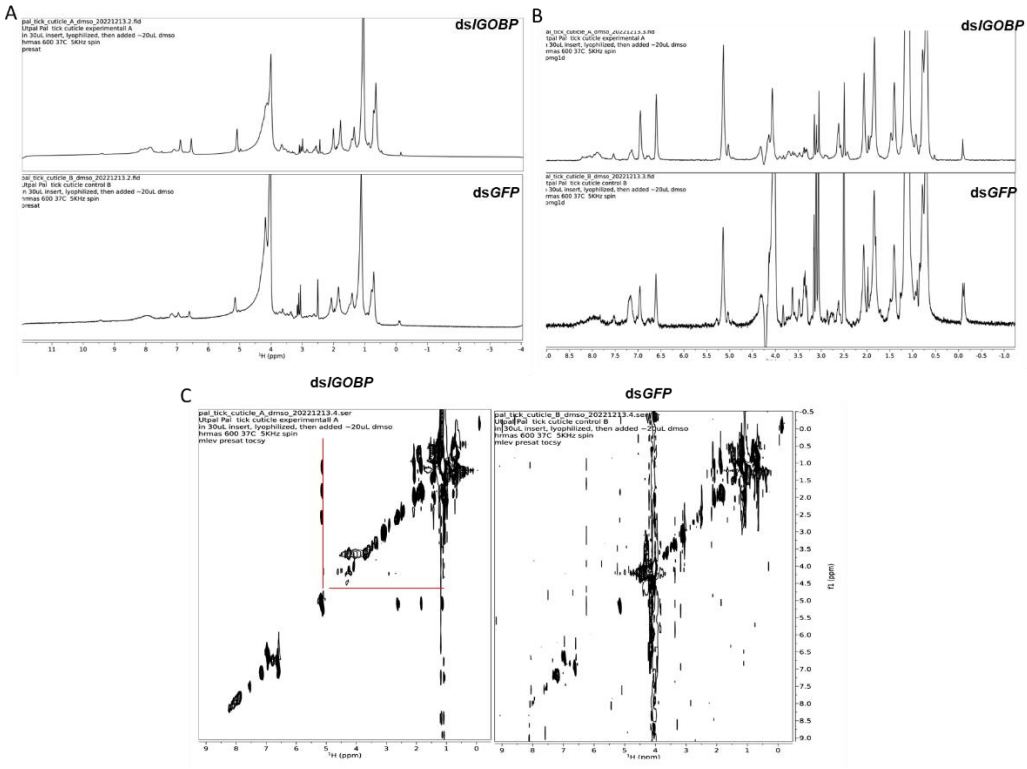

Figure. 5.1. Preliminary NMR spectra of adult female cuticles. **(A)** Cuticles suspended in DMSO-d6 and proton spectra were generated by HRMAS 600. Some minor differences in the relative intensity of peaks are observed. **(B)** 1D proton spectra from CPMG in HRMAS 600. Signals from species that are more mobile and soluble may be detected, and minor variations in the relative intensity of peaks are observed**. (C)** 2D proton TOCSY performed in HRMAS 600. Preliminary interpretation suggests the detection of additional soluble/mobile components at 5.1 ppm in the ds*IGOBP* group denoted by the red lines.

Our study thus characterized and established the significance of a tick protein that is essential for vector physiology and may be potential target for preventing tick

feeding and transmission of tick-borne diseases. By immunizing mice with recombinant IGOBP, we determined if it is a suitable vaccine target. Although ELISA and immunoblot results confirm that vaccinated mice develop appreciable antibodies, they were unable to prevent either tick feeding or transmission of *B. burgdorferi*. In fact, IGOBP antibodies injected directly into the tick gut were also unable to prevent ticks from transmitting pathogens to mice as well. We discovered that mammalian hosts like mice recognize the protein and produce antibodies in response to tick exposure, suggesting that ticks secrete this protein to the bite sites during feeding, which is also supported by the detection of IGOBP in tick salivary glands. Together, these observations may explain why repeated tick infestations do not prevent ticks from feeding mice despite the presence of IGOBP antibodies, suggesting that tick vector has strategies to avoid host antibodies against its exposed proteins. However, note that mice are ticks' natural hosts, and they typically do not develop acquired tick resistance (ATR) like other non-natural hosts do (e.g., guinea pigs, rabbits, cows, and most likely humans). We therefore cannot exclude the possibility that the protein may induce protective immunity in non-reservoir or incidental hosts, such as guinea pigs or humans.

RNAi knockdown of *IGOBP* in unfed nymphal ticks does not prevent pathogen transmissions (bacterial transmission from infected ticks to naive mice) or acquisitions (pathogen intake by naive ticks from infected mice), indicating that this protein may not be involved in the persistence of pathogens in ticks. However, to suppress IGOBP transcripts via RNAi, the ticks were treated with dsRNA 16 to 18

hours before being placed on mice for transmission studies. Although RNAi knockdown severely reduced IGOBP transcript copies, it does not affect premade proteins. Hence, pre-existed IGOBP proteins may persist in ticks and are able to aid in pathogen transmission or acquisition, which remains a subject of future investigation.

We further performed RNA-Seq of the control and knockdown ticks to determine IGOBP's roles in underlying mechanisms. Comparison of the transcriptome analysis in the control and *IGOBP* knockdown ticks identified several differentially expressed genes that are linked to *IGOBP's* expression changes. However out of 104 upregulated genes and 75 downregulated genes, respectively, 89% and 83% or more of the genes are either uncharacterized or have undefined roles, further indicating unique and unknown biological properties of tick proteins and signaling pathways that impact tick biology, highlighting fundamental information gaps in the field of ticks. Nevertheless, our findings indicate a key role of IGOBP and functionally associated proteins in tick hematophagy and development that can potentially be targeted for anti-tick vaccines and whose roles can be understood through additional further research. These studies will entail exciting opportunities for new discoveries on the fundamental mechanistic aspects of tick biology, its intriguing hematophagy and developmental episodes, including ones that can directly impact the persistence and transmission of invading pathogens, and together these efforts will contribute to the development of novel anti-tick strategies to curb many serious vector-borne illnesses.

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