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Elevated Ambient Carbon Dioxide Levels Induce Attraction but Not Attachment of Adult *Ixodes scapularis* in Artificial Membrane Feeding

Abstract

Numerous feeding studies on tick species have explored disease transmission, vector interactions, and acaricide testing. Traditionally, these studies used animals for feeding. However, artificial membrane feeding offers several advantages including increased standardization of experiments, decreased costs, and improved animal welfare. *In vitro* conditions must closely mimic natural environments to promote successful feeding attachment. Kairomones produced by the host are strong stimulants that encourage attachment. An important kairomone detected by ticks is carbon dioxide (CO_2). Previous studies have shown elevated CO_2 levels stimulate host identification and attraction and potentially improve artificial feeding rates in some tick species. The objective of this study was to use an artificial membrane feeding chamber prototype to explore the effects of ambient CO_2 in inducing *Ixodes scapularis* attachment. Differences in attachment rate were explored at an air-typical ambient CO_2 level of 0.04% and an elevated CO_2 level of 4.0%. Tick attachment was not detected in either ambient CO_2 condition during the incubation period, indicating ambient CO_2 does not impact the attachment rate under the presented condition. However, *I. scapularis* contact with the artificial membrane occurred at an increased rate of 0.014 female ticks in contact with the membrane per hour in the elevated CO_2 condition (4%) compared with a rate of 0.01 ticks per hour in the air-typical CO_2 condition (0.04%) (p=0.048) suggesting that the ambient CO_2 level affects attraction to the blood but does not directly stimulate attachment of *I. scapularis*.

Introduction

Ticks are responsible for over 95% of vector-borne disease cases in the United States[1]. The most prevalent, Lyme disease, is caused by the spirochete bacterium *Borrelia burgdorferi*. When transmitted to humans, *B. burgdorferi* induce inflammatory responses causing systemic symptoms[2,3]. Most cases of Lyme disease can be treated with antibiotics. However, as a result of late diagnosis or failed antibiotic therapy, chronic manifestations of Lyme disease have been documented[4,5]. Bacterial dissemination denotes late disease and is characterized by persistent pain and impaired cognitive function[6].

The black-legged tick, *Ixodes scapularis*, is the vector of several human pathogens, including those causing Lyme disease[7]. The life cycle of *I. scapularis* is approximately two to four years[8]. During this time, ticks cycle through four stages: egg, larva, nymph, and adult. *I. scapularis* are three-host ticks, requiring a blood meal at each life stage[9]. Although literature often states a low risk of transmission for infected adult ticks removed before 72 hours, experimental data using animal models has indicated the transmission time for *B. burgdorferi* is frequently less than 24 hours[10]. Expansion of the geographic range of *I. scapularis* and subsequent increase in incidents of Lyme disease over the past two decades makes this an increasing public health concern[7]. As temperatures warm due to climate change, the range of appropriate tick habitat and active periods are expanding[11].

Numerous studies on tick species explored disease transmission, vector interactions, and acaricide testing. Traditionally, these studies used animals for feeding. Currently, animal-derived or artificial membranes have replaced these methods and increased standardization of experiments, decreased costs, and improved animal welfare[12]. Early studies used membranes constructed from thin slices of cattle, rabbit, or mouse skin. However, these membranes were prone to rapid degradation[12]. The first report of voluntary feeding of ixodid ticks on an artificial membrane was the use of modified Baudruche membrane made from processed animal intestine to study the feeding activity of *Rhipicephalus microplus* ticks[13]. A comparison on feeding and survival of *R. microplus* on cattle versus through a Baudruche membrane showed that membrane conditions were capable of facilitating feeding outcomes comparable to natural conditions[14]. Another study demonstrated efficient artificial feeding of the *Ornithodoros coriaceus* tick through a Parafilm membrane using various stimulants, including covering the membrane with animal hair and an optimal feeding temperature of 37-39 °C. Bovine red blood cells, fetal calf serum, and glutathione solutions promoted high engorgement rates[15].

Many current studies involving artificial tick feeding find success with silicone membranes. However, in vitro conditions must incorporate critical stimuli present in natural environments to promote attachment and subsequent feeding[16]. Attractants continue to be explored for optimization of successful feeding using membranes. Host hair is an important stimulus as it provides both olfactory and tactile stimuli[16]. Volatiles and glandular secretions also assist in recognition of the host. These cues are unique to different tick species and their choice of hosts. For example, *I. scapularis* were found to exhibit an arrestant response to secretions associated with the glands of the white-tailed deer[17]. Ticks also release pheromones to facilitate aggregation (when ticks gather on a host in large numbers) and mating. Multiple reproductive pheromones have been identified in metastriate ticks, such as *Amblyomma* and *Dermacentor* species, which engage in on-host aggregative mating[18]. For example, attraction-aggregation-attachment

pheromone (AAAP) is produced by male *Amblyomma* ticks to attract unfed males and females[19]. Prostriate ticks, including *I. scapularis*, use nest-based mating strategies, with no consistent identification of volatile sex pheromones[18]. However, evidence of assembly pheromones have been documented in *I. scapularis*[20].

Haller's organ, a chemosensory structure located on the front legs of ticks, serves as a primary means of receiving host cues including heat, odors, and CO_2[21]. Due to the ability of ticks to detect radiant heat, accepted optimal membranes temperature tends to be 37 °C, the typical body temperature of hosts. Kairomones produced by the host are strong attractants that encourage attachment. An important kairomone detected by ticks is CO_2. In the 1950s, several studies found CO_2 to be a stimulant or attractant for bloodsucking arthropods such as mosquitos, mites, and fleas[22–24]. In the following decade, similar responses were shown for certain tick species[25]. CO_2 generated from dry ice has since been used to attract and subsequently capture ticks. Increased CO_2 concentrations have been found to stimulate behavioral responses in tick species *A. americanum* and *D. variabilis* at rates as low as 9 ppm above the average ambient level[26]. Elevated CO_2 levels have also been shown to improve feeding success in some tick species. For example, Krull et al. found a significant increase in engorgement mass and fertility of female *D. reticulatus* ticks at 5% CO_2 compared to ambient CO_2 levels[27]. Recent research has indicated that CO_2 activates *I. scapularis* and initiates responses that resemble host-seeking[28]. This study found that *I. scapularis* responded to CO_2 stimuli even with a disabled Haller's organ. Further research is needed to understand the relationship between CO_2 and *I. scapularis* behavior, attraction, and attachment.

The objective of this study was to use an artificial membrane feeding chamber prototype to explore the effects of ambient and elevated CO_2 levels as a stimulant to induce *I. scapularis* attachment. Optimizing feeding chamber parameters to allow for successful artificial feeding is required to set the stage for further experiments exploring the transmission of *B. burgdorferi* in these settings.

Methods

Membrane Optimization

The artificial membrane feeding technique used in this study was modified from Oliver et al. (2015) and based on methods developed by Kröber and Guerin (2007)[29,30]. Microscope lens paper (Fisher Scientific, Waltham, MA) was infiltrated with silicone rubber to produce a membrane mimicking the epithelial layer of skin. Lens paper was taped on a smooth sheet of Saran® wrap. Parts A and B of Ecoflex 00-30 (Smooth-On, Macungie, PA) were mixed in a 1:1 ratio to a total volume of 10 mL. Hexane was added to the silicone to temporarily thin the solution and ease the spread over the lens paper. The solution was mixed vigorously for three minutes per the manufacturer's instructions. The silicone solution was evenly spread over the lens paper and allowed to fully saturate the paper. Excess silicone was scraped off with a plastic kitchen scraper and membranes were allowed to cure for 24 hours. Each lens paper produced membranes for six chambers (Figure 1A).

Membrane thickness was measured using two different points on each membrane with a micrometer. Optimal membrane thickness is between 50 and 100 µm for adult *I. scapularis* as this allows for successful access of the hypostome to the blood through the membrane[29]. Ecoflex silicone is available in different shore hardness (00-XX, where XX represents the shore hardness), which indicates the resistance to indentation. Silicone with higher shore hardness produces thicker membranes, while those with lower shore hardness produce thinner membranes. Ecoflex 00-20 resulted in membranes with thickness consistently less than 50 µm, while Ecoflex 00-30 produced membranes typically between 50 and 100 µm. Several factors were investigated to reduce variation in membrane thickness, including shore hardness of the silicone, saturation time, and amount of hexane added to solution.

A two factorial design was conducted to determine if the saturation time (8, 12 min) and the amount of hexane added to the silicone to thin the solution (1.5, 1.75, 2.0 mL) would reduce variation in membrane thickness while still producing membranes in the optimized thickness range. One lens paper was used for each condition. All conditions used Ecoflex 00-30. Each membrane was cut into six equivalent squares signified in Figure 1A as the "Region." The thickness of the membrane was measured at two points in the center of the square.

Damaged membranes and membranes with thickness below or above the optimal range were discarded. Before using these membranes to construct the feeding chambers, they were pressed with hands to reduce tackiness.

Chamber Assembly

Feeding chambers were constructed by cutting a 1.5-inch section of transparent vinyl tubing (outer diameter = 1.5 inches, inner diameter = 1.25 inches) and placing them in individual wells of a 6-well plate. A rubber band encircling the tubing determined the depth at which the feed chamber would be submerged in the blood. This prevented the chamber from being flush against the bottom of the well plate, limiting access to blood (Figure 1B). Chambers were attached to the membranes using All Purpose Krazy Glue®. The adhesive was applied to the end of the chamber and the membrane was centered over the chamber. Tweezers were pressed along the edge to ensure a constant seal around the entire edge of the chamber. The chambers were allowed to dry for 24 hours. Excess membrane was carefully removed using a scalpel. Chambers were tested for damage by adding a 5 mL solution of water and food coloring. Chambers that demonstrated leaks were discarded.

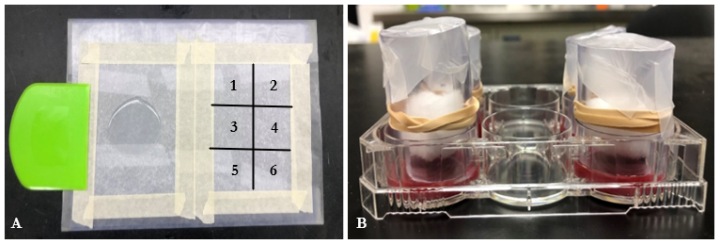

Figure 1. Infiltration of microscope lens paper with silicone (A) and artifical feeding chamber set-up (B). Cotton wool was placed in the chamber for clarity in the image and was not used during the testing process.

I. scapularis Maintenance and Feeding

Four mL of sterile, mechanically defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) and 90 µL of ampicillin/streptomycin solution (Gibco, Thermo Fisher Scientific Inc, Waltham, MA) was added to the four wells of a six well plate. The major component of the aggregation attachment pheromone, 2-nitrophenol (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), was dissolved in hexane to a final concentration of 10 mM[31]. Each well was supplemented with 4.5 µL of the 2-nitrophenol solution to encourage attachment. Additional attractants described in literature such as tick frass or deer hair extract were not used due to resource constraints.

Feeding chambers were placed into the wells to ensure direct contact between the membrane and blood. The well plate was placed in the NAPCO Automatic CO_2 Incubator (Series 5400 11V Model) set at 37 °C and the

designated ambient CO_2 level. The humidity in the incubator was approximately 100%. Attachment rate was evaluated at an assumed air-typical ambient CO_2 level of 0.04% and an elevated ambient CO_2 level of 4.0%. Four female and four male ticks were placed in each feeding chamber for the air typical CO_2 condition and five female and five male ticks were placed in each feeding chamber for the elevated CO_2 condition. New ticks were used for each treatment. *I. scapularis* adults, obtained from Atlanta CDC, were unfed, free of known human pathogens, and approximately 4 months post-molted. Ticks were held in air equilibrated with saturated K_2HPO_4 with relative humidity around 96% before transfer to the feeding chambers. Two sheets of parafilm sealed the top of the chamber to prevent ticks from escaping. A photoperiod of 16:8 (light:darkness) hours was implemented in the incubator to mimic outdoor conditions.

Feeding chambers were removed every 8 hours to check for evidence of attachment. Attachment was detected through visual assessment of the hypostome piercing the membrane and supported by a subsequent increase in body diameter as feeding occurred. Images of membranes were taken using the Nikon D 7500 digital camera with the AF-S DX Micro NIKKOR 40 mm lens (Nikon, Tokyo, Japan). *I. scapularis* that climbed up the sides of the chambers were returned to the surface of the membrane. Every 12 hours, feeding chambers were removed, rinsed with Milli-q water, and submerged in amphotericin B (Cytiva, Thermo Fisher Scientific Inc, Waltham, MA) for three minutes to prevent fungal contamination. Chambers were placed in new six well plates with fresh blood. Trials were conducted for 48 hours or until membrane failure (i.e., when tears or leakage occurred).

Results

Membrane Optimization

Great variation in thickness was seen in different lens papers and even in different membranes on the same lens paper. Therefore, saturation time and amount of hexane added to thin the solution were explored as potential parameters to reliably produce membranes in the optimized thickness range of 50 to 100 µm. A two-way ANOVA was performed to analyze the effect of saturation time (8, 12 min) and amount of hexane added to the silicone (1.5, 1.75, 2.0 mL) on membrane thickness. There was not a statistically significant interaction between the effects of saturation time and amount of hexane ($F(2, 15)$=0.6672, p=0.53). Simple main effects analysis showed that saturation time did not have a statistically significant effect on membrane thickness (p=0.58). Simple main effects analysis showed that amount of hexane did have a statistically significant effect on membrane thickness (p=0.0009). Upon further analysis, adding 1.75 mL hexane to the solution more reliably produces membranes below 50 µm (Mean, $\overline{x}$ = 26.67 µm ± 5.27 µm for 8 minutes, $\overline{x}$ = 36.83 µm ± 3.50 µm for 12 minutes) when compared to 1.5 mL hexane added ($\overline{x}$ = 67.31 µm ± 19.06 µm for 8 minutes, $\overline{x}$ = 75.78 µm ± 24.65 µm for 12 minutes) and 2.0 mL hexane added ($\overline{x}$ = 70.70 µm ± 30.16 µm for 8 minutes, $\overline{x}$ = 63.50 µm ± 27.96 µm for 12 minutes).

I. scapularis Attachment Rate

Differences in attachment rate were explored at an air-typical ambient CO_2 level of 0.04% and an elevated CO_2 level of 4%. Neither ambient CO_2 condition induced visible attachment of *I. scapularis* during the 48-hour incubation period. However, a direct relationship was seen between percent of female *I. scapularis* in contact with the membrane (i.e., on the membrane surface and not on the chamber walls) and incubation time in both CO_2 conditions (Figure 2 and Figure 3). A t-test comparing the slopes of the two regression lines in Figure 3 was statistically significant ($t_{(10)}$=2.25, p=0.048).

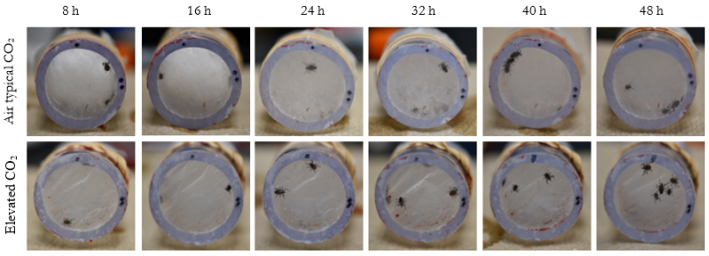

Figure 2. Progression of *I. scapularis* in contact with the membrane over the incubation period at 8, 16, 24, 32, 40, and 48 hour intervals (left to right) in a singular feeding chamber. Air typical CO_2 condition (top) and elevated CO_2 condition (bottom) are shown.

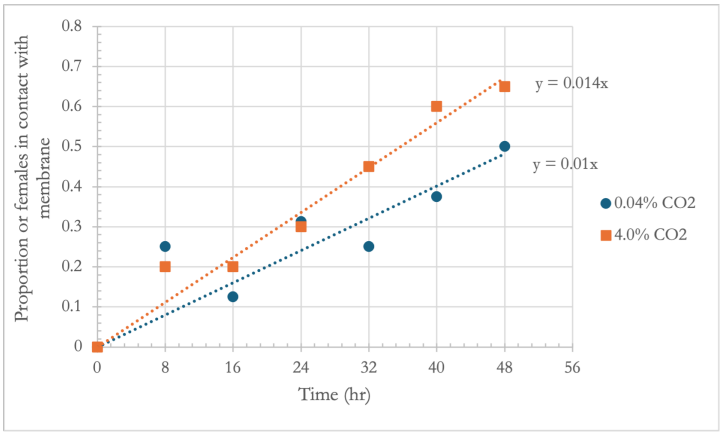

Figure 3. Proportion of females in contact with the membrane over a 48-hour incubation period for both air-typical CO_2 levels (0.04%) and elevated ambient CO_2 levels (4.0%). n=20 females at each CO_2 level from pooling all female ticks from chambers at the same CO_2 level. A significant difference between the slopes suggests that an elevated CO_2 level promoted tick contact with the membrane over time.

Discussion

Membrane Optimization

Membrane thickness must be between 50 and 100 µm for adult *I. scapularis* to successfully access the blood through the membrane via the hypostome. Consistency in membrane thickness is thus essential in standardizing experiments. Saturation time (8, 12 min) and the amount of hexane added to thin the silicone solution (1.5, 1.75, 2.0 mL) were explored as potential factors to reduce variability. The amount of hexane was shown to have a statistically significant effect on membrane thickness. Specifically, adding 1.75 mL hexane to the solution more reliably produces membranes below 50 µm. Adding 1.5 and 2.0 mL hexane produced thicker membranes with greater variability. Therefore, 1.75 mL of hexane appears to be the optimal volume to achieve the required membrane thickness and consistency. The saturation time was not found to have a statistically significant effect on membrane thickness either alone or in conjunction with hexane volume.

I. scapularis Attachment Rate

I. scapularis attachment to the artificial membrane was not detected in either ambient CO_2 condition during the incubation period, indicating ambient CO_2 does not impact the attachment rate under the presented condition. However, *I. scapularis* contact with the artificial membrane occurred at an increased rate of 0.014 female ticks in contact with the membrane per hour in the elevated CO_2 condition (4%) compared with a rate of 0.01 ticks per hour in the air-typical CO_2 condition (0.04%) (p=0.048) suggesting that the ambient CO_2 level affects attraction to the blood but does not directly stimulate attachment of *I. scapularis*. This phenomenon is consistent with

several previous studies in various arachnids species that demonstrate the ability of ticks to sense and react to CO_2 output from potential hosts. For example, adult *A. americanum* and *D. variabilis* demonstrated behavioral responses including questing, initial movement, and activity rate, to CO_2 concentrations as low as 9 ppm above the average ambient background[26]. Another study found CO_2 to consistently attract the highest number of host-seeking ticks (*A. americanum* and *D. variabilis*) when compared to several semiochemicals[32]. There has also been some suggestion that CO_2 must be present in combination with certain pheromones for successful attraction of ticks to the host. Maranga et al. studied the response of *A. variegatum* ticks to AAAP at various distances in the presence or absence of elevated CO_2. A significant majority (up to 90%) of ticks released were attracted to AAAP in the presence of CO_2 while CO_2 alone was unattractive[33]. Although, the effects of CO_2 on metastriate ticks are well documented, these findings cannot be applied to the *Ixodes* genus where less is known about stimulants that encourage aggregation, mating, and feeding. Recent research has shown CO_2 is a potent stimulant for *I. scapularis* in regard to walking behavior[28]. In this study, there was no clear concentration preference. Ultimately, the presence of CO_2 has been shown to be essential in the tick life cycle from questing to feeding. However, the exact mechanism is not entirely understood.

Male ticks did not appear to engage in attachment or attraction. This is unsurprising as the genus *Ixodes* are prostriate ticks in which copulation can occur off the host and unfed females can be inseminated prior to host attachment[34]. Although males can be found on hosts, they do not require an adult bloodmeal[35]. Therefore, they were not the focus of our study. It is also important to note that our results are limited to snapshots at different time points throughout the experiment. Whether the ticks moved about randomly in between data collection or were more arrested on the membrane over time is unclear and warrants further study using a camera with live-recording capabilities. Another consideration is that the effects of CO_2 were explored in an ambient setting rather than emitted directly from the blood. This suggests that increased ambient CO_2 levels facilitate attraction to the blood via an indirect mechanism.

Possible explanations for the lack of attachment include the following. The rapid failure of the membrane, resulting in leakage of blood into the chamber, and contamination with fungal growth only allowed for potential attachment and feeding window of 48 hours. It is also possible that attachment was accomplished but substantial feeding with an increase in body diameter was not yet achieved. This prospect is supported by previous studies that have established several days are required for *I. scapularis* attachment and feeding to reach fulfillment. For example, a study on in vitro feeding of *I. scapularis* adults found 45% attachment rate with 19 of the 49 attached ticks engorged after 13 days[36]. Oliver et al. revealed a minimum of three days were required for *I. scapularis* nymph to reach engorgement[29]. In that study, between 30-50% of female adult *I. scapularis* and approximately 50% of nymphs completed engorgement when given seven days to feed[29]. Another concern is the small number of ticks used in the study, as well as the limitation of a single trial. As noted above, a substantial proportion of ticks fail to successfully feed using artificial membrane techniques. This phenomenon may be compounded by a low sample size. Therefore, our results require careful interpretation and should be reanalyzed with an increased number of ticks and trials. Nonetheless, these results show a promising prototype of an artificial membrane feeding chamber to study tick attachment and feeding behavior under different conditions once further improvements to the design and fabrication of the chambers are successfully created to allow the experiments to proceed beyond 48 hours. Ideally, chambers would function for over one week in order to visualize attachment and subsequent feeding of ticks.

In conclusion, *I. scapularis* attachment to the artificial membrane was not affected by ambient CO_2 concentrations. However, ambient carbon dioxide levels appeared to affect attraction to the blood as suggested by an increase in tick contact with the membrane over the incubation period as well as a higher percentage of ticks in contact with the membrane in the elevated CO_2 condition compared with the air-typical CO_2 condition. Multiple studies have successfully implemented membrane feeding through additives to the blood, as well as stimulants present on the membrane. Potential factors that could be added to our experimental set-up include incorporating tactile and olfactory stimuli such as the addition of cow hair extract, deer hair extract, or tick frass extract to the membrane[29,30,37]. Further research should be conducted to explore the stimulants which promote attachment and feeding in *I. scapularis*.

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