Effects of Density and Linoleic Acid Exposure on the Chemical Profiles of Cercarial Echinostome Parasites

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Thesis Abstract

For many 2- and 3-host life cycle trematode parasites, transmission between hosts requires cercariae, a free-swimming stage, to emerge from the first intermediate host to find and penetrate a second intermediate host or a definitive host. As the role of chemicals in cercarial-host interactions has only been investigated in a few trematode species and in limited contexts, it is unclear if ecological factors affect the diversity of types and concentration of emissions. To address these gaps, I investigated the influence of cercarial density and host exposure to linoleic acid on the oxylipin emissions of the trematode parasite, *Echinostoma trivolvis* lineage c. I collected cercariae as they emerged from the first intermediate host snail, *Ladislavella elodes*, rinsed and pooled them at different densities, and allowed them to condition water for 4 hours. I then extracted lipids and used high performance liquid chromatography-tandem mass spectrometry to characterize oxylipins. A total of 40 oxylipins were quantified from nine samples ranging from 363-1621 cercariae per 10 mL, with seven oxylipins being present in ≥75% of the samples. Neither the diversity of types of oxylipins nor their concentrations showed a monotonic relationship with cercarial density, and no linear trends were observed. Forty-seven oxylipins were quantified from four samples of cercariae that each originated from infected snails that were exposed or unexposed to linoleic acid (385-674 cercariae/10 mL and 470-640 cercariae/10 mL, respectively), with 29 oxylipins being quantified in ≥75% of the samples in the unexposed, exposed, or both groups. Among these, oxylipin concentrations between the groups did not differ. Although linoleic acid appeared to be higher in the tissues of exposed snails, this increase did not affect the chemical profiles of the cercariae themselves. My thesis is the first to demonstrate that cercariae of *E. trivolvis* lineage c emit oxylipins. In addition, this work is the first to test whether density and host diet influence the chemical emissions of echinostome

cercariae. By understanding how ecological factors may influence oxylipin emissions of parasites, we can better understand how these chemicals mediate transmission in nature, ultimately shaping patterns of infection, host-parasite dynamics, and ecosystem-level interactions.

Contributions of Authors

This thesis is the original work of Joshita Sehgal. Joshita designed the study, conducted the experiments, analyzed the data, and wrote the thesis.

Dr. Jillian Detwiler provided supervision throughout the project, including providing guidance on the study design, assisting with data analysis and interpretation, as well as offering feedback and suggestions to refine the writing, structure, and clarify of the thesis content.

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List of Abbreviations

ALA – Alpha-linolenic acid (C18:3)

ADA – Adrenic acid (C22:4)

ARA – Arachidonic acid (C20:4)

COX – Cyclooxygenase

CYP – Cytochrome P450

DGLA – Dihomo-gamma-linolenic acid (C20:3)

DHA – Docosahexaenoic acid (C22:6)

DNS – De novo synthesis

DNS/DS – De novo synthesis/dietary sources

DS – Dietary sources

EFA – Essential fatty acid

EPA – Eicosapentaenoic acid (C20:5)

FA – Fatty acid

GLA – Gamma-linolenic acid (C18:3)

HPLC-MS/MS – High-performance liquid chromatography-tandem mass spectrometry

LA – Linoleic acid (C18:2)

LCFA – Long-chain fatty acid

LOX – Lipoxygenase

MCFA – Medium-chain fatty acid

MUFA – Monounsaturated fatty acid

n-3 – Omega-3 fatty acids

n-6 – Omega-6 fatty acids

PUFA – Polyunsaturated fatty acid

SCFA – Short-chain fatty acid

SFA – Saturated fatty acid

Thesis Introduction

Host-parasite interactions are a fundamental component of ecological systems (Thieltges et al., 2008; Fischhof et al., 2020, influencing both the structure as well as the function of host communities (Wood et al., 2007). Most often, this ecological approach focuses on the effect that parasites have on their hosts. For instance, parasitism can lead to host mortality, altering both the diversity (i.e., number and evenness of host species) and the composition (i.e., identity and relative abundance of host species) of the community (Johnson et al., 2008). In addition, parasites can also affect the behaviour of their hosts often in ways that benefit the parasite (Lafferty & Shaw, 2013). The ecology of host-parasite interactions can also be approached from the parasite's perspective and focus on elucidating fundamental natural history life characteristics such as host specificity (i.e., range and diversity of hosts that are used by a particular parasite life stage; Wells & Clark, 2019). In studies of host specificity, the host species that are infected or uninfected is what is observed, but the mechanisms underlying the patterns of host specificity are not. In other words, what mediates encounters and compatibility between parasites and their hosts is typically unknown (Lafferty & Shaw, 2013).

One potential mechanism underlying ecological interactions between hosts and parasites involves chemicals, either produced by the parasite or the host. In chemical ecology, what is often studied is how organisms interact with their environment and other organisms through the production, emission, and reception of chemical compounds (Charpentier et al., 2012). Hosts and parasites emit chemicals that potentially serve as cues in a variety of different ecological processes, including but not limited to communication, defense, foraging, reproduction, development, mate and habitat selection, feeding choices, movement patterns, and energy and nutrient transfer within ecosystems (Hay, 2009; Charpentier et al., 2012; Bortolotti & Costa,

2014). However, relative to the diversity of types and concentrations of chemicals produced by hosts and parasites, their functions and roles in key aspects of host and parasite biology, such as parasite-modified host behaviour and host specificity, remain unclear.

Chemical signaling molecules from hosts and parasites include different groups, some with distinct and some with overlapping functions. For instance, host immune function is affected by amines, amino acids, peptides, and proteins (Sato et al., 2009; Bunte et al., 2022; Mladineo et al., 2023) as well as lipid-based chemicals, such as fatty acids and their derivatives (Gao & Kolomiets, 2009; Kuźniak & Gajewska, 2024). However, some of the same chemical groups, like amines and amino acids, are also involved in metabolic interactions between hosts and parasites (Tielens et al., 2010; Ren et al., 2018). Similarly, host recognition by parasites is also mediated by some volatile organic compounds, such as aldehydes and ketones, by serving as attractants or repellents that either guide parasites towards hosts or help hosts avoid parasites (Poldy, 2020; Tawich, 2022; Makhlouf et al., 2024; Ni et al., 2024; Hotermans, 2024). Lipid-derived molecules are also implicated in host-seeking behaviours for some parasites (Gao & Kolomiets, 2009; Kuźniak & Gajewska, 2024).

Among this diversity of chemicals, lipid-derived molecules, particularly oxylipins, have been suggested to play a potential role in mediating interactions between helminths (i.e., nematodes, trematodes, cestodes, acanthocephalans) and their vertebrate and invertebrate hosts (Noverr, 2003). Oxylipins, bioactive molecules derived from the oxidation of polyunsaturated fatty acids (PUFAs), have been documented to be emitted from a wide array of hosts in helminth life cycles including humans, birds, and gastropods (Haas, 1992; Haas, 1994) as well as from some helminth parasites including different species of trematodes (Friesen & Detwiler, 2021). These molecules, produced through mono- or dioxygen-dependent reactions primarily, are

synthesized via 3 enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) (Gabbs et al., 2015). The COX pathway catalyzes the conversion of PUFAs into prostanoids, including prostaglandins and thromboxanes, which play roles in inflammation, vascular homeostasis, and immune regulation in mammals (Gabbs et al., 2015). The LOX pathway transforms PUFAs into hydroxy fatty acids and their metabolites, such as leukotrienes, lipoxins, and resolvins, which are involved in inflammation, immune responses, and the cardiovascular health in mammals (Gabbs et al., 2015). The CYP pathway, primarily recognized for xenobiotic metabolism, produces epoxy fatty acids and hydroxy fatty acids, which play roles in vascular, cardiac, and renal functions in mammals (Gabbs et al., 2015).

As most research on oxylipins has studied their effects on vertebrates, with only some studies extending to invertebrates, there remains a gap in our understanding regarding the synthesis, emission, and function of oxylipins from parasites (Gabbs et al., 2015; Friesen & Detwiler, 2021). Recent work has characterized the oxylipins from freshwater snails that are either uninfected or infected with trematodes and found differences based on infection status with infected hosts emitting a greater diversity of types of oxylipins and higher concentrations of some oxylipins than uninfected individuals (Friesen et al., 2022a, 2022b, 2024). However, this work did not isolate which and how much of the oxylipins were produced by the trematode parasites within or emerging from the infected snails. Determining the origin of these molecules, whether they are host-derived or parasite-derived or a combination of both, is particularly important as it will provide insights into whether these molecules originate from the host's immune or metabolic system, or if they are being synthesized de novo by the parasite. To address this gap, this thesis aims to characterize the oxylipins from a trematode parasite from one of

these prior studies (Friesen et al., 2022a) and test whether ecologically relevant factors such as parasite density and host condition, influence their emission.

Parasite transmission may be density-dependent or density-independent (Wojdak et al., 2014). Density-dependent transmission refers to the probability of contact between hosts and parasites being affected by the number of parasites or hosts within a given space (Wojdak et al., 2014). Density independent transmission, also referred to as frequency dependent transmission, is influenced by the number of parasites or hosts independent of space (Wojdak et al., 2014). A laboratory-based study exposing the same dose of heterophyid cercariae to common carp (*Cyprinus carpio*) hosts in containers with different volumes of water (i.e. densities) found that prevalence and intensity of infection were independent of cercarial density (Boerlage et al., 2014). If the cercariae were seeking out hosts using host-derived chemical cues, then that may explain why the rate of encounter between host and parasite did not increase with increasing density (Sousa, 1990). Sensing host chemical cues may allow parasites, even when host density is low, to locate hosts according to increasing concentration gradients (Haas, 1995). In response to host cues, parasites can also produce their own chemical cues. When cercariae of the human blood fluke (*S. mansoni*) were exposed to increasing concentrations of the fatty acid linoleate, the emission of two eicosanoids (leukotriene (LT) and hydroxyeicosatetraenoic acid (HETE) from the parasite also increased. Furthermore, the cercariae exhibited a higher frequency of penetration and transformation behaviour, which indicates that transmission would also increase (Fusco et al., 1985). However, it is currently unknown whether cercarial density influences the types and concentrations of chemicals. Thus, investigating whether parasite-derived chemical emissions, particularly oxylipins, are influenced by the density of parasites remains is an

important area to explore, as variation in the density of parasites could have an effect on the diversity of types and concentrations of molecules released by parasites.

Host diet can affect the chemical and physiological environment of host tissues, which in turn may modify the chemical environment of the parasites developing within the host (Babaran et al., 2021; Poudyal & Paul, 2022; Zhou et al., 2022). Host condition may influence parasite development and transmission, where factors such as nutritional status, immune activity, and physiological state of the host could influence the resources available for the parasites (Babaran et al., 2021; Poudyal & Paul, 2022; Zhou et al., 2022). For example, hosts fed diets rich in protein or fatty acids may provide a more diverse array of biochemical precursors for the parasite, potentially altering the types and concentrations of metabolites, such as oxylipins, that parasites may be able to produce and emit (Babaran et al., 2021; Poudyal & Paul, 2022; Zhou et al., 2022). On the contrary, hosts with diets deficient in proteins or fatty acids may pose restrictions for parasites from accessing key substrates, hindering parasite metabolism and potentially altering the chemical emissions of the parasites (Babaran et al., 2021; Poudyal & Paul, 2022; Zhou et al., 2022). Thus, examining how host condition influences parasite-derived chemical emissions can help highlight the contributions of both the host and parasite to the overall chemical profile of parasites.

I focused on characterizing the oxylipins from the cercariae of the freshwater trematode, *Echinostoma trivolvis* lineage c because they can be obtained from laboratory infected hosts, eliminating the possibility that field-collected, first intermediate hosts, freshwater water (*Ladislavella elodes*), are infected with more than one echinostome species. For instance, *L. elodes* field-collected from one wetland were infected with 3 species of echinostome trematodes (*Echinoparyphium* sp. C, *Echinoparyphium* lineage 2, and *Echinostoma revolutum* sensu lato

(Eliuk et al., 2020) are able to obtain laboratory-infected first hosts that are only exposed to that lineage of parasite. Certain species, such as *E. trivolvis* and some of its genetic lineages (*E. trivolvis* lineage a, b, and c sensu Detwiler et al., 2010), have well-defined life cycles and are relatively easy to maintain in controlled laboratory conditions, which makes them particularly suitable for laboratory studies (Kanev et al., 2009; Hodinka & Detwiler, 2024). The life cycle of *E. trivolvis* involves several life stages, each with unique characteristics and interactions. The first intermediate host is typically a freshwater snail, which becomes infected with the miracidial stage and subsequently the sporocyst and redial stages (Esch & Fernandez, 1994; Schumacher 2025). The second intermediate host, often an amphibian or a freshwater invertebrate, hosts the cercarial stage, an actively swimming larval form of the parasite (Esch & Fernandez, 1994; Schumacher 2025). Finally, the definitive host, usually an aquatic bird or mammal, harbours the adult trematode (Esch & Fernandez, 1994; Schumacher 2025). Echinostomes are widely distributed and ubiquitous in aquatic ecosystems throughout the world (Detwiler et al., 2010; Huffman & Fried, 2012). The genetic lineages are found throughout North America and use some of the same host species at different points in their life cycle but can also differ in their host use (Detwiler et al., 2010; Detwiler et al., 2012). However, like most parasites, the mechanisms underlying these differences in host specificity remain unknown.

Understanding the origin of chemical cues in host-parasite interactions is often challenging to decipher, particularly when attempting to disentangle whether the chemicals are originating from the host, the parasite, or a combination of both. Among the different life stages of echinostomes, the cercarial stage is ideal for chemical analysis. This larval stage can be easily isolated from its host as it is free-swimming (i.e., not confined within a host) compared to other stages such as rediae, a tissue-dwelling stage (i.e., confined with a host). As rediae reside within

the tissues of the host, distinguishing rediae-derived oxylipins from host-derived chemicals can be challenging. Although the rediae of some species can survive for some time outside of the host, it is unclear whether this would affect their chemical emissions (Garcia-Vedrenne et al., 2016). Moreover, although there are other stages such as eggs and miracidia that are also free within the environment (i.e., not confined within a host), cercariae are larger in size and thus, this stage is easier to isolate compared to the other larval stages. Furthermore, as echinostome cercariae play a role in transmission, it is important to characterize the chemical emissions from this particular stage to determine the role of chemical signalling molecules in first to second host transmission.

Echinostome cercariae exhibit specialized host-finding behaviours, allowing them to increase their transmission success to second intermediate hosts by responding to both environmental cues as well as host-derived signals (Haas, 1994; McCarthy et al., 2002). These behavioural adaptations include certain responses to environmental factors including light, temperature, and gravity, as well as reactions to stimuli (i.e. shadows, water turbulence, and chemical compounds) from potential hosts (Haas, 1994). The cercariae show energy-efficient swimming behaviour, select favourable microhabitats, and avoid unsuitable stimuli, optimizing their ability to survive and find an appropriate host (Haas, 1994). Additionally, their responses to host-specific signals, such as the release of chemical cues, are important for locating as well as invading suitable second intermediate hosts (Haas, 1994). In the case of certain trematodes such as echinostomes, these parasites can potentially manipulate the behaviour of their second intermediate hosts, increasing their attraction to infected first intermediate hosts (Gray et al., 2009; Eliuk et al., 2020). Despite findings on the various types of manipulations that echinostome cercariae may be employing to progress through their life cycles, not much is

known about the chemical mechanisms underlying these interactions. Specifically, as the chemical profiles of echinostomes themselves have never been exclusively characterized, it is challenging to determine the potential chemicals or cues responsible for manipulating its host.

However, recent studies have shown distinct oxylipin profiles in infected versus uninfected snails, suggesting oxylipins as one potential chemical cue mediating these interactions (Friesen & Detwiler, 2021; Friesen et al., 2022a). Specifically, infected snail hosts emitted a higher number of overall oxylipins and higher concentrations of certain oxylipins compared to uninfected snails, suggesting that oxylipins may be a chemical cue responsible for mediating these interactions (Friesen & Detwiler, 2022a). However, the origin of these oxylipins-whether derived from the host, the parasite or both are currently not known. By characterizing the oxylipin profiles of echinostome cercariae, this study aims to understand the origin of oxylipins in mollusc-echinostome interactions.

To characterize the oxylipin emission profiles of echinostome cercariae, it is important to understand ecological factors that may influence these emissions. One such factor is the density of cercariae, defined as the number of cercariae within a specific amount of volume (Welsh et al., 2017). As infected snail densities fluctuate in natural environments, the number of cercariae released into aquatic environments may also vary (Orlofske et al., 2015). These shifts could change the composition and concentrations of chemical cues present in the water, potentially influencing cercarial survival, host-finding, and overall transmission potential. I hypothesize that density may affect the diversity of types and concentration of chemicals in the environment. If the amount emitted per individual parasite remains consistent regardless of group size, a larger group of parasites may collectively release a higher diversity of types and concentration of chemicals. As cercariae are short-lived, energetically limited (rely on energy reserves from redial

stage), and must locate suitable second intermediate hosts to continue their life cycles (Haas, 1994; McCarthy et al., 2002), higher densities can increase the probability of encountering a host, enhancing transmission success, whereas lower densities may reduce encounter rates, potentially limiting parasite reproduction. Additionally, as cercariae can respond behaviourally to conspecifics, they may adjust their swimming patterns or aggregation in response to chemical cues emitted by other cercariae, which could have broader implications on cercarial performance as well as chemical signalling, potentially influencing the survival and host-finding efficiency cercariae (Haas, 1992). Therefore, understanding how cercarial density affects oxylipin emissions provides insights not only into the chemical signalling between hosts and parasites, but also into ecological interactions including parasite transmission dynamics.

Another potential factor that may be affecting the oxylipin emission of cercarial parasites is the diet of the host. When molluscs were fed diets that differed in fatty acid profiles, the overall fatty acid composition (%) between the mollusc tissue and mollusc tissue containing sporocysts (progenitors of cercariae) also differed according to diet. The % PUFA content was higher in the trematode-containing tissue compared to the diet or snail tissues suggesting the ability of the parasites to synthesize fatty acids de novo (Babaran et al., 2021). While the omega-3 fatty acids tended not to differ between tissue types, there were several omega-6 fatty acids such as arachidonic acid (ARA) and dihomo-gamma-linolenic acid (DGLA) that were in a higher percentage in trematode-containing tissues of molluscs (Babaran et al., 2021). However, it remains unclear whether the PUFA composition of the sporocysts affects the emission of oxylipins from the cercariae. Rather than through the diet, free-swimming schistosome cercariae, (*Schistosoma mansoni*), a trematode parasite, were directly exposed to linoleic acid (LA), an omega-6 fatty acid, and been found to alter the amounts of certain eicosanoids such as PGE1 and

5-HETE (Fusco et al., 1985). In contrast, the present study exposed another trematode cercariae, (*Echinostoma trivolvis)*, indirectly to LA via their snail hosts. If LA is ingested and incorporated into the gonad tissues of the snail hosts, then *E. trivolvis* rediae (the parasitic stage that develops within the host before cercariae emerge while feeding on its tissues) may metabolize LA to produce oxylipins. Consequently, emerging cercariae could emit a greater diversity and concentrations of specific oxylipins derived from LA or other fatty acid precursors present in host tissues. LA is an omega-6 PUFA that serves as a precursor for a variety of oxylipins through both enzymatic and non-enzymatic pathways (Hennebelle et al., 2017). In mammals, LA can be converted via Δ6-desaturation into gamma-linoleic acid (GLA). GLA undergoes elongation to form DGLA, which subsequently goes through Δ6-desaturation to produce ARA (Yang & Chapkin, 2000). ARA can be metabolized by COX, LOX or CYP enzymes to produce prostaglandins, thromboxanes, leukotrienes, and other ARA-derived oxylipins. In addition, LA can be directly converted by LOX or CYP enzymes into hydroxy-, hydroperoxyl-, epoxy-, and dihydroxy-octadecadienoic acids (e.g., HODEs, HpODEs, EpOMEs, DiHOMEs), and can undergo non-enzymatic oxidation to form other oxylipins such as isoprostanes and keto-fatty acids (Gabbs et al., 2015). Other PUFAs can likewise to undergo non-enzymatic oxidation to produce oxylipins (Gabbs et al., 2015). As differences in the host diet and nutritional condition, particularly host fatty acid composition, may affect the diversity of types and concentrations of oxylipins emitted by cercariae, it may potentially alter the ability of cercariae to interact with second intermediate hosts, affecting their transmission success. As the chemical profiles from cercarial parasites may be context dependent, factors such as the density of cercariae and the diet of the host may play an important role on the chemical profiles of cercarial parasites themselves.

Thus, it is important to study how these factors may potentially influence the types of diversity and concentrations of oxylipins emitted by cercariae.

This study aims to characterize the oxylipins of cercariae from an echinostome parasite and determine whether there are changes in emissions depending upon a parasite-related factor (density), and a host-related factor (host diet). The first chapter investigates whether density influences the oxylipin emissions from *E. trivolvis* lineage c cercariae. I predict that as cercarial density (number of cercariae within a given volume) increases, the emission of oxylipins will also increase. As there will be a higher number of cercariae present within the same volume of water, I expect that they will collectively release a greater diversity of types and concentrations of oxylipins into their surroundings. Ecologically, at higher densities, cercariae-emitted chemicals may act as stronger attractants, increasing the likelihood of encountering a host and enhancing transmission, whereas at lower densities, weaker chemical cues could reduce encounters, potentially limiting contact and reproduction. The second chapter examines whether host exposure to LA, an omega-6 fatty acid, influences the oxylipin emissions from *E. trivolvis* lineage c cercariae. I predict that cercariae emerging from hosts exposed to LA will emit a greater diversity of types and concentrations of oxylipins derived from LA or other fatty acid precursors present in host tissues.

In summary, this thesis helps fill a knowledge gap by characterizing the oxylipin profiles of a parasite and determining whether ecological factors, like parasite density and host diet, influence their emission. Understanding what oxylipins the parasite synthesizes is a step towards a more comprehensive understanding of the origin of oxylipins emitted from echinostome-infected snail hosts. As these molecules may potentially play a role in mediating interactions between trematode cercariae and their freshwater snail hosts, understanding their origin is crucial

for understanding the chemical mechanisms underlying host-parasite interactions. Ultimately, by investigating the effects of density and host exposure to a fatty acid on the oxylipin emission of cercariae, this thesis aims to understand how trematode cercarial parasites influence the biochemical pathways and chemical composition of one group of lipid-derived molecules, the oxylipins involved in mollusc-echinostome interactions within freshwater systems.

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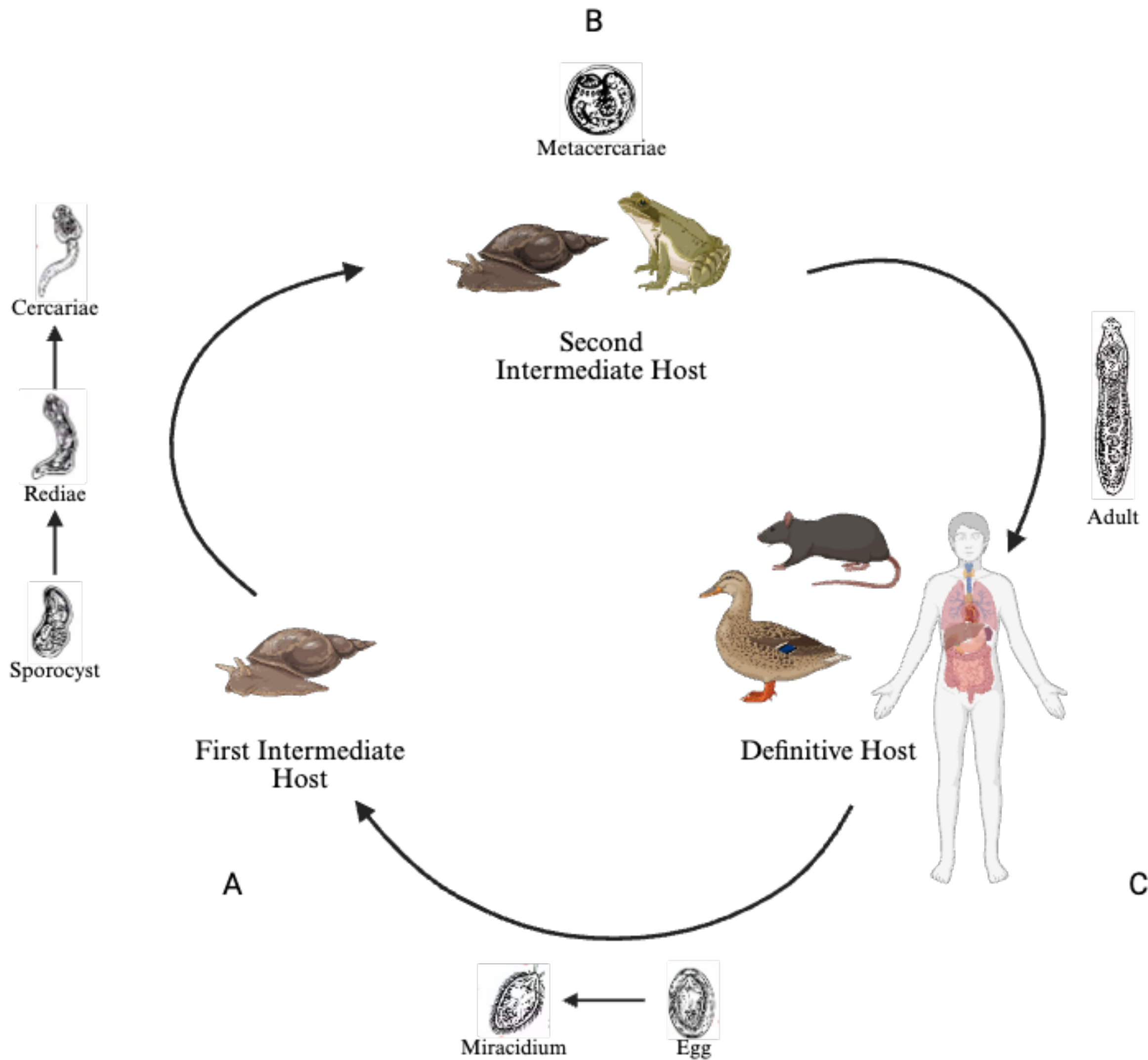

Figure 1.1. Generalized life cycle for lineages of the trematode *Echinostoma trivolvis*. A). First intermediate hosts are freshwater snails, which are penetrated by miracidia that asexually reproduce to form sporocysts and rediae. The latter stage produces cercariae, which can remain within the snail and encyst or emerge into the aquatic environment. B) Second intermediate hosts, including amphibians, snails, and other invertebrates are penetrated by cercariae, which then encyst as metacercariae. C) Definitive hosts, including mammals (e.g., muskrats) and aquatic waterfowl (e.g., ducks and geese) become infected upon consuming infected second intermediate hosts allowing the metacercariae to develop into an adult fluke.

Chapter 1: The influence of density on the oxylipin emissions from trematode cercariae of *Echinostoma trivolvis* lineage c

Abstract

Mollusc-trematode interactions are mediated by chemical signaling molecules, such as oxylipins, which play an important role in host-parasite communication. However, the influence of ecological factors, such as density, on oxylipin emissions remains poorly understood. This chapter investigates whether the density (i.e., number of individuals per volume of water) affects the emission of oxylipins from the cercarial parasites (i.e., the free-swimming stage that emerges from first intermediate hosts). I predicted that as parasite density increased, the diversity of types and concentrations of oxylipins emitted from cercariae would also increase. Cercariae were obtained from lab-raised freshwater snails (*Ladislavella elodes*) that were lab-exposed to *Echinostoma trivolvis* lineage c miracidia. Nine pools of cercariae at different densities (363-1621 cercariae per 10 mL) were allowed to condition the water with their chemical emissions for 4 hours. Lipids were extracted and the oxylipin profiles were analyzed using high-performance liquid chromatography/tandem mass spectrometry. Among the 158 oxylipins that were scanned for, 40 total oxylipins were quantified across the nine cercarial density samples. Among these oxylipins, 22 were lipoxygenase (LOX)-derived, 8 cyclooxygenase (COX)-derived, and 10 cytochrome P450 (CYP)-derived. Among these, a total of seven oxylipins were quantified in at least 75% of samples (≥7 of 9), where 5 belonged to the LOX pathway, 1 to COX, and 1 CYP. Cercarial density did not significantly predict the diversity of types nor concentrations of oxylipins, with no linear or monotonic relationships observed. However, the seven oxylipins quantified showed non-linear patterns, with concentrations that were low at the lowest densities, higher at subsequent densities, lower at intermediate densities, high again at higher densities, and then low again at the highest densities. Among these oxylipins, concentrations were generally

low (<0.01 ng/mL), with the exception of two linoleic acid-derived LOX oxylipins (9,10,13-triHOME and 9,12,13-triHOME), which were higher than the other five oxylipins. Notably, the majority of these seven consistently quantified oxylipins had linoleic acid (LA) as their precursor, suggesting that LA-based metabolic pathways might be a central component for cercarial oxylipin emissions. Overall, these findings confirm that *E. trivolvis* lineage c cercariae emit a range of LOX-, COX-, and CYP-derived oxylipins, with limited evidence that they are influenced significantly by density alone. Interestingly, the predominance of LA-derived oxylipins being the most consistently quantified oxylipins across all cercarial density samples suggests that LA-based pathways may be an important component of cercarial chemical emissions, potentially influenced by host diet and tissue composition.

Introduction

Trematode parasites have complex life cycles with various life stages, which each contribute to the survival and transmission of the parasite (Zemmer et al., 2020). Some of these life stages, such as cercariae, are relatively short-lived (e.g., < 24 hours, Esch et al., 2002; Studer & Poulin, 2012) and possess a limited ability to control their dispersal relative to their external environment. For instance, cercariae of aquatic trematodes tend to be weak swimmers that cannot swim against the flow of water to find the next host required to progress through their life cycle (Fitzpatrick et al., 2016). Similarly, cercariae emerging from terrestrial molluscs may not be able to actively seek out a host due to their limited mobility or the absence of water, constraining their ability to navigate towards their host. Instead, they may rely on passive transmission strategies, such as being embedded in snail-emitted slime trails that attract the next host in their life cycle, as exhibited by cercariae of the lancet liver fluke, *Dicrocoelium dendriticum* (Unrau, 2019). Another characteristic of cercariae that influences their survival and transmission is the number at which they are emitted from their first intermediate hosts. Depending upon the species, the daily output of cercariae that are emitted from snail hosts into the external environment can range from anywhere between tens to thousands of cercariae per snail per day (Poulin, 2006; Rosenkranz et al., 2018). The density of cercariae in the environment is one factor that may influence the odds of individual parasites encountering and attaching and penetrating the next host in their life cycle. Higher densities may potentially increase these odds, but it remains unclear whether density interacts with cues in the environment that shape cercarial survival and transmission.

Cercariae may respond to a variety of external stimuli, including thermal, mechanical, visual, and chemical cues, influencing the ability of parasites to locate and infect suitable hosts

(Haas, 1992). Among these cues, chemical stimuli have been hypothesized to be particularly important for the cercarial stage of trematodes, which must find and infect slow-moving mollusc hosts (Haas, 1992). To detect and recognize potential hosts via chemical cues, cercariae behave in a variety of different ways including through attachment, remaining stationary, directed creeping, and penetration onto the host (Haas, 1992). Among some species of trematodes, penetration behaviour is influenced by fatty acids or a combination of fatty acids and other chemicals (Haas, 1992; Haas, 1994). A focus on lipid-derived components has been driven in part by the presence of such chemicals in extracts from mammalian skin (Stanley-Samuelson, 1994). Schistosome cercariae, such as those of *Schistosoma mansoni* (human blood fluke), which use humans and other mammals as definitive hosts, rely on these cues to initiate skin penetration. However, cercariae have also been shown to use fatty acids to penetrate birds, fish, and amphibian hosts (Haas, 1992; Haas 1994a; Haas 1994b; Motzel & Haas, 1985).

The fatty acids emitted from vertebrate hosts include polyunsaturated fatty acids (PUFAs), which have two or more double bonds within their carbon chain. PUFAs are typically obtained from the diet as essential fatty acids (EFAs) or converted endogenously from other PUFAs in enzymatic and non-enzymatic pathways to non-essential fatty acids (non-EFAs). The outer surface of human skin (lipid mantle) contains EFAs, which led to the prediction that these chemicals would be important in shaping aspects of cercariae penetration behaviour by the trematode *S. mansoni* as humans are the primary next host in the life cycle (Haas & Schmidt, 1982). As predicted, schistosome cercariae initiated a higher penetration response when exposed to EFAs such as linoleic acid (C18:2) and linolenic acid (C18:3), but not to non-EFAs such as oleic acid (C18:1), suggesting a difference in the response of parasites to essential and non-EFAs (Salafsky et al., 1984). EFAs like linoleic acid are also important in that they are part of

the pathways for the synthesis of other fatty acids. C18:2 is part of the pathway for the synthesis of arachidonic acid (C20:4), which is a PUFA that is a precursor to secondary metabolites known as the oxylipins. These bioactive molecules are the product of PUFAs undergoing oxygenation via enzymatic and nonenzymatic pathways. For example, PUFAs in the C18 to C22 series are precursors to oxylipins known as the eicosanoids, which include prostaglandins (PGs), thromboxanes (TBs), leukotrienes (LTs), and hydroxyeicosatetraenoic acid (HETEs). Some of these eicosanoids are produced by hosts and affect the behaviour of cercariae (Stanley-Samuelson, 1994).

In addition to hosts, some trematode cercariae can synthesize fatty acids and oxylipins (Stanley-Samuelson, 1994; Hambrook & Hanington, 2021). From pools of ~13,000 cercariae, Fusco et al., (1985) found that *S. mansoni* emitted several eicosanoids including prostaglandins (PGE2, PGD2), leukotrienes (LTC4, LTB4) and hydroxyeicosatetraenoic acids (5-HETE, 15-HETE). Although very little is known about the factors that influence the oxylipin emissions of cercariae, exposure to certain fatty acids has been associated with the synthesis of certain oxylipins. The tissue of schistosome cercariae contained certain oxylipins after exposure to C18:2, but not when exposed to C18:1 (Fusco et al., 1985). In addition to certain oxylipins being present while others being absent, concentrations of oxylipins can vary in their emission over time with the highest concentrations recovered after 1 min compared 10 and 60 minutes (Fusco et al., 1985). These examples suggest that the diversity of types and concentrations of oxylipins emitted from cercariae vary over space and time and could be further affected by density of either hosts or the parasites if that factor influences oxylipin emissions. However, it is yet unclear whether the density of cercariae alters the oxylipin profiles of cercariae as most studies

have based their results on large pools of cercariae instead of comparing oxylipin profiles from pools of cercariae in different densities (e.g., Fusco et al., 1985).

This chapter aims to characterize the oxylipin profiles from the cercarial stage of a lineage of the echinostome species, *Echinostoma trivolvis* lineage c. This lineage serves as a useful contrast to oxylipin profiling of schistosomes trematodes due to differences in their life cycle and host use. In contrast to the 2-host life cycle of schistosomes, echinostomes have a 3-host life cycle where the cercariae must colonize a second intermediate host before they are able to infect a definitive host. The cercariae of *E. trivolvis* can penetrate a taxonomically diverse set of hosts including various species of tadpoles, annelids, and molluscs (Kanev et al., 1995; Johnson & McKenzie, 2009; Szuroczki & Richardson, 2009). At present, the second intermediate host specificity of *E. trivolvis* lineage c is unclear other than that the first host, freshwater snail (*Ladislavella elodes*) can also be a second intermediate host (Hodinka & Detwiler, 2024). It is unclear whether oxylipin emissions from these hosts differ and how that affects the synthesis of oxylipins of echinostome cercariae. Another aspect in which echinostomes differ from schistosome cercariae is their size and densities of cercariae emitted from first intermediate host snails. Echinostome cercariae tend to be larger (300-350 x 150-250 µm, Kanev et al., 2009) compared to schistosome cercariae (500 x 64 µm, Braun et al., 2018). Further, the number of cercariae emitted from the first intermediate host snails differ between parasite species. The daily cercarial output from snails of various schistosome species was between 291-4908 whereas echinostome species was between 320-1981 (see sources in Poulin, 2006). Therefore, if size and density of cercariae affect oxylipin emission, there may be differences in the chemical profiles (oxylipins) between echinostomes and schistosomes.

I hypothesize that density will affect oxylipin emission from cercariae and predict that as density increases, both the diversity of types and concentrations of oxylipins will increase. This prediction assumes that a higher quantity of cercariae in the same volume of water will lead to a cumulative increase in the release of oxylipins. If the density of cercariae influences their chemical profiles (emission of oxylipins), then we can better understand the role that the diversity of types and concentrations of oxylipins serves in the survival and transmission of this trematode parasite stage.

Methods

I characterized the oxylipins produced by the echinostome trematode *Echinostoma trivolvis* lineage c. This lineage is found in invertebrate and vertebrate hosts that occur naturally in wetlands within North America including Manitoba, Canada and several states within the U.S.A. (Sultana, 2018; Detwiler et al., 2010). *Ladislavella elodes* (syn *Stagnicola elodes)*, the marsh pond snail, serves as its first intermediate host (Friesen et al., 2022b). Although the entire life cycle of this parasite has not been elucidated in the laboratory, the methods for obtaining lab-infected first intermediate hosts were recently described (Hodinka & Detwiler, 2024). These methods allowed us to obtain varying densities of cercariae from lab-infected snail hosts.

Obtaining infected first intermediate hosts started with the isolation of eggs from adult worms acquired from muskrats (*Ondatra zibethicus*). Carcasses trapped within 24-48 hours were donated from a fur trapper at 3 different times (October 2023, April 2024, and October 2024). The small intestine, ceca, and large intestine were divided into ~10 cm lengths and examined for adult echinostomes under a stereomicroscope. These trematodes were putatively identified based on the presence of a collar with spines surrounding the oral sucker (Fried et al., 1997) and their total body length (mean = 16.32 mm, ranging from 9.93 to 23.85 mm; Sultana, unpublished).

Pools of eggs were created by teasing apart the uteri from approximately 5 worms each in petri dishes filled with autoclaved, non-chlorinated water. The anterior 1/3 portion of each worm (oral sucker to start of uterus) was removed and stored in 100% ethanol at -20 °C for genetic identification using methods outlined in Hodinka & Detwiler (2024). Each petri dish containing eggs was incubated in the dark at approximately 22 °C to allow for miracidia to develop and hatch from the eggs. After a 12-day incubation period, each pool of eggs was examined under a stereomicroscope to check for free-swimming miracidia. If miracidia were not observed, the incubating eggs were exposed to overhead lamps (100 watt incandescent light bulb) placed 38 cm above the dishes to stimulate hatching, and they were checked every 2 hours or less until a sufficient number of miracidia were observed for snail exposures.

Approximately 200 lab-raised *L. elodes* snails, ranging in shell size from 1-21 mm (spire to aperture), were haphazardly selected from the breeding colony maintained in the Animal Holding Facility (AHF) at the University of Manitoba. The size of each snail was measured for shell length (tip of spire to aperture) using a digital caliper (accuracy of 0.1 mm), and shell and body mass was determined using a scale (accuracy of 0.01 g). Each snail was placed individually into a well of a 6-well plate, which was filled to approximately three-quarters of non-chlorinated water, and each snail was exposed to either 2, 5 or 10 miracidia. Following at least 12 hours of exposure, snails were transferred back to their containers, which were filled with clean water and fresh lettuce. This process was repeated three times (October 2023, April 2024, and October 2024) to collect a range of cercarial densities, as infection rates in the snail colony were low. Infection prevalence was determined by monitoring the snails exposed to miracidia for cercarial shedding. The proportion of snails that shed cercariae was used to calculate prevalence: 13

infected out of 150 snails exposed (8.7%), 12 of 108 snails (11.1%), and 16 of 330 (4.8%), respectively).

Following approximately 18 hours of exposure to miracidia, snails were grouped into 600 mL circular plastic containers (top diameter of 130 mm, bottom diameter of 80 mm, and height of 80 mm), with 6 snails per container and filled with non-chlorinated water. They were fed *ad libitum* with green leaf lettuce, with one piece of lettuce provided per snail (e.g., for 6 snails, 6 pieces of lettuce were given). The lettuce was rinsed thoroughly with non-chlorinated water before placing in the container. The water in each container was replaced twice a week, and any uneaten lettuce was removed to keep the amount of food per container the same. Each container also contained a small piece of white dustless chalk (Crayola Sanigene) approximately 15.60 mm in length and weighing 2 g, which was a source of calcium carbonate to help maintain the thickness of the shells and prevent mortality due to calcium deficiency (Wacker & Baur, 2004; Egonmwan, 2008).

Approximately 6-8 weeks following exposures to miracidia, snails were assessed to determine whether cercariae had developed by placing individual snails into each well of 6-well plates filled to three-quarters with non-chlorinated water, with lamps positioned 38 cm above the plates. Snails were placed under light at approximately 09:00, with each well being examined under a stereomicroscope every 30 minutes to determine whether cercariae were emerging into the water. Cercarial emergence was typically observed around 12:00, after which cercariae from infected snails were collected and pooled into beakers over approximately 3 hours, typically from 12:00 to 15:00. The number of cercariae within each beaker was estimated from the mean of three 0.5 mL aliquots, which were each placed in 100% ethanol within a gridded petri dish to make them immobile during counting and improve the accuracy of the cercarial density estimate.

The number of counted cercariae was subtracted from the total number of cercariae in the pool to ensure the mean number of cercariae reflected the cercariae killed to determine the number of cercariae within the pool.

Once the number of cercariae was estimated for each pool, cercariae were passed through a 70 µm mesh that was stacked on top of a 20 µm mesh and secured over a glass beaker. Both meshes were pre-wet with filter-sterilized water to facilitate the flow of water. The 70 µm mesh filtered out larger particles (e.g., snail feces/mucus) and allowed the cercariae to pass through, while the 20 µm mesh trapped the cercariae. While trapped on top of the 20 µm mesh, the cercariae were rinsed 3 times to separate them from any source of host chemicals and transferred to a glass beaker filled with 10 mL of non-chlorinated filter-sterilized water by having the mesh come into contact with the surface of the autoclaved non-chlorinated water. Each sample of pooled cercariae was placed under lights (100 watt incandescent bulb set 38 cm above sample) for 4 hours to allow the cercariae to condition the water with their emissions. This water conditioned with and containing cercariae was transferred into a 15 mL falcon tube, and flash frozen and stored in the -80 °C freezer.

To extract lipids from the water samples conditioned with cercariae, samples were thawed on ice for 0.5-2 hours. To each sample, 0.1 mL of a standard oxylipin mixture was added, which was composed of 0.2 mg/mL BHT, 0.2 mg/mL EDTA, 100 µM indomethacin, and 100 µM trans-AUCB in 50:50 methanol:water (v/v) (Rund et al., 2018). This mixture also contained a deuterated oxylipin (8-iso-prostaglandin $F_{2}\alpha$), which was used as an internal standard to correct for extraction efficiency and instrument variability. Then, the solution was filtered using a syringe fitted with a 0.2 µm nylon membrane (Thermo Fischer Nalgene) to remove any snail feces, parasite larvae, or other debris. The volume of the filtered sample plus the standard was

recorded and passed through a Strata-X SPE Phenomenex column placed on a vacuum filter apparatus. A weak vacuum was used to avoid drying out the column, and the column was preconditioned with 2 mL of 100% MeOH followed by 2 mL of pH 3 water. Next, the filtered cercariae water sample was applied to the preconditioned column, allowing the sample to run through with a weak vacuum, ensuring the column did not go dry at any point. After the sample passed through the column, the sample tube was rinsed with 1 mL of 10% MeOH in pH 3 water, which was run through the column. To elute the lipids from the column, 1 mL of 100% MeOH was added, and was allowed to drip through the column for at least 1 minute into a 1.5 mL microtube. Gentle pressure was applied to the plunger of the syringe attached to column to maintain a continuous flow, followed by flushing the column dry with nitrogen for 30 seconds. The sample was then placed in the -80 °C freezer prior to being shipped for HPLC-MS/MS analysis. Please refer to Figure 1.2 for a visualization of the methods.

To determine the diversity of types of oxylipins emitted, I examined the data for all oxylipins quantified among the samples. The full list of oxylipins screened by HPLC-MS/MS (n = 158 oxylipins) is provided in the Appendix (Table 3.1). Samples that appeared as outliers either in terms of the lack of oxylipins quantified or in terms of unusual amounts (e.g., negative amounts in comparison to controls). Using this dataset, I then removed any oxylipins if they were not quantified in at least 75% of the samples (≥7 of 9). To examine whether density affected the oxylipin profiles of cercariae, regression analyses were performed on the reduced dataset to determine whether ≈ were linearly related to cercarial density. For oxylipin diversity, I examined the data for all oxylipins quantified among the samples as well as for a reduced set with oxylipins removed if they were not quantified in 75% of the samples (≥7 of 9). For oxylipin concentration, I only analyzed the relationship with cercarial density for oxylipins that met the

threshold rule. Furthermore, I grouped individual oxylipins according to their enzymatic pathways (LOX, COX, and CYP) to qualitatively compare whether cercarial density influenced oxylipin diversity according to pathway.

Results

Diversity of Types of Oxylipins

Using high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS), 158 oxylipins were scanned across 10 samples, with cercarial densities ranging from 363 to 1621 cercariae per 10 mL (Figure 1.2). Two of the samples were analyzed in a separate batch from this chapter (363 and 584 cercariae/10 mL) alongside chapter 2 samples. The same internal standard was added to all samples to help mitigate any potential variation in detection and quantification of oxylipins. One sample (544 cercariae/10 mL) was omitted from the analysis due to fewer oxylipins being quantified in comparison to the other samples and for those that were quantified, several consisted of negative values (i.e., less concentrated than the negative controls). Oxylipins were considered quantifiable when their chromatographic peak exceeded five times the baseline signal (lower limit of quantification, LLOQ). Upon omitting the precursors and other oxylipins that were below the LLOQ, 40 oxylipins were quantified across the nine samples (Figure 1.3). Of these, 22 belonged to the lipoxygenase (LOX) pathway, 8 to the cyclooxygenase (COX) pathway, and 10 to the cytochrome P450 (CYP) pathway (Figure 1.3). Across the nine samples, some oxylipins appeared in only one sample while others were present in all nine, and the number of oxylipins quantified in each sample ranged from 11 in the sample with the fewest to 26 in the sample with the most (Figure 1.3). Upon applying a detection threshold that oxylipins must be quantified in at least 75% of samples (≥7 of 9), the total number of oxylipins decreased to 7 oxylipins (Figure 1.4). Of these, 5 belonged to the LOX pathway, 1

to the COX pathway, and 1 to the CYP pathway (Figure 1.4). The majority of consistently, quantified oxylipins were derived from LA, particularly among those originating from the LOX pathway, suggesting that LA-associated pathways may dominate the oxylipin profiles of *E. trivolvis* cercariae. It is important to note that as the number of samples analyzed was relatively low, and some samples had oxylipins with concentrations below the LLOQ, these results should be regarded as preliminary and should be interpreted as observations rather than statistically robust findings.

To assess whether cercarial density influences the diversity of types of oxylipins, I performed both Spearman correlation and linear regression. Spearman correlation was used to test for a monotonic relationship that does not assume linearity or normality, while linear regression assessed the presence of a linear trend. Across the nine cercarial density samples (363-1621 cercariae/10 mL), there was no significant monotonic relationship between the number of oxylipins quantified and cercarial density (Spearman's rho (ρ) = -0.24, p = 0.53) and no significant linear relationship (regression coefficient = -0.0033, t_7 = -0.87, p = 0.41; $F_{1,7}$ = 0.76, R^2 = 0.098, adjusted R^2 = -0.031) (Figure 1.5). Similarly, when focusing on the subset of oxylipins quantified in at least 75% of samples (≥7 of 9), there was no significant monotonic relationship between the number of oxylipins quantified and cercarial density (Spearman's rho (ρ) = 0.29, p = 0.45) and no significant linear relationship (regression coefficient = 0.00017, t_7 = -0.20, p = 0.485; $F_{1,7}$ = 0.041, R^2 = 0.006, adjusted R^2 = -0.14) (Figure 1.6).

Concentrations of Oxylipins

To determine whether cercarial density influenced oxylipin concentrations, I conducted Spearman's rank correlations for all oxylipins quantified across the nine cercarial density samples (361-1621 cercariae/10 mL). Among the 40 oxylipins quantified, none of them showed

a significant monotonic relationship with cercarial density (all $p > 0.05$; Table 1.1), indicating that oxylipin concentrations did not consistently increase or decrease as cercarial density changed. Along with statistical analyses, oxylipin concentrations were visualized across the nine cercarial density samples (Figure 1.7). Most oxylipins were present at lower concentrations (often below 0.01 ng/mL), while two linoleic acid (LA)-derived oxylipins belonging to the lipoxygenase (LOX) pathway (9,10,13 triHOME and 9,12,13 triHOME) exhibited higher concentrations (Figure 1.7A) and were removed to allow for better visualization of the other 38 oxylipins (Figure 1.7B). Full concentration plots for each oxylipin across cercarial densities have been provided in the Appendix (Figures 3.1-3.40), allowing for a more detailed evaluation beyond the visualization shown in Figure 1.7. Focusing on the subset of oxylipins quantified in at least 75% of samples (≥7 of 9), seven oxylipins were visualized across the nine cercarial density samples (Figure 1.8A). Similarly to Figure 1.7, two LA-derived oxylipins from the LOX pathway (9,10,13 triHOME and 9,12,13 triHOME) showed higher concentrations and thus, were excluded to allow for clearer comparison of the other 5 oxylipins (Figure 1.8B). Visually, the concentrations of some of these oxylipins appeared to be low at the lowest densities, increased at subsequent densities, declined at intermediate densities, increased again at higher densities, and then declined again at the highest densities, highlighting variable concentration patterns across densities.

Discussion

This study confirms that, in the absence of any hosts, echinostome trematode cercariae emit oxylipins. Prior to this study, it was known that infection with echinostomes alters the oxylipin profiles of snail hosts, but whether those changes were due directly or indirectly to parasites was unclear (Friesen et al., 2022a; 2022b). This study represents the first step towards

teasing apart the emissions from parasites and snails by isolating emissions from parasites only. I have provided evidence that cercariae of *E. trivolvis* lineage c synthesize oxylipins, primarily through one enzymatic pathway, the LOX pathway. Snails infected with the progenitor stage of cercariae for echinostome parasites (rediae) also primarily emitted oxylipins stemming from this LOX pathway along the CYP pathway (Friesen et al., 2022a; 2022b). In comparison to cercariae alone, *L. elodes* snails infected with rediae of *Echinoparyphium* lineage emitted more than double the number of oxylipins (90) compared to cercariae alone (41) (Friesen et al., 2022a). In the same study, uninfected *L. elodes* snails emitted a total of 50 oxylipins, which is more similar to cercariae alone, but both ratios (number of number of oxylipins quantified divided by the number scanned for) are drastically lowered once the threshold rule was applied to cercariae. The reasons for the differences in the number of oxylipins emitted between cercariae and infected snails are unclear as they could relate to several factors such as differences in the ability of snails and parasites to synthesize fatty acid precursors, differences in diet between snails and parasites, differences in body size between snails and parasites, the combined oxylipins from hosts and parasites, and the fact that parasites were exposed to host chemical cues during water conditioning in the previous studies.

To my knowledge, this is the first study to explore whether density affects the oxylipin emissions of cercarial parasites. Given that cercariae emerge from individual snails in clusters of 10s-1000s of individuals, it is ecologically relevant to determine whether this affects the diversity of types and concentration of the oxylipins, to better understand how these chemicals may influence a cercaria(e) ability to find a host in a complex environment. In contrast to my predictions, I found that the diversity of types and concentrations of oxylipins did not increase linearly with increased density. Among the 7 oxylipins quantified in at least 75% of the samples

(≥7 of 9), 5 oxylipins exhibited a pattern where their concentrations were low at the lowest densities, increased at subsequent densities, declined at intermediate densities, then increased again at higher densities, then declined again, often times to zero at the highest densities. Interestingly, the precursor for the majority of these consistently, quantified oxylipins was linoleic acid, suggesting the need to further study the role of linoleic acid on the production and release of oxylipins by cercarial parasites of *E. trivolvis*. The lack of linear relationship suggests that cercariae were regulating their emissions as otherwise increases should have occurred if individual parasites were emitting the same concentrations of oxylipins as their group density increased. Contrary to predictions, as increased cercarial density did result in higher diversity of types and concentrations of oxylipins in a linear pattern, it suggests that oxylipin production may be regulated rather than passively increasing along with the number of cercariae. It is possible that since lipid mediators are involved in chemical signaling and host interactions, excessive oxylipin emissions may be energetically costly to this parasitic stage, which relies on energy reserves, and cercariae at higher densities may indeed downregulate their synthesis through feedback inhibition. High densities of cercariae may also influence individual cercarial behaviour or physiology, resulting in stress, competition, or other metabolic activities, which may reduce their production. Furthermore, methodological factors, such as a small sample size or limited replication, variation in extraction efficiency procedure or detection limits, may have contributed to these findings.

In addition to density, there is evidence that other factors like time since cercarial emergence and duration of exposure to chemical cues influence oxylipin emissions. For instance, cercariae of *Schistosoma mansoni* showed the ability to regulate the production of some eicosanoids (PGE1, 5-HETE) which decreased in concentration over a short time interval (1, 10,

and 60 minutes) when exposed to linoleate, a form of linoleic acid, metabolizing it into arachidonate-derived compounds through the LOX and COX pathway (Fusco et al., 1985). This latter study suggests that these cercariae may have the ability to activate their COX and LOX systems upon being exposed to skin essential fatty acids from the skin of the next host in the life cycle. It remains unclear if echinostome cercariae alter the emissions of oxylipins over time as our samples were conditioned over a longer time frame. Also, in contrast to this work, I did not explore whether oxylipin emissions from cercariae change in the presence of host cues.

Thus, questions remain about what endogenous or exogenous factors may be driving the oxylipin synthesis in echinostome cercariae in the absence of non-parasite cues. The patterns observed in the oxylipin profiles of *E. trivolvis* cercariae in this study could reflect either natural precursor turnover, biosynthetic regulation over time, or density-dependent effects. Future studies should explore whether *E. trivolvis* cercariae produce different types or concentrations of oxylipins across various time intervals to better understand how oxylipin production and release may fluctuate over time in the absence of external stimuli.

I characterized the oxylipin profiles from much smaller numbers of cercariae than previously used. To date, most studies pooled 10,000s of cercariae to characterize their PUFAs or oxylipins (Babaran et al., 2021; Fusco et al., 1985), so my study is also unique in that oxylipins were quantified from much lower numbers of cercariae than previously used making future explorations of the factors that influence oxylipins from cercariae much more feasible. I established methods to determine a range of densities at which oxylipins could be quantified using HPLC-MS/MS, which was previously unclear. Interestingly, while the absolute limit of detection or lowest possible threshold at which oxylipins from cercariae can be quantified cannot be determined through this study, this study highlights that it is possible to quantify oxylipins

from hundreds to lower thousands of cercariae, highlighting the sensitivity of this tool in detecting chemicals at lower cercarial densities and concentrations. I attempted to obtain even higher densities of cercariae, however, only a limited number of infected snails were obtained through lab exposures. Higher prevalence may be achieved if snails were exposed to greater than 5 miracidia though exposing to too many miracidia could increase snail mortality (Hodinka & Detwiler, 2024). Higher prevalence may also be achieved if measures were implemented to reduce mortality over the 6-8 weeks that the parasite requires for development. In this study, snails were housed at a density of 6 individuals per container, with each container holding 600 mL of water. However, future studies should consider maintaining snails at lower densities, as crowding of snails has been shown to reduce cercarial production. As shown by another study, cercarial shedding was highest at a density of 5 snails per litre (1451 ± S.E. 142), followed by 10 snails per litre (1160 ± S.E. 237), and lowest at 20 snails per litre (712 ± S.E. 59) (Coles, 1973). Thus, future studies could benefit from having a higher number of infected snails by housing snails at lower densities.

Conclusion

In this chapter, I found that cercariae of *E. trivolvis* lineage c have the ability to produce oxylipins. Furthermore, I demonstrated that by using HPLC-MS/MS, it is possible to quantify oxylipins being produced by cercariae at relatively low densities (fewer than 500 cercariae per 10 mL). I found that density influences the concentrations of some oxylipins in a non-linear pattern, suggesting that interactions among cercariae, whether through crowding effects, metabolic changes, or density-dependent release of cues, may affect the chemical profiles of cues being emitted into the aquatic environment. However, it is important to note that the interpretation of these density patterns is limited by cercariae being pooled rather than measured at the individual

level and replication across density treatments being limited. A key finding of this chapter was that LA-derived oxylipins, particularly those produced through the LOX pathway, were consistently, quantified oxylipins of *E. trivolvis* cercariae. Although echinostome cercariae infect different species of hosts compared to schistosomes (i.e. aquatic invertebrates and amphibians vs. humans, other mammals, and birds), in which-LA associated oxylipin pathways have described, the prominence of LA-derived oxylipins in *E. trivolvis* suggests that LA may an important role in shaping the oxylipin profiles of *E. trivolvis* and warrants further investigation of its influence in snail-echinostome trematode.

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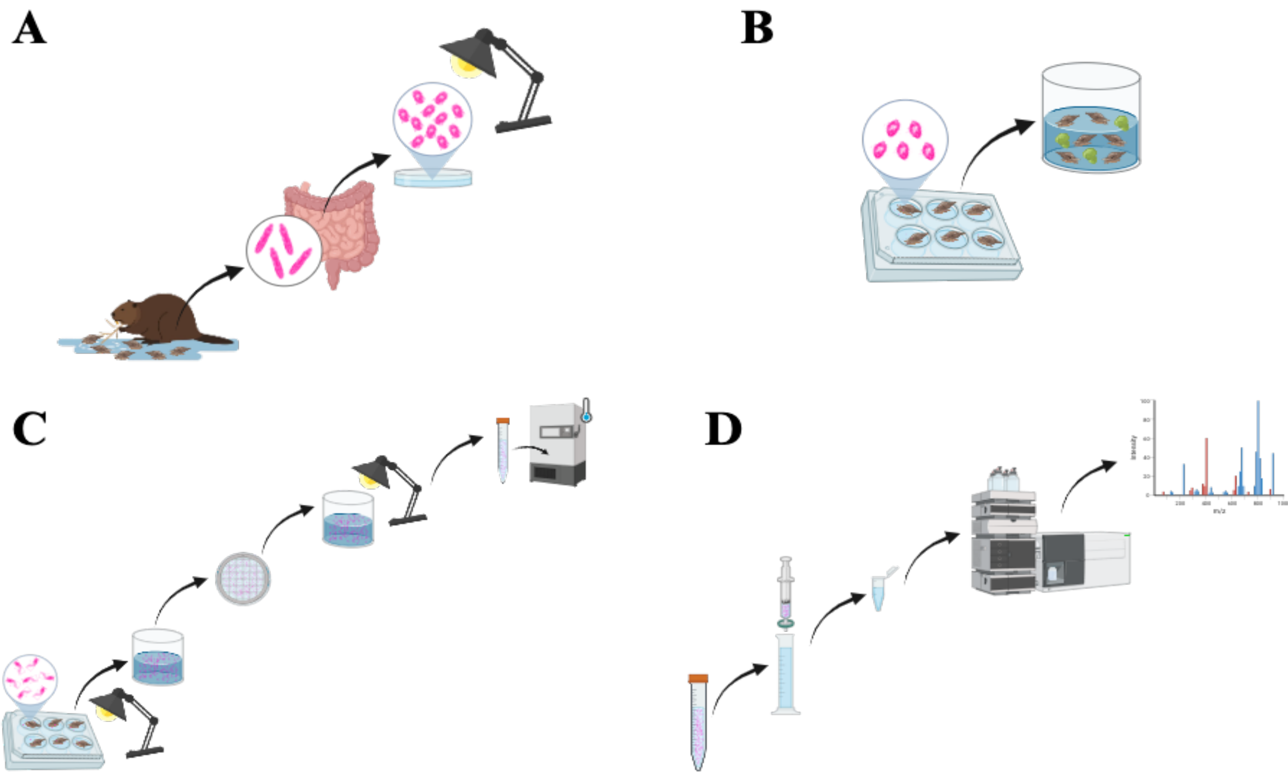

Figure 1.2. Overview of experimental methods to assess oxylipin emissions of *Echinostoma trivolvis* cercariae at different densities. (A) Obtained miracidia from incubated eggs from adult echinostomes from wild-caught muskrats. (B) Exposed lab-raised *Ladislavella elodes* to miracidia. (C) Isolated and estimated cercarial densities, and conditioned water with cercariae. (D) Extracted and scanned for oxylipins from the cercariae-conditioned water using HPLC-MS/MS

Figure 1.3. Diversity of types of oxylipins across cercarial densities (n = 9 samples; 363-1621 cercariae/10 mL). Each coloured tile represents the presence of a specific oxylipin in a particular sample. Oxylipins are grouped by pathway: COX (orange), CYP (blue), and LOX (purple). Numbers above each column indicate the total number of oxylipins quantified in a sample, and numbers to the right of each row represent the total number of samples in which each oxylipin was quantified.

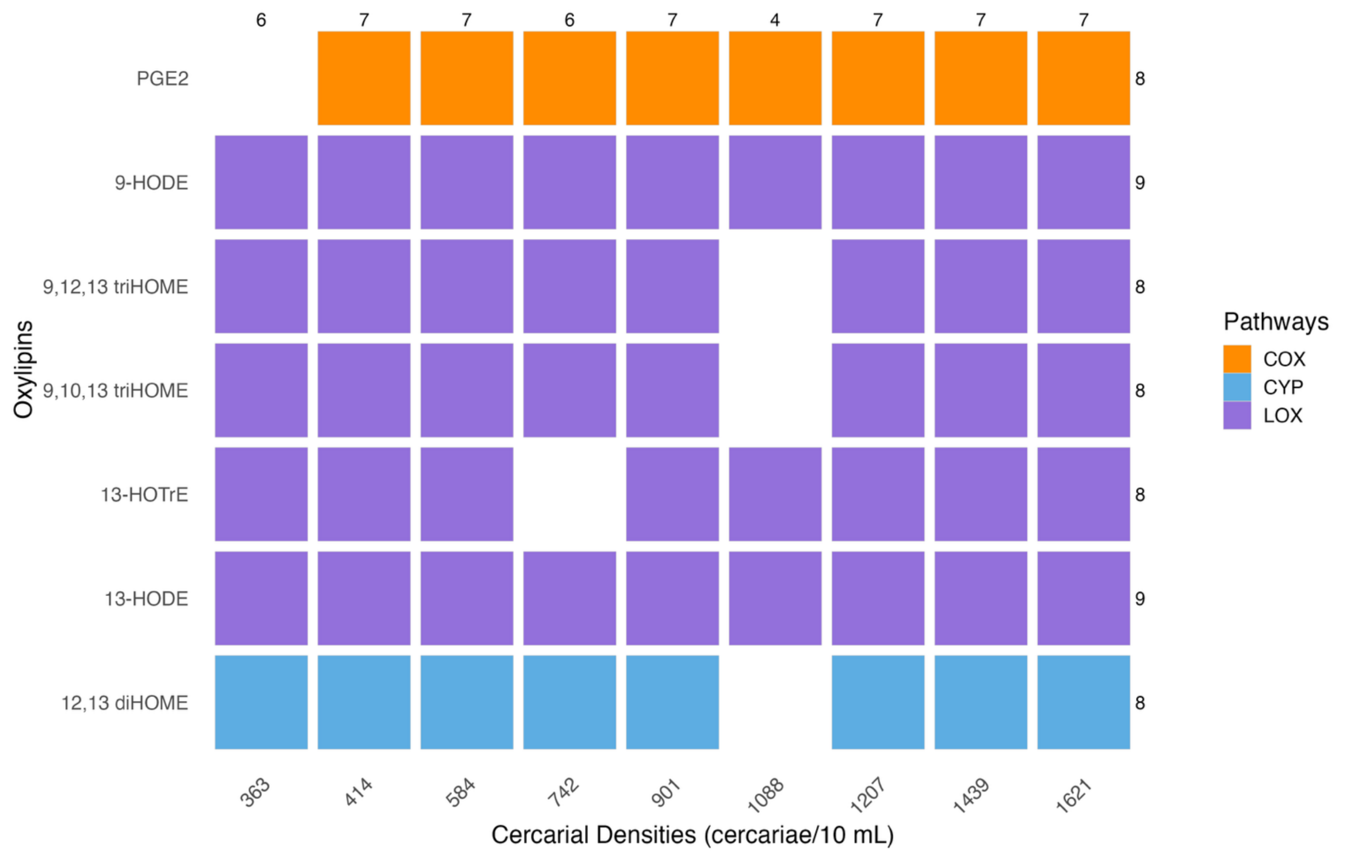

Figure 1.4. Diversity of types of oxylipins (n = 9 samples; 363-1621 cercariae/10 mL), showing only oxylipins quantified in at least 75% of samples (≥7 of 9). Each coloured tile indicates the presence of an oxylipin quantified in a sample. Oxylipins are grouped according to pathway: COX (orange), CYP (blue), and LOX (purple). Numbers above each column are the total number of oxylipins quantified in a sample, and numbers beside each row represent the total number of samples in which each oxylipin was quantified.

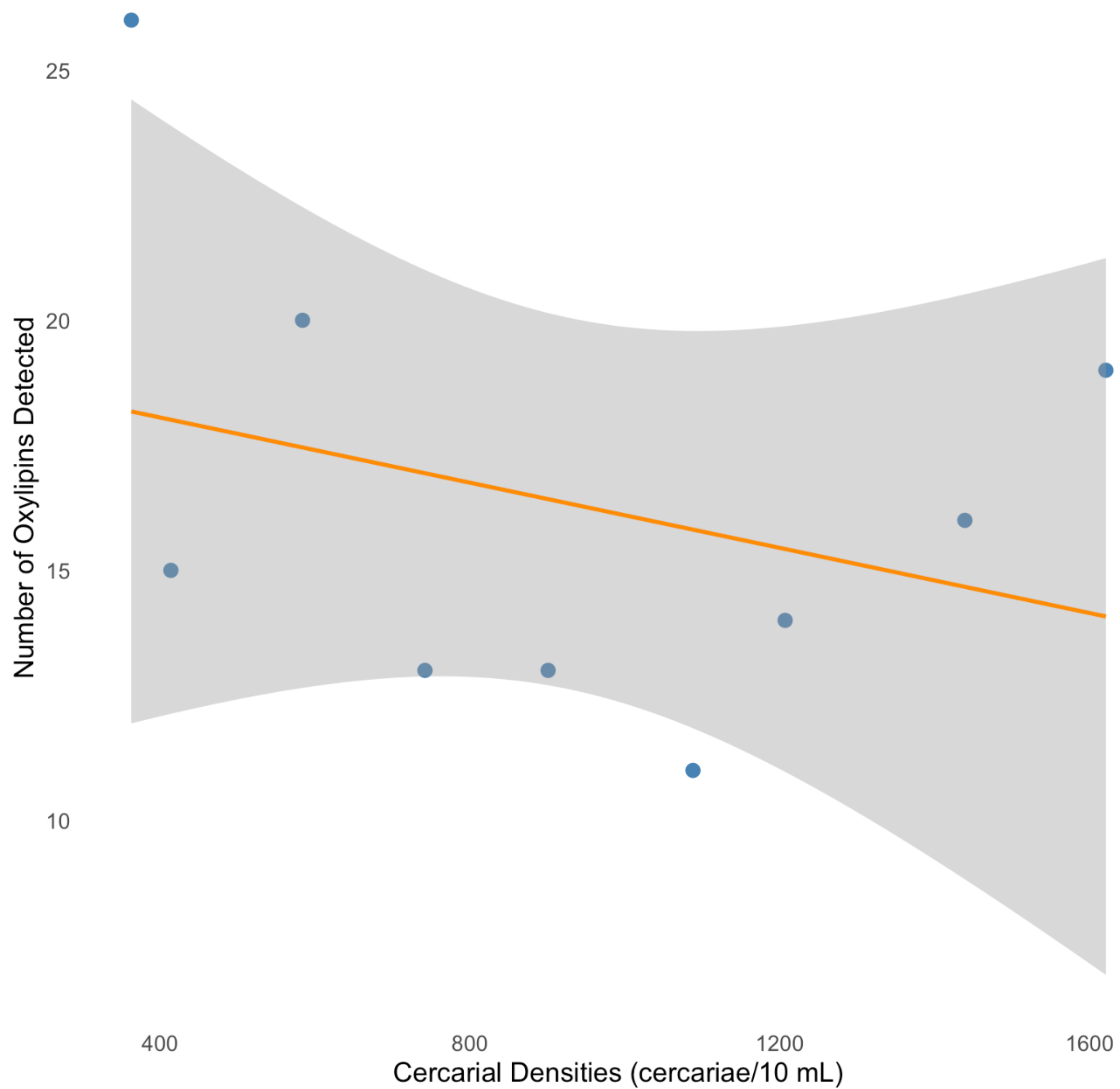

Figure 1.5. Number of oxylipins quantified across nine cercarial density samples (363-1621 cercariae/10 mL). The orange line shows the linear regression fit with 95% confidence interval.

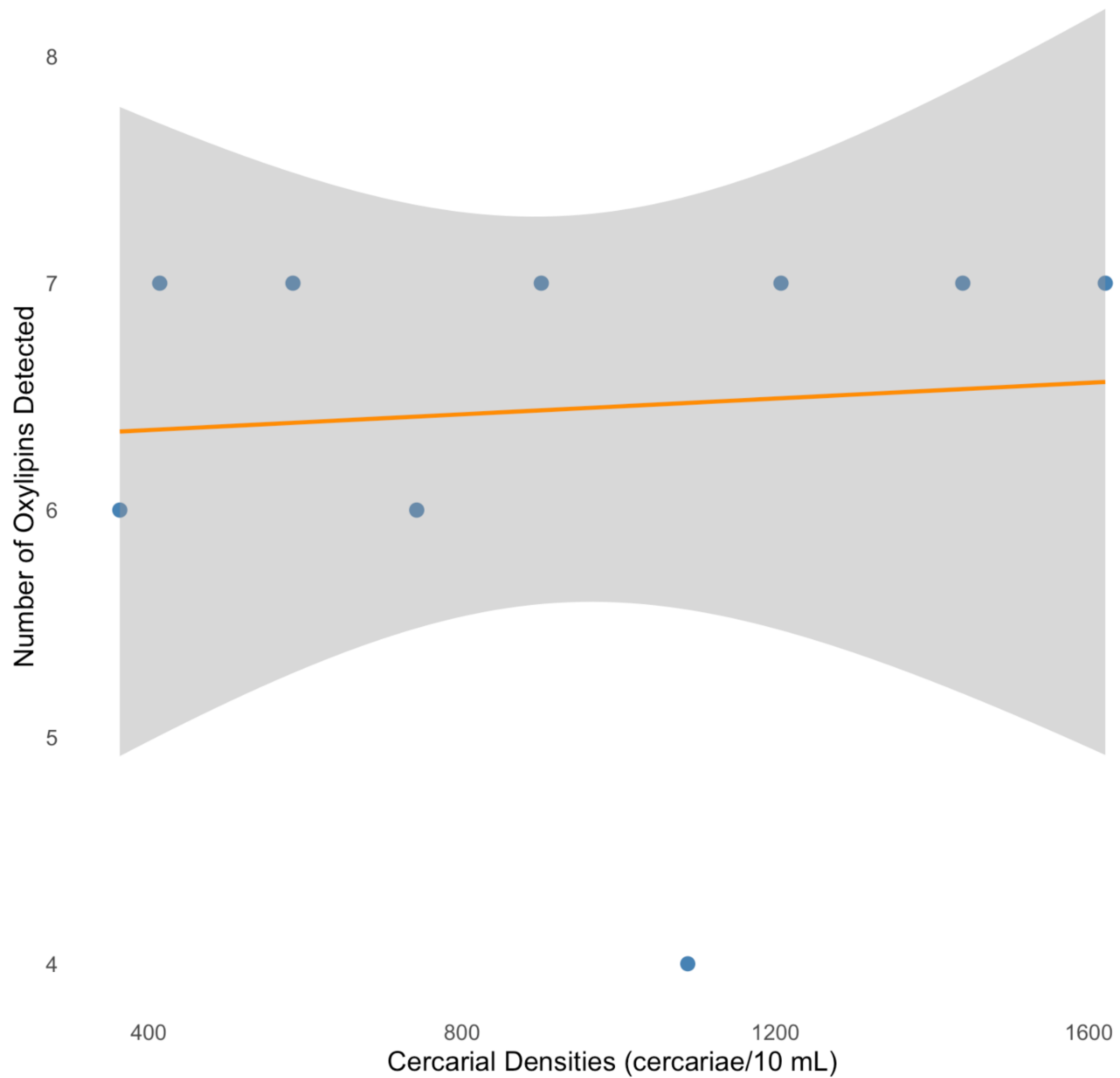

Figure 1.6. Number of oxylipins quantified across nine cercarial density samples (363-1621 cercariae/10 mL), showing only oxylipins quantified in at least 75% of samples (≥7 of 9). The orange line shows the linear regression fit with 95% confidence interval.

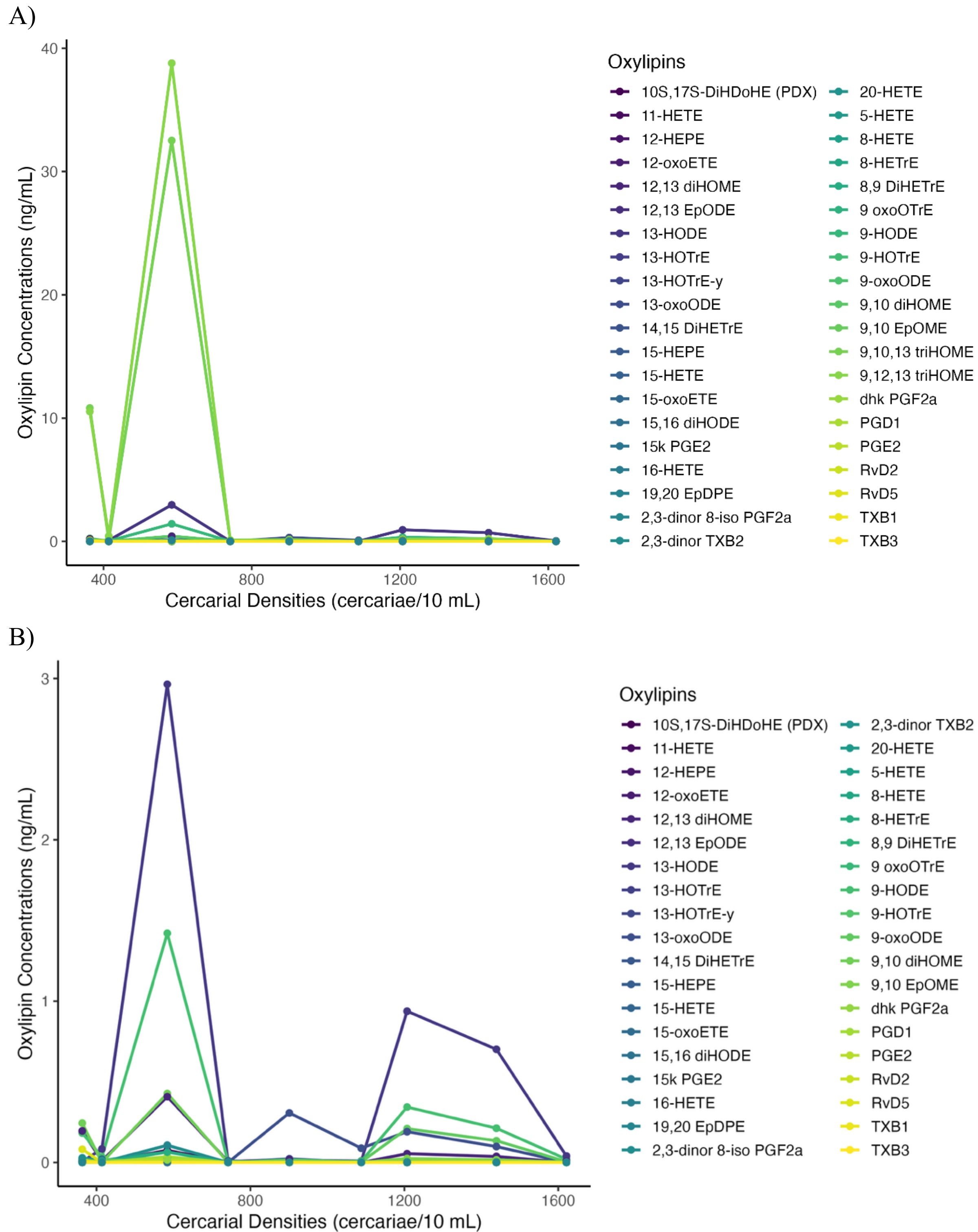

Figure 1.7. Oxylipin concentrations (ng/mL) across nine cercarial density samples (363-1621 cercariae/10 mL). Panel A includes all 40 quantified oxylipins, while panel B excludes 2 oxylipins (9, 10, 13 triHOME and 9, 12, 13 triHOME), whose extreme concentrations limited the visualization of the other oxylipins.

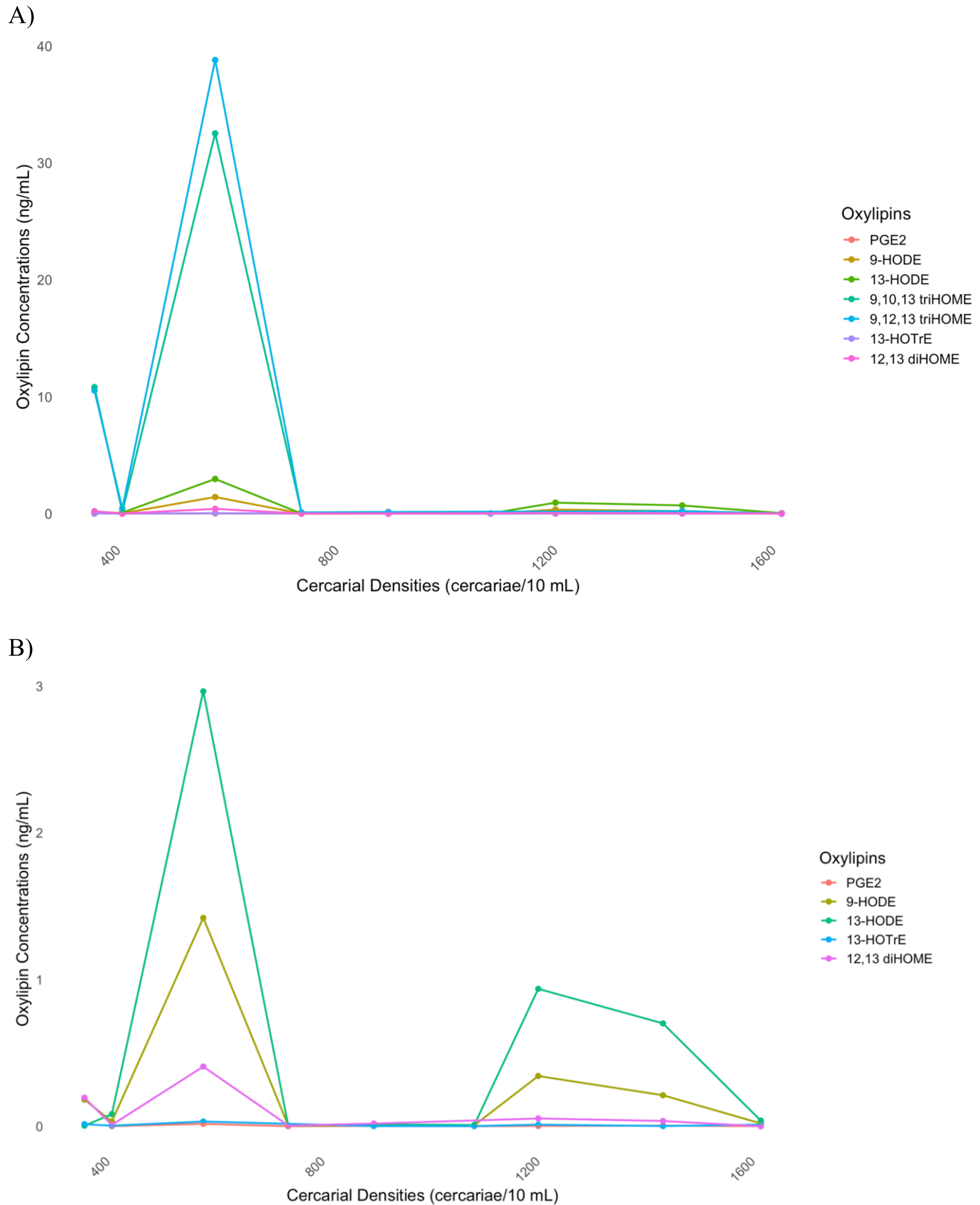

Figure 1.8. Oxylipin concentrations (ng/mL) across nine cercarial density samples (363-1621 cercariae/10 mL), showing only oxylipins quantified in at least 75% of samples (≥7 of 9). Panel A includes 7 oxylipins, while panel B includes 5 oxylipins (2 oxylipins - 9, 10, 13 triHOME and 9, 12, 13 triHOME were omitted), whose extreme concentrations limited the visualization of the other oxylipins.

Tables

Table 1.1. Oxylipin concentrations (ng/mL) for individual cercarial density samples (cercariae/10 mL) and mean concentrations (ng/mL ± SE), shown with their corresponding parent PUFA (polyunsaturated fatty acid) and enzymatic pathway. Spearman's ρ (rho; df = 7) indicates the rank correlation between oxylipin concentrations and cercarial density. Abbreviations: Precursors: LA = linoleic acid (C18:2); GLA = gamma-linolenic acid (C18:3); DGLA = dihomo-gamma-linolenic acid (C20:3); ARA = arachidonic acid (C20:4); ALA = alpha-linolenic acid (C18:3); EPA = eicosapentaenoic acid (C20:5); DHA = docosahexaenoic acid (C22:6). Pathways: LOX = lipoxygenase; COX = cyclooxygenase; CYP = cytochrome P450.

		Concentrations (ng/mL) at Cercarial Density Samples (estimated density ± standard error)											
Precursors	**Oxylipins**	**363 ± 77 cercariae/10 mL (n = 1)**	**414 ± 94 cercariae/10 mL (n = 1)**	**584 ± 96 cercariae/10 mL (n = 1)**	**742 ± 179 cercariae/10 mL (n = 1)**	**901 ± 122 cercariae/10 mL (n = 1)**	**1088 ± 155 cercariae/10 mL (n = 1)**	**1207 ± 92 cercariae/10 mL (n = 1)**	**1439 ± 116 cercariae/10 mL (n = 1)**	**1621 ± 278 cercariae/10 mL (n = 1)**	**Mean Concentrations (ng/mL) ± Standard Errors**	**Spearman's ρ (rho)**	***P***
						LOX Pathway							
LA	9-HODE	0.18267	0.03335	1.42072	0.00585	0.00712	0.00728	0.34322	0.21212	0.02007	0.24804 ± 0.15196	-0.033	0.948
LA	9-oxoODE	0	0.01922	0	0	0.01627	0.00347	0.20945	0.13485	0.00463	0.0431 ± 0.02528	0.525	0.146
LA	13-HODE	0.00493	0.08345	2.96381	0.00517	0.01448	0.00953	0.93735	0.70145	0.03939	0.52884 ± 0.32589	0.3	0.437
LA	13-oxoODE	0	0.00173	0	0	0.30688	0.08838	0.19048	0.09858	0	0.07623 ± 0.03646	0.357	0.346
LA	9,10,13-triHOME	10.81833	0.1395	32.51726	0.1051	0.1333	0	0.1769	0.1974	0.00962	4.89971 ± 3.64856	-0.433	0.25
LA	9,12,13-triHOME	10.53249	0.43216	38.78998	0.08106	0.14166	0	0.17246	0.21776	0.0041	5.59685 ± 4.30433	-0.617	0.086
GLA	13-HOTrE-y	0	0.00458	0	0	0.00335	0	0	0	0	0.00088 ± 0.00059	-0.342	0.367
ALA	13-HOTrE	0.01512	0.00484	0.03318	0	0.00224	0.002	0.01208	0.00227	0.01221	0.00933 ± 0.00349	-0.2	0.613
EPA	15-HEPE	0	0.01945	0	0	0.0228	0	0	0	0	0.00469 ± 0.00312	-0.274	0.476
ARA	8-HETE	0.00152	0	0	0	0	0.00106	0	0	0.00187	0.00049 ± 0.00026	0.149	0.703
ALA	9-HOTrE	0.00891	0.00042	0.01375	0	0	0.00389	0.00165	0	0.00465	0.0037 ± 0.0016	-0.237	0.539
ALA	9-oxoOTrE	0.0112	0	0	0	0	0.00856	0	0.0041	0.00436	0.00314 ± 0.00142	0.11	0.779
ARA	5-HETE	0.00327	0	0	0.00565	0	0	0	0.00298	0.0078	0.00219 ± 0.00098	0.219	0.571
ARA	12-oxoETE	0	0	0	0.01044	0	0	0	0	0.00819	0.00207 ± 0.00138	0.251	0.515
ARA	15-HETE	0.0078	0.00471	0	0.00928	0	0	0	0	0.00424	0.00289 ± 0.00125	-0.475	0.197
ARA	15-oxoETE	0	0	0.00302	0.00591	0	0	0	0	0	0.00099 ± 7e-04	-0.297	0.438
EPA	12-HEPE	0.00357	0	0	0.001	0	0	0.00343	0.00074	0.00235	0.00123 ± 5e-04	0.122	0.755
ARA	11-HETE	0.0022	0	0	0	0	0	0	0	0.00051	3e-04 ± 0.00024	-0.091	0.815

DHA	RvD$_2$	0.01299	0	0	0	0	0	0	0	0	0.00144 ± 0.00144	-0.548	0.127
DHA	RvD$_5$	0.08155	0	0	0	0	0	0	0	0	0.00906 ± 0.00906	-0.548	0.127
DHA	10S,17S-DiHDoHE (PDX)	0.00903	0	0.07553	0	0	0	0	0	0	0.0094 ± 0.00833	-0.593	0.092
DGLA	8-HETrE	0	0	0.06415	0	0	0	0	0	0	0.00713 ± 0.00713	-0.274	0.476
							COX Pathway						
ARA	PGE$_2$	0	0.00182	0.01755	0.00148	0.00339	0.0015	0.00306	0.00496	0.00165	0.00393 ± 0.00177	0.267	0.493
ARA	15-keto-PGE$_2$	0	0	0	0	0	0	0	0.00181	0	2e-04 ± 2e-04	0.411	0.272
ARA	2,3-dinor-TXB$_2$	0.00971	0	0.00712	0	0	0	0	0	0	0.00187 ± 0.00126	-0.639	0.064
DGLA	PGD$_1$	0.01809	0	0.03591	0	0	0	0	0	0	0.006 ± 0.00424	-0.593	0.092
DGLA	TXB$_1$	0.0026	0	0.00209	0	0	0	0	0	0	0.00052 ± 0.00035	-0.639	0.064
EPA	TXB$_3$	0.00182	0	0.00072	0	0	0	0	0	0	0.00028 ± 0.00021	-0.639	0.064
ARA	2,3-dinor 8-iso-PGF$_{2\alpha}$	0.02437	0	0.10699	0	0	0	0	0	0	0.0146 ± 0.01186	-0.593	0.092
ARA	dhk-PGF$_{2\alpha}$	0	0	0.01551	0	0	0	0	0	0	0.00172 ± 0.00172	-0.274	0.476
							CYP Pathway						
LA	9,10-diHOME	0.24362	0	0.42724	0	0.00389	0	0.01434	0.01734	0	0.07849 ± 0.05096	-0.252	0.512
LA	12,13-diHOME	0.19539	0.01027	0.40757	0.00601	0.01938	0	0.05414	0.03699	0.00068	0.08116 ± 0.04562	-0.4	0.291
ARA	14,15-DiHETrE	0	0	0	0	0.00049	0	0	0	0	0 ± 0	0	1
ARA	8,9-DiHETrE	0	0	0	0.00018	0	0	0	0	0	2e-05 ± 2e-05	-0.137	0.725
LA	9,10-EpOME	0	0.00338	0	0.00289	0	0.00109	0.02462	0.0161	0.00226	0.00559 ± 0.00291	0.458	0.215
ARA	16-HETE	0.02137	0	0	0	0	3e-05	0	0	0	0.00238 ± 0.00237	-0.365	0.334
ARA	20-HETE	0.02838	0.01209	0.06618	0	0	0	0.00356	0.00016	0.00086	0.01236 ± 0.00743	-0.458	0.215
ALA	12,13-EpODE	0.0041	0	0	0	0	0	0	0	0.00322	0.00081 ± 0.00054	-0.091	0.815
LA	15,16-diHODE	0.01139	0	0	0	0	0	0	0	0	0.00127 ± 0.00127	-0.548	0.127
DHA	19,20-EpDPE	0	0	0.10577	0	0	0	0	0	0	0.01175 ± 0.01175	-0.274	0.476

*n refers to the number of samples

Chapter 2: The effect of host exposure to linoleic acid on the oxylipin emission from cercariae of *Echinostoma trivolvis* lineage c

Abstract

Host diet is a factor that can influence the chemical ecology of host-parasite interactions, particularly through the availability of fatty acid precursors for oxylipin synthesis. This chapter explores how host exposure to linoleic acid, an omega-6 fatty acid and a key precursor for the production/synthesis of certain oxylipins, affects the oxylipin emission of *Echinostoma trivolvis* cercariae. Specifically, this study investigates whether exposure to linoleic acid alters the chemical composition of host tissues, which are consumed by *E. trivolvis* rediae, potentially influencing the chemical profile of the developing cercariae. Freshwater snails (*Ladislavella elodes*) were assigned to one of two groups: an experimental group maintained on a lettuce diet and exposed to linoleic acid and a control group on a lettuce diet. Cercariae from both groups were collected and analyzed using high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS). Across all eight samples (control: n = 4 samples: 470-640 cercariae/10 mL; experimental: n = 4 samples: 385-674 cercariae/10 mL), out of the 158 oxylipins scanned for, 47 oxylipins were quantified. Of these, 26 belonged to the lipoxygenase (LOX) pathway, 11 to the cyclooxygenase (COX) pathway, and 10 to the cytochrome P450 (CYP) pathway. Among these, 29 oxylipins were quantified in at least 75% of the samples (≥3 out of 4 samples) either in the control group only, experimental only, or both. Of these, 16 belonged to the LOX pathway, 6 to COX, and 7 to CYP. No statistically significant differences were observed in oxylipin concentrations between treatment groups. Fatty acid analysis revealed higher levels of linoleic acid in experimental snail tissues to controls. Interestingly, experimental snails released more cercariae than controls across weeks (mean: control = 450 cercariae;

experimental: 1618 cercariae) and per infected snails (mean: control = 164 cercariae; experimental: 485 cercariae). Thus, these findings suggest that while exposing snail hosts to linoleic acid did not result in significant differences between the oxylipin emission profiles of *E. trivolvis* cercariae between the control and experimental groups, it may influence cercarial production, highlighting the role of host nutritional ecology in shaping parasite population dynamics in natural environments.

Introduction

Diet plays an important role in shaping not only the physiological condition of hosts, but also that of their parasites (Schlotz et al., 2013). Through its effects on host growth, host diet can impact various aspects of parasite biology including reproduction (Sandland & Minchella, 2003). Since host-parasite interactions are often regulated by host nutritional state (Schlotz et al., 2013), the relationship between host diet and parasite physiology is an important and growing area of research within chemical ecology. It remains unknown whether the production and emission of chemicals released by parasites changes if host diet affects the host tissues that the parasites are consuming.

Host diet can be manipulated by altering the quantity (i.e., how much food is available) as well as the quality (i.e., what is in the food). Both aspects have been shown to influence parasite condition. When the freshwater snail host *Lymnaea stagnalis* was starved, not only did stressed hosts experience a higher mortality rate, but their parasites also produced fewer and poorer quality cercariae (larval parasite stage that emerges from snails to infect next host in the life cycles) (Seppälä et al., 2008). If the reduced number and condition of cercariae affects their ability to encounter a host, and successfully penetrate, migrate, and become infective within the host, then these negative effects affect the survival of the parasite as a whole as cercariae are essential for transmission from one host to another.

In addition to quantity, diet quality of the host can vary depending upon levels of macronutrients such as proteins and lipids. Several studies have manipulated the amount of protein in snail host diets and found that hosts fed more protein had more cercariae that emerged from them (Keas et al., 1997; Sandland & Minchella, 2003). Cercarial emergence is a measure of parasite reproduction as the cercariae are the products of asexual reproduction within the snail.

However, this increase in asexual larval parasite reproduction was not directly due to host diet, but rather due to the high protein diet resulting in increased host size (Sandland & Minchella, 2003). In other words, more cercariae emerged from larger hosts, and more hosts tended to be larger on a higher quality diet. Host size can also affect the diversity of types and concentrations of lipid-based chemicals being emitted from hosts (Friesen et al., 2024). Lipids, are a diverse group of molecules, including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acid (PUFAs), which can vary in the length of their chains, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and long-chain fatty acids (LCFAs). When PUFAs become oxidized, they lead to the production of oxylipins, which are bioactive molecules known to affect the reproduction and immunity of snails (Stanley-Samuelson, 1994). The oxylipin profiles from the freshwater snail *Planorbella duryi* differed based on size with the greatest differences being between small, non-reproductive snails to either medium or large reproductive snails (Friesen et al., 2024). This study maintained snails on the same diet (i.e., low protein-low lipid lettuce-based diet), so it remains unknown how differences in host diet would affect oxylipin emissions from uninfected compared to trematode-infected snails.

In general, fatty acids in animals originate from two different sources: dietary sources (DS) or de novo synthesis (DNS). Among vertebrates, essential fatty acids such as linoleic acid (LA) and alpha-linoleic acid (ALA) belong to the DS category because they cannot be synthesized de novo and must be obtained primarily through their animal or plant diet (Das, 2006; Whelan & Fritsche, 2013). Consuming LA is important as it is a precursor for other omega-6 fatty acids and metabolizes either directly or indirectly into other omega-6 fatty acids such as gamma-linoleic acid (GLA) and arachidonic acid (ARA), respectively (Tortosa-Caparrós

et al., 2017; Innes & Calder, 2018). Other fatty acids such as certain SFAs and MUFAs can be synthesized internally by the organism (i.e., made by DNS). In addition, fatty acid synthesis may differ between vertebrates and invertebrates as some invertebrates, including freshwater snails, may have a limited ability to synthesize certain PUFAs from dietary precursors. Understanding these origins is important when examining the fatty acid profiles of animals, as dietary manipulations may result in changes in the tissue composition potentially due to a shift in DS and DNS.

Trematode sporocysts and rediae absorb and consume mollusc tissue, respectively, so their tissues, including the developing cercariae inside of them, could include PUFAs synthesized from snail host diet. In one of the few studies on this topic, snail tissue containing sporocysts of the trematode *Plagiorchis* sp. was enriched in several PUFAs relative to snail tissue including some omega-6 fatty acids like eicosadienoic acid (EDA) and dihomo-gamma-linolenic acid (DGLA) (Babaran et al., 2021). Given that PUFAs are precursors to oxylipins, this result suggests that the oxylipin emissions of cercariae would also be affected. Oxylipins are formed when fatty acids undergo oxidative processes via enzymatic pathways involving lipoxygenases (LOX), cyclooxygenases (COX), and cytochrome P450 enzymes (CYP) (Gabbs et al., 2015). For instance, the oxygenation of LA gives rise to certain oxylipins, such as 13-hydroxyoctadecadienoic acid (13-HODE) and its isomers via more than one pathway. Oxylipins, such as 13-HODE are known to regulate inflammation, immune responses, and cellular signaling in vertebrate hosts (Gabbs et al., 2015). The role of PUFAs and oxylipins in cercarial biology, remains limited largely to the schistosome trematodes, which use PUFAs and oxylipins emitted from their hosts as cues to undergo penetration behaviour (Haas, 1992; Haas 1994). The functions of PUFAs and oxylipins emitted from cercariae is less clear, but they are hypothesized

to induce skin penetration behaviour and transformation to the next developmental stage (i.e., schistosomules) as well as serve as immunomodulators that allow migrating parasites to evade the host immune response (Salafsky & Fusco, 1987). The focus on the cercariae from this group of trematodes is warranted as the cercariae penetrate the skin of humans and then develop into adult worms whose eggs cause schistosomiasis over 200 million people annually (Chitsulo et al., 2000; Dougherty et al., 2013). However, these trematodes represent a few species and one family relative to the 18,000 nominal species found in over 140 families of trematodes (Gibson et al., 2002; Jones et al., 2005; Bray et al., 2008). It remains unclear how taxonomically conserved oxylipin profiles would be among cercariae especially if they differ in host use as well as diet.

In this study, I tested the hypothesis that exposure to an omega-6 fatty acid alters the chemical composition of snail host tissues, which in turn influences the oxylipin profiles of cercarial parasites. I exposed freshwater snail hosts (*Ladislavella elodes*) to one type of omega-6 fatty acid, LA, that were infected with the trematode parasite *E. trivolvis* lineage c. The snail host *L. elodes* is both a grazer and a scraper, that feeds on organic material, algae, and detritus that is present in its aquatic environment (Harrold & Guralnick, 2010). If LA is present in the water or on surfaces of the water, these snails may ingest LA due to their grazing behaviour (Boiché et al., 2011; Babaran et al., 2021).

The life cycle of *E. trivolvis* lineage c is not fully described, though it is likely similar to that of *Echinostoma trivolvis* (Cort, 1914), which was redescribed by Kanev et al., (1995). (i.e., *E. trivolvis* lineage as described by Detwiler et al., 2010). Based on what is known about *E. trivolvis,* the cercariae develop from a series of progenitor stages including the miracidia, the sporocyst, and the mother and daughter rediae. Briefly, miracidia hatch from eggs and penetrate a snail host and transform into sporocysts within a few days post-infection (Kanev et al., 1995).

As sporocysts continue developing within the snail's tissues, they do not feed on the host's tissues, but instead, they absorb nutrients directly from the host through their tegument as other species of trematodes have been shown to do (Bibby & Rees, 1971; Køie, 1971). During the second week, sporocysts give rise to mother rediae, which actively start feeding on the host's tissues, particularly the digestive gland (Kanev et al., 1995; McKee, 2018), while also reproducing asexually to produce daughter rediae (Preston et al., 2013; Neal et al., 2024). During the third week, daughter rediae have emerged and continue to feed on the host's tissues (Krejci & Fried, 1994; Probst & Kube, 1999). As the daughter rediae mature, they give rise to cercariae in about 6-8 weeks (Krejci & Fried, 1994; Hodinka & Detwiler, 2024). As cercariae begin developing within the daughter rediae and emerging from them (Hodinka & Detwiler, 2024), cercariae begin relying on the energy reserves they accumulate during the redial stages as cercariae do not feed on host tissues (McKee, 2018: Koprivnikar et al., 2023).

By exposing snails LA, I expect the fatty acid profiles of snails to be different from unexposed snails. In addition to increased levels of LA (C18:2) I expect increased levels of GLA (C18:3n6), DGLA (C20:3n6), ARA (C20:4), ADA (C22:4), and DPA (C22:5n6). In addition, because snails may prioritize uptake and metabolism of dietary linoleic acid, they may downregulate de novo synthesis of saturated and monounsaturated fatty acids including palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1). Furthermore, as omega-3 (n-3) and omega-6 (n-6) fatty acids often compete for the same enzymatic pathway, increased levels of supplemented, dietary omega-6 fatty acid (LA) in host tissues may result in suppressed/reduced levels of omega-3 fatty acids including ALA (C18:3), EPA (C20:5), and DHA (C22:6). Thus, by experimentally exposing snail hosts to linoleic acid, I expect the above changes to be reflected in their tissues.

I also hypothesize that exposure of *L. elodes* snails to linoleic acid will affect both the diversity of types and concentrations of oxylipins emitted by *E. trivolvis* cercariae, particularly oxylipins where linoleic acid serves as the precursor for their synthesis such as hydroxy fatty acids (9-HODE and 13-HODE), epoxy fatty acids (9,10-EpOME and 12,13-EpOME. I predict that the oxylipin profiles of cercariae emerging from snails exposed to linoleic acid will consist of an increased diversity of types and concentrations of oxylipins emitted that have linoleic acid as their precursor, compared to cercariae emerging from snails that were not exposed to linoleic acid. This study provides insights into how the nutritional state of hosts (host diet quality) influences the chemical emissions of their developing parasites.

Methods

To obtain *Echinostoma trivolvis* lineage c miracidia, adult worms were isolated and dissected within 24-48 hours from muskrats (*Ondatra zibethicus*) collected by a local fur trapper. Upon examining the small intestine, ceca, and large intestine of the carcasses under a stereomicroscope, adult echinostomes were identified based on the presence of a collar of spines around the oral sucker, and their uteri were teased apart to collect pools of eggs in petri plates with autoclaved, non-chlorinated water.

After preserving the anterior ends of the adult worms in 100% ethanol at –20 °C for genetic verification (Hodinka & Detwiler, 2024), petri plates containing eggs were incubated in the dark at ~22 °C for 12 days to allow miracidia to develop. If miracidia had not hatched, the dishes were placed under a 100-watt incandescent lamp at 38 cm above the dish and checked every 2 hours until miracidia were observed swimming.

To obtain infected snails, a total of 144 *L. elodes* snails (1-5 mm in shell length) were individually exposed to 5 *E. trivolvis* lineage c miracidia in 6-well plates for 12 hours following

methods detailed in Chapter 1 (Figure 2.1). Following exposure, snails were divided into 24 plastic containers (top diameter of 130 mm, bottom diameter of 80 mm, and height of 80 mm), with 6 snails per container and 600 mL of non-chlorinated water. These containers were randomly assigned to either the control group or the experimental group, with 12 containers in each group (Figure 2.1). The control group received green leaf lettuce and chalk, while the experimental group received green leaf lettuce, chalk, and linoleic acid. For both groups, one circular piece of green leaf lettuce p (16.60 mm in diameter and weighing an average of 0.072 g) was provided 3 times per week per snail). For the experimental group, 30 µL of linoleic acid (Thermo Scientific, 99% purity) was directly pipetted into each container twice per week, and water in the containers was also changed twice per week.

Approximately 6-8 weeks post-exposure, infected snails were identified by placing each snail into a well of a 6-well plate under a 100-watt lamp 38 cm above the plate. Each well was observed under a stereomicroscope every 30 minutes to check for cercarial emergence. Snails were shed a total of five times throughout the experiment, with a one-week interval between each session. To reduce variation in cercarial density between treatment groups, four samples were selected per group (control and experimental). Cercariae were collected into beakers, and the total number of cercariae per pool was estimated by averaging counts from three 0.5 mL aliquots, with these cercariae immobilized in 100% ethanol in gridded petri dishes for a more precise estimate of the number of cercariae present in the pool. The cercariae were then filtered to remove host-derived compounds using a 70 µm mesh (filtered later particles such as mucus and feces) placed above a 20 µm mesh (retained the cercariae). After rinsing the cercariae three times and transferring them into 10 mL of non-chlorinated, filter-sterilized water, each pool was placed under a 100-watt incandescent lamp at 38 cm for 4 hours to allow cercariae to condition

the water with their chemical emissions. The cercariae-conditioned pools were each transferred to 15 mL Falcon tubes, flash-frozen, and stored at -80°C.

Lipids were extracted from the cercariae-conditioned water samples following the same procedure detailed in Chapter 1. Samples were thawed on ice for 0.5-2 hours, and 0.1 mL of an oxylipin standard mixture was added to the sample. The solution was filtered through a 0.2 µm nylon syringe membrane (Thermo Fisher Nalgene) to remove any additional debris and then passed through a Stata-X solid-phase extraction (SPE) column (Phenomenex) using a vacuum. Columns were preconditioned with 2 mL of 100% methanol followed by 2 mL of pH 3 water. After applying the filtered sample to the column, they were rinsed with 1 mL of 10% methanol in pH 3 water, followed by eluding with 1 mL of 100% methanol. The eluate was collected into 1.5 mL microtubes, filled with nitrogen gas for 30 seconds, and stored at -80°C until high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) was conducted.

To compare oxylipin profiles between treatment groups, several analyses were performed using RStudio (version 2023.09.1+494) using the packages dplyr, ggplot2, tidyr, car, purrr, broom, tidyverse, vegan, viridis, ggforce, lmtest, MASS, pathchwork, tibble, and ggrepel. A total of 8 cercarial density samples were analyzed (4 control, 4 experimental), with 158 oxylipins scanned using HPLC-MS/MS. Mann-Whitney U tests were used to determine whether there were differences in the concentrations of oxylipins between the control and experimental groups. Non-metric multidimensional scaling (NMDS) was performed to visualize differences between the oxylipin profiles of cercarial samples from control and experimental groups. To determine whether the oxylipin profiles differed between the treatment groups, a permutational multivariate analysis of variance (PERMANOVA) test was performed.

To determine whether exposure to linoleic acid affected the fatty acid (FA) composition of the snail tissues, I prepared tissue samples from both the control and experimental groups to run gas chromatography (GC). To obtain an adequate amount of tissue (~1 g of tissue) for FA analysis through GC, I placed snails from the control and experimental groups in petri plates under a dissecting microscope to check for infection. I gently crushed snails to remove their shells, and divided tissue from uninfected snails into two sections: (1) the head-foot region and (2) the middle section, which included the gonads and digestive gland, similar to methods outlined in Babaran et al., (2021). Tissue from snails that were found to be infected with parasites during dissections were excluded to ensure that the FA composition reflected snail tissue only.

I pooled tissue from multiple snails to reach the target mass for each sample. For the control group, I obtained the head-foot sample (1.1627 g) and gonad sample (1.1905 g) each from 20 snails. For experimental group, I prepared two head-foot samples, where one sample (0.9946 g) was prepared using 14 snails and one sample (0.3306 g) was created from 6 snails. Similarly, for the experimental group, I prepared two gonad samples, where one sample (1.1905 g) was prepared from 14 snails and one sample (0.3285 g) was reached using 6 snails. I wrapped each combined tissue sample in aluminum foil, flash froze in liquid nitrogen and stored them at -80 °C until GC was conducted at the Food and Human Nutritional Analytical Services at the University of Manitoba. Individual fatty acids were identified by comparing retentions times with known standards. The fatty acid composition was expressed as area percent, calculated as the proportion of the chromatographic peak area of each fatty acid relative to the total integrated fatty acid peak area within each sample. For each tissue type, replicate samples from the experimental group (n = 2 samples) were analyzed, and the mean area percent ± standard error

(SE) was calculated from these replicates (i.e., peak area of a fatty acid divided by the total peak area of all detected fatty acids).

Results

Diversity of Types of Oxylipins

A total of 158 oxylipins were scanned for across a total of 8 samples. The samples selected for oxylipin analysis had cercarial densities ranging from 470-640 cercariae per 10 mL for the control group (n = 4 samples, average density = 562 cercariae/10 mL) and 385-674 cercariae per 10 mL for the experimental group (n = 4 samples, average density = 559 cercariae/10 mL). Oxylipins were considered present at quantifiable levels when their chromatographic peak exceeded five times the baseline signal (lower limit of quantification, LLOQ). After excluding oxylipins below the LLOQ, a total of 47 oxylipins were quantified (Figure 2.2). Of these, 26 belonged to the lipoxygenase (LOX) pathway, 11 to the cyclooxygenase (COX) pathway, and 10 to the cytochrome P450 (CYP) pathway (Figure 2.2). Within the control samples, the number of oxylipins quantified per sample ranged from a minimum of 27 to a maximum of 32, whereas in the experimental samples, the range was from 18 to 35 oxylipins quantified across samples (Figure 2.2). Upon applying a detection threshold that oxylipins must be quantified in at least 75% of the samples (≥3 out of 4 samples) in 1) the control group only, 2) the experimental group only, or 3) either the control or experimental groups (including oxylipins quantified in both groups), 29 oxylipins met the criteria (Figure 2.3). Of these, 16 belonged to the LOX pathway, 6 to the COX pathway, and 7 to the CYP pathway (Figure 2.3). Within this set of oxylipins, the number of oxylipins quantified in each treatment group ranged from 21 to 27 in the control samples and 15 to 28 in the experimental samples (Figure 2.3). Using a threshold that oxylipins must be quantified in at least 75% of the samples

irrespective of the treatment group (≥6 out of 8 samples), the total number of oxylipins decreased to 22 oxylipins (Figure 2.4). Of these, 13 belonged to the LOX pathway, 4 to the COX pathway, and 5 to the CYP pathway (Figure 2.4). The number of oxylipins quantified within the control samples ranging from 14 to 22 and 14 to 21 in the experimental samples (Figure 2.4).

Concentrations of Oxylipins

To determine whether oxylipin concentrations differed between control and experimental cercarial density samples, oxylipins quantified in at least 75% of samples (≥3 out of 4 samples in each group) were compared using Mann-Whitney U tests (Table 2.1). The control group consisted of 4 samples with cercarial densities ranging from 470-640 cercariae per 10 mL, while the experimental group included 4 samples with densities ranging from 385-674 cercariae per 10 mL. Across all pathways, individual oxylipin concentrations varied widely, ranging from <0.001 to 28.57 ng/mL. Of the 29 oxylipins quantified in at least 75% of the samples (≥3 out of 4 samples either in the control group, experimental group, or both groups), 13 oxylipins had higher mean concentrations (ng/mL) in the control group compared to the experimental group including 12,13 EpODE, 12,13 diHOME, 12-HEPE, and 10S,17S-DiHDoHE (PDX). However, none of these differences showed statistical significance ($P > 0.05$; Table 2.1). For the remaining 16 oxylipins, mean concentrations were higher in the experimental group compared to the control group such as 11-HETE, 13-HODE, 20-HETE, RvD5, and TXB3. However, similarly, none of the differences were statistically significant either ($P > 0.05$; Table 2.1).

Oxylipin Profiles (Diversity of Types and Concentrations of Oxylipins)

To complement these comparisons, a non-metric multidimensional scaling (NMDS) analysis was performed to examine overall patterns in oxylipin profiles samples. The analysis was performed on a matrix of concentrations of all oxylipins quantified in samples. Bray-Curtis

dissimilarity was used to quantify differences among the samples, which captures differences in both the diversity of types and concentrations of oxylipins. The NDMS ordination based on Bray-Curtis in oxylipin profiles resulted in a low stress value (2.45×10^{-13}), indicating a good fit of the data. No distinct clustering was apparent according to treatment group (Figure 2.6). PERMANOVA was used to test whether treatment group explained differences in oxylipin profiles, with the analysis being set to 999 permutations. PERMANOVA confirmed that treatment group did not explain a significant portion of the variation in oxylipin profiles ($F = 0.55$, $R^2 = 0.084$, $P = 0.55$), with only 8.4% of the variation explained by the treatment group.

Cercarial Production

Cercarial production was monitored across five shedding weeks (weeks 9, 10, 11, 13, and 15 post-exposure). At 9 weeks post-exposure, four *Ladislavella elodes* snails from the control group and four from the experimental snails were found to be infected with *Echinostoma trivolvis* lineage c. At 15 weeks post-exposure, no control snails shed, whereas three experimental snails shed cercariae. The total number of cercariae summed across all pools per week was 1618 and 450 cercariae/week in the experimental and control group, respectively (Figure 2.5A, Table 3.3.). The mean number of cercariae released per infected snail across weeks was 485 and 164 cercariae/snail for the experimental and control group, respectively (Figure 2.5B, Table 3.3.).

Fatty Acid Analysis

Control samples were limited to a single analysis (n = 1 sample) as tissue availability was insufficient to generate replicates, whereas sufficient tissue mass available in the experimental group allowed multiple samples to be generated for fatty acid analysis. According to the fatty acid (FA) analysis, there appeared to be a difference in the amount of linoleic acid (C18:2) in

snail tissues between the experimental and control group (Table 2.2). The mean area percent ± SE of linoleic acid (LA; C18:2) in the head-foot tissue of the experimental group was 36.51 ± 13.84 compared to the mean area percent of 7.70 from the control group. A similar pattern was observed in the fatty acid composition of the gonad tissue, where the experimental group had a mean area percent ± SE of LA of 35.12 ± 8.78 compared to the control group at area percent of 10.49.

Discussion

This study investigated how host dietary exposure to linoleic acid, an omega-6 fatty acid and precursor for the synthesis of certain oxylipins, influences the chemical profiles of *Echinostoma trivolvis* lineage c. Across all samples, 47 oxylipins were quantified from 158 scanned, including 26 from the lipoxygenase (LOX) pathway, 11 from the cyclooxygenase (COX) pathway, and 10 from the cytochrome P450 (CYP) pathway. Applying a detection threshold of ≥75% of samples per group, 29 oxylipins, were used for quantitative comparisons, including 16 from LOX, 6 from COX, and 7 CYP.

Despite measurable differences in linoleic acid levels within the snail host tissues, where experimental snails had higher linoleic acid levels in head-foot tissue (mean ± SE: control: 7.70; experimental: 36.51 ± 13.84) and gonad tissue (mean ± SE: control: 10.49; experimental: 35.12 ± 8.78), oxylipin concentrations emitted from cercariae did not different significantly between control and experimental groups. Interestingly, there was no effect of linoleic acid on the prevalence of infection as 5.6% (8 infected snails out of 144 exposed; 4 from each treatment group) of exposed snails were infected. This number limited my ability to collect samples especially for the control group as I had no infected snail remaining to shed cercariae for week 15. In contrast, linoleic acid had a positive effect on the parasites as a higher number of cercariae

emerged from exposed snails compared to unexposed, with weekly totals of 450 cercariae (control) compared 1,618 cercariae (experimental) per week, and mean numbers of 164 cercariae (control) than 485 cercariae (experimental) per infected snail. As the treatment groups had the same number of infected snails and were exposed to the same dose of 5 of miracidia, my results suggest that exposure of snail hosts to linoleic acid enhanced the production and release of *E. trivolvis* lineage c cercariae. As I did not keep track of changes in snail size (weight or shell length) over time, I am unable to determine whether linoleic acid had any effect on snail size. Similar to the effect of a higher protein diet on snail growth, linoleic acid may have indirectly influenced the asexual reproduction of the cercariae by allowing exposed snails to grow larger than unexposed (Keas et al., 1997; Sandland & Minchella, 2003). Given that the rediae reside and feed on gonad tissue, if the linoleic acid allows infected snails to replace tissue and create more tissue that would provide more habitat and food for the rediae and may allow them to generate more cercariae. A study conducted on *Biomphalaria glabrata*, a type of freshwater snail, showed that the quality of the diet and host density influenced both snail growth and cercarial shedding (Coles, 1973). They found that snails fed dried lettuce experienced slower growth rates and cercarial output compared to those maintained on higher-protein diets such as Tetramin or Philips fish food (Coles, 1973).

Although we maintained a consistent host density within each treatment group (6 snails per 600 mL container, with dead snails replaced as needed), host density is a factor that may have impacted the overall cercarial production and potentially the chemical emissions of cercariae. Coles (1973) found that crowding of snails negatively affected both snail growth and cercarial output, where snails maintained at higher densities shed fewer cercariae per individual, likely as a result of reduced growth or possible pheromonal effects on parasite development

(Coles, 1973). Interestingly, shedding rates improved when snail density was reduced, suggesting that optimal cercarial yields may require lower snail density rearing conditions (Coles, 1973). Snail density in our experiments was most similar to the density that led to an intermediate level of cercarial shedding in Coles (1973). Interestingly, Coles (1973) speculated that in addition to limited resources, chemical cues, particularly pheromones released by snails raised in crowded conditions, may suppress parasite development or shedding (Coles, 1973). These pheromones may potentially alter the development of daughter sporocysts or formation of germinal cells, which give rise to cercariae, instead of directly impacting the cercariae existing within the daughter sporocysts (Coles, 1973). Future studies should monitor snail size post-exposure and maintain snails at lower densities than 6 snails per 600 mL containers to determine whether linoleic acid directly enhances parasite development or improves host conditions that enable increased cercarial shedding. Despite apparent differences in the cercarial output between the control and experimental groups, host-exposure to linoleic acid did not significantly affect the oxylipin profiles of the cercariae. For the 29 oxylipins that met the less conservative cutoff, the diversity of types of oxylipins between the control and experimental group was the same with all 29 oxylipins being quantified in at least 3 of the 4 samples for either of the treatment groups or when combined. Although a number of oxylipins appeared to be in a higher concentration in one group compared to the other such as 12, 13 EpODE and PDX in the control groups and 20-HETE, RvD5, and TXB3 in the experimental groups, none of these differences were statistically significant. Multivariate analysis also revealed no significant clustering of oxylipin profiles according to treatment group. The low R^2 for the PERMANOVA suggests that most of the variation in oxylipin profile is unexplained by treatment group. The lack of clustering in oxylipin profiles within groups could be due to the range of densities chosen for this experiment. Based

on Chapter 1 results, the concentrations of some oxylipins, especially those that are synthesized from linoleic acid in the LOX pathway (i.e. 9, 12, 13, triHOME, 9, 10, 13 triHOME, 9-HODE, 13-HOTrE, and 13 HODE) were lower at ~500 cercariae per 10 ml compared to other densities. When comparing oxylipin quantification between both chapters, it's important to note that oxylipin profiles at similar cercarial densities in chapter 1 were based on a single sample at each density, whereas in chapter, there were replicates of samples at comparable densities within a similar range. It is possible that this increased replication in chapter 2 likely improved the quantification of oxylipins near the limit of quantification compared to chapter 1, while additional factors such as biological variability among cercarial density samples and some methodological differences may have also contributed to the observed differences between the samples with similar densities across both chapters. However, this density of cercariae was used as it was challenging to obtain replicate samples at higher densities from the control group. Increasing sample size would bolster the confidence in these results as the current sampling was limited by several biological and technical challenges. Biologically, the number of samples was limited by the low prevalence in both treatment groups especially for the control group as there were fewer cercariae shed per snail than in the exposed group. In addition, creating replicates with the same cercarial density within and between treatment groups was challenging as the cercariae were free-swimming making the goal of achieving a particular density within a certain time frame difficult.

The lack of a difference in oxylipin profiles between cercariae in the treatment groups could also reflect that the tissue of exposed snails was not enriched in linoleic acid. After applying the linoleic acid to the containers, I observed droplets of linoleic acid floating at the surface of the water due to its hydrophobic nature. Although statistical comparisons were not

possible due to sample size (limited by the number of snails required for 1 g samples for GC analysis), the amount of linoleic acid in the head-foot and gonad tissues were 5x and 3.5x in exposed snails relative to unexposed, respectively, confirming that exposed snails were likely enriched in linoleic acid.

However, due to a scarcity of data, it is currently unclear how similar the oxylipin profiles of echinostome cercariae are compared to other echinostome species or other types of trematode cercariae beyond schistosomes. Some of the same oxylipins, such as PGE2, are emitted by schistosome cercariae (Fusco et al., 1985). Though schistosome cercariae in the presence of linoleic acid also produced PGE1, 5-HETE and 15-HETE, which we did not quantify from echinostome cercariae (Fusco et al., 1985). Our study additionally included several linoleic acid-derived oxylipins that Fusco et al. (1985) did not report. The observed discrepancies between our findings and Fusco et al. (1985) may reflect species-specific variation, differences in exposure method (direct exposure of schistosome cercariae in the Fusco et al., 1985 study versus indirect exposure of echinostome cercariae via the snail hosts in our study), or variations in analytical detection. Overall, these findings highlight the need for further studies across multiple trematode species to better the regulation of cercarial oxylipin emissions.

Future studies should directly examine cercarial fatty acid composition by isolating whole cercariae or cercarial tissue for targeted GC analysis though obtaining enough tissues for a 1 g sample would likely require >10 000 cercariae similar to Babaran et al., (2021). Another alternative hypothesis is that linoleic acid's effects on cercarial oxylipin production and emission may not operate through altering downstream oxylipin pathways within the cercariae themselves, but rather through systemic physiological changes in the snail host (i.e., altered energy allocation, immune modulation or reproduction-linked pathways). For example, as snails

exposed to linoleic acid released more cercariae than unexposed snails in our study, suggesting that host physiological condition may influence parasite reproduction, which should be explored in future studies.

Conclusion

Our findings suggest that snails incorporate linoleic acid into their tissues via exposure in their aqueous environment and that tissues enriched in linoleic acid benefit the parasite by increasing the number of cercariae produced via asexual reproduction. There appeared to be no effect on the oxylipin emissions from the cercariae, but given the limited context of this study, there are many future avenues to explore including the effect of density on the synthesis of oxylipins using other precursor fatty acids. Additionally, while our GC analysis focused primarily on host tissues, future work should consider isolating and analyzing tissue from cercariae directly, which could provide better insights into whether and how dietary fatty acids have been incorporated by the developing cercariae while residing in the host. These investigations would help elucidate whether exposure of the host to dietary fatty acids have direct or indirect or a combination of both effects on the oxylipin production and release of the parasites, providing insights about the mechanisms underlying these interactions.

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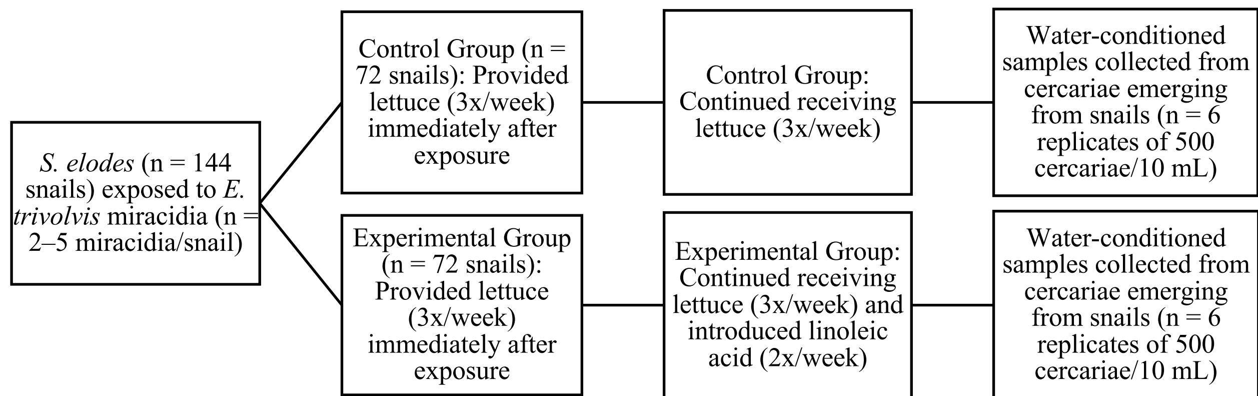


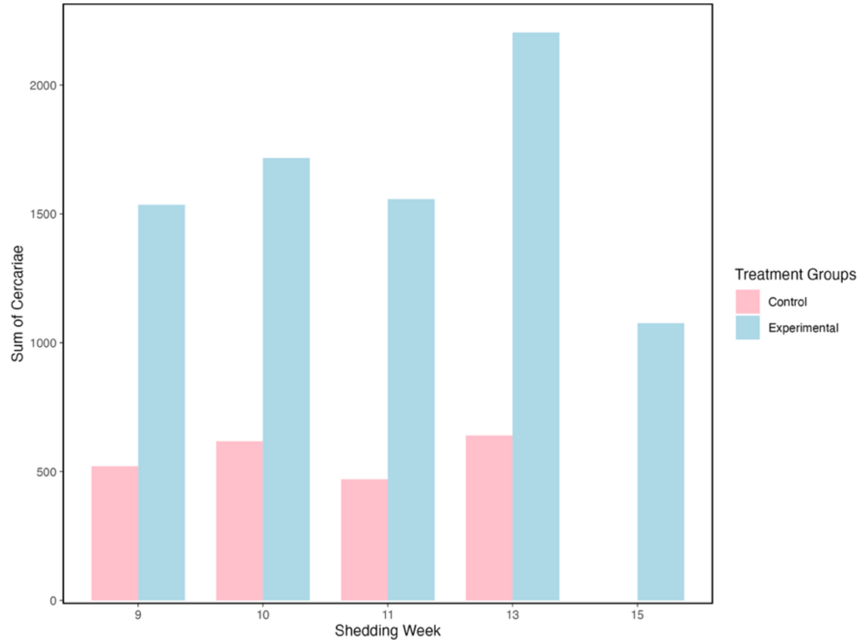
Figure 2.1. Experimental design examining the influence of snail hosts exposed to linoleic acid on the oxylipin emission of *Echinostoma trivolvis* (lineage c) cercariae.

Figure 2.2. Diversity of oxylipins quantified in control (n = 4 samples; 470-640 cercariae/10 mL) and experimental groups (n = 4 samples; 385-674 cercariae/10 mL). Every coloured tile indicates the presence of an oxylipin within a sample. Oxylipins are grouped according to pathway: COX (orange), CYP (blue), and LOX (purple). Tallies above each column are the total number of oxylipins quantified in a sample, and tallies to the right of each row show the total number of samples in which each oxylipin was quantified.

Figure 2.3. Diversity of oxylipins quantified in at least 75% of all samples (≥3 of 4) in either the control group only (n = 4 samples; 470-640 cercariae/10 mL), experimental group only (n = 4 samples; 385-674 cercariae/10 mL), or both groups. Every tile that is coloured indicates that the oxylipin was quantified within that sample. Oxylipins have been grouped according to pathway: COX (orange), CYP (blue), and LOX (purple). Tallies above columns represent the total number of oxylipins quantified within a sample, and tallies next to rows show the total number of samples in which each oxylipin was present.

Figure 2.4. Diversity of oxylipins quantified in at least 75% of all samples (≥6 of 8), irrespective of treatment group, from control (n = 4 samples; 470-640 cercariae/10 mL) and experimental group (n = 4 samples; 385-674 cercariae/10 mL). Each coloured tile shows the presence of an oxylipin in a sample. Oxylipins are grouped based on pathway: COX (orange), CYP (blue), and LOX (purple). Tallies above columns show the total number of oxylipins quantified in a sample, and tallies next to rows represent the total number of samples in which each oxylipin was quantified.

A)



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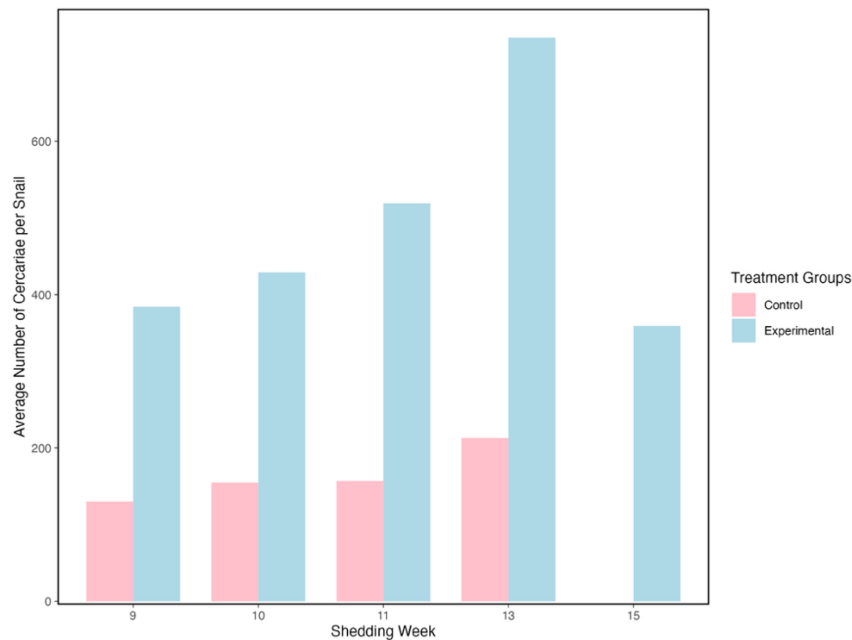

Figure 2.5. Cercarial production across five shedding weeks for control (pink) and experimental (blue) groups. A) Total number of cercariae summed across all pools per shedding week (means across weeks: control = 450 cercariae; experimental = 1618 cercariae). B) Average number of cercariae released per infected snail per shedding week (means across weeks: control = 131 cercariae; experimental = 485 cercariae). The control group value for week 15 represents missing data, as no infected snails shed cercariae during this shedding period.

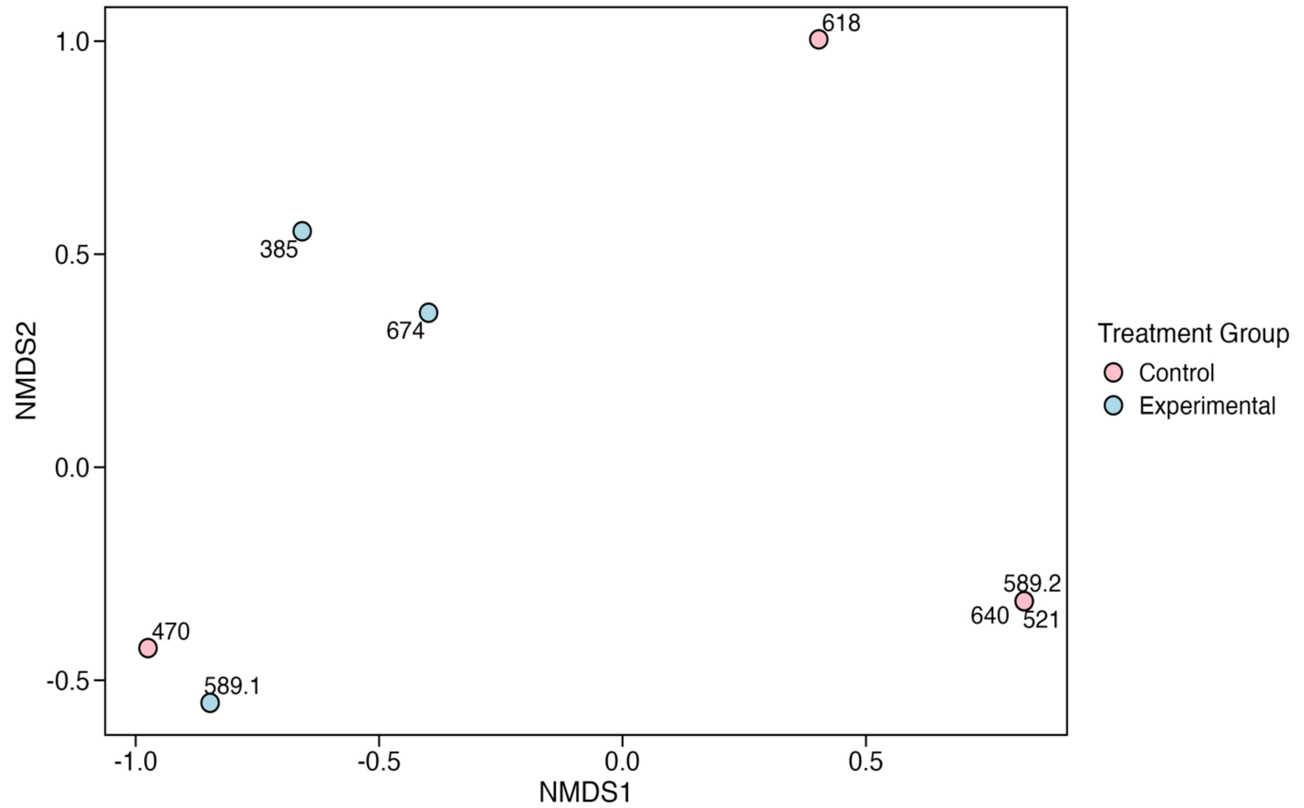

Figure 2.6. Non-metric multidimensional scaling (NMDS) ordination of oxylipin profiles of cercariae across treatment groups. Points represent samples, with distances in the ordination reflecting differences in overall oxylipin profiles based on Bray-Curtis dissimilarity. Control samples (pink) and experimental samples (blue) form distinct clusters, indicating treatment-related differences among oxylipin profiles.

Tables

Table 2.1. Results of Mann-Whitney U tests conducted on the concentrations of oxylipins quantified in at least ≥75% of samples (≥3 of 4) within either the control, experimental or both groups. Abbreviations: Precursors: ARA = arachidonic acid (C20:4); LA = linoleic acid (C18:2); ALA = alpha-linolenic acid (C18:3); EPA = eicosapentaenoic acid (C20:5); DHA = docosahexaenoic acid (C22:6). Pathways: LOX = lipoxygenase; COX = cyclooxygenase; CYP; cytochrome P450.

		Control Group					Experimental Group						
		Concentrations (ng/mL) for Cercarial Density Samples ± Standard Error					Concentrations (ng/mL) at Cercarial Density Samples ± Standard Errors						
Precursors	**Oxylipins**	**470 ± 141 cercariae/10 mL (n = 1)**	**521 ± 64 cercariae/10 mL (n = 1)**	**618 ± 139 cercariae/10 mL (n = 1)**	**640 ± 159 cercariae/10 mL (n = 1)**	**Mean Control Concentrations (ng/mL) ± Standard Errors**	**385 ± 93 cercariae/10 mL (n = 1)**	**589.1 ± 70 cercariae/10 mL (n = 1)**	**589.2 ± 98 cercariae/10 mL (n = 1)**	**674 ± 205 cercariae/10 mL (n = 1)**	**Mean Experimental Concentrations (ng/mL) ± Standard Errors**	**U Statistic**	***P***
							LOX Pathway						
ARA	11-HETE	0.0035	0.00713	0.0082	0.00738	0.00655 ± 0.00104	0.00474	0.00523	0.02385	0.00286	0.00917 ± 0.00492	10	0.66500554
EPA	12-HEPE	0.00521	0.01077	0.00726	0.00952	0.00819 ± 0.00123	0.00315	0.00143	0	0	0.001145 ± 0.000749	16	0.02940105
ARA	12-HETE	0.01498	0.02643	0.0236	0	0.01625 ± 0.00594	0.02594	0.03276	0	0	0.01468 ± 0.00859	8	1
LA	13-HODE	0	1.11513	0.35228	1.41123	0.71966 ± 0.32756	0	0	3.40363	0	0.85091 ± 0.85091	10.5	0.53847238
ALA	13-HOTrE	0.01645	0.03303	0.07011	0.04716	0.04169 ± 0.01136	0.00517	0.01509	0.03465	0.00719	0.01553 ± 0.00672	14	0.1123512
ARA	15-HETE	0.01662	0.01483	0.0157	0.01318	0.01508 ± 0.00073	0.00326	0.01065	0.03007	0	0.01100 ± 0.00674	12	0.31232142
ARA	15-oxoETE	0.02725	0.03145	0.01531	0	0.01850 ± 0.00705	0.00868	0.01889	0.00994	0	0.00938 ± 0.00386	11.5	0.38363033
ARA	5-HETE	0.00613	0.01371	0.01152	0.01159	0.01074 ± 0.00162	0.00243	0.01063	0.02216	0.0101	0.01133 ± 0.00407	10	0.66500554
ARA	8-HETE	0.00311	0.00598	0.00268	0.0036	0.00384 ± 0.00074	0.00236	0.00379	0.00552	0.00161	0.00332 ± 0.00086	10	0.66500554
ALA	9-oxoOTrE	0.02913	0.0476	0.22052	0.02771	0.08124 ± 0.04665	0.02038	0.03537	0.0714	0	0.03179 ± 0.01506	11	0.47048642
LA	9-HODE	0.51264	0.31533	0.48289	0	0.32772 ± 0.11755	0.13625	1.12932	0.12521	0	0.34770 ± 0.26237	9.5	0.77150341
ALA	9-HOTrE	0.01262	0.04095	0.11106	0.03143	0.04902 ± 0.02150	0.01308	0.01939	0.05212	0.00367	0.02207 ± 0.01053	11	0.47048642
DHA	RvD$_5$	0.00307	0.18947	0.03753	0.05573	0.07145 ± 0.04083	0.00253	0.22912	0.0815	0	0.07829 ± 0.05372	9	0.88523391
DHA	10S,17S-DiHDoHE (PDX)	0.03346	0.00794	0	0	0.01035 ± 0.00793	0.0006	0.01889	0.00626	0	0.00644 ± 0.00438	8	1
LA	9,10,13-triHOME	24.98009	12.70232	15.84786	0	13.38257 ± 5.16508	2.43149	28.57489	5.82452	0	9.20773 ± 6.56527	9.5	0.77150341
LA	9,12,13-triHOME	0.01225	25.9505	12.64088	16.39249	13.74903 ± 5.36804	2.56221	34.99448	6.24766	0	10.95109 ± 8.11638	10	0.66500554
							COX Pathway						

ARA	2,3-dinor TXB$_2$	0.01634	0.09175	0.04197	0	0.03752 ± 0.02004	0.04433	0.02475	0.04903	0	0.02953 ± 0.01116	7.5	1
DGLA	PGD$_1$	0.03074	0.00589	0.01135	0	0.01199 ± 0.00666	0.00344	0.02311	0.00777	0	0.00858 ± 0.00510	9.5	0.77150341
DGLA	TXB$_1$	0.00161	0.00468	0.00824	0.01336	0.00697 ± 0.00252	0.00478	0.00928	0.01313	0	0.00680 ± 0.00284	8	1
EPA	TXB$_3$	0.0048	0.0024	0.00643	0.00829	0.00548 ± 0.00125	0.00488	0.02044	0.00364	0.01125	0.01005 ± 0.00384	5	0.47048642
ARA	dhk PGF$_{2\alpha}$	0	0.01042	0.03299	0.02838	0.01795 ± 0.00771	0	0	0.03154	0	0.00789 ± 0.00789	11.5	0.35617033
ARA	2,3-dinor-6k PGF$_{1\alpha}$	0.01634	0.09175	0.04197	0	0.04098 ± 0.04098	0.04433	0.02475	0.04903	0	0.12442 ± 0.04522	3.5	0.21860374
CYP Pathway													
ALA	12,13-EpODE	0.01409	0.00455	0.01286	0.00252	0.00969 ± 0.00227	0.01355	0	0	0.00725	0.00402 ± 0.00323	13	0.19126699
LA	12,13-diHOME	0.00551	0.31702	0.16811	0.27722	0.19197 ± 0.06967	0.07616	0.00992	0.54272	0.06617	0.17374 ± 0.12385	9	0.88523391
ARA	14,15-DiHETrE	0.00068	0.00096	0	0.00275	0.00110 ± 0.00059	0	0	0.00258	0	0.00065 ± 0.00065	11.5	0.35617033
ARA	16-HETE	0.01827	0.02408	0.01921	0.01425	0.01895 ± 0.00202	0.01684	0.016	0.02068	0.02265	0.01904 ± 0.00158	8	1
ARA	20-HETE	0.0276	0.00491	0.02425	0.03281	0.02239 ± 0.00609	0.02767	0.02893	0.01851	0.05987	0.03375 ± 0.00901	5	0.47048642
LA	9,10-diHOME	0.00753	0.34266	0.18619	0.30662	0.21075 ± 0.07555	0.06686	0.01501	0.5693	0.07606	0.18181 ± 0.12986	9	0.88523391
LA	9,10-EpOME	0.00557	0	0	0	0.00139 ± 0.00139	0.0038	0.00006	0.039	0	0.01072 ± 0.00947	4.5	0.35617033

*n refers to the number of samples

Table 2.2. Gas chromatography analysis of omega-3 and omega-6 fatty acids from snail tissues belonging to control (lettuce only) and experimental (lettuce and linoleic acid-exposed) groups. Peak area percentages, measured as the proportion of a fatty acid's chromatographic area relative to the total detected fatty acid peak area within a sample. Abbreviations: SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, SCFA = short-chain fatty acid, MCFA = medium-chain fatty acid, LCFA = long-chain fatty acid, n-3 = omega-3, n-6 = omega-6, DNS = de novo synthesis (synthesized by organism), DS = dietary sources (obtained from diet), and DNS/DS = de novo synthesis/dietary sources (not synthesized by organism, but can be synthesized from precursors obtained from diet).

			Head-Foot Area (%)		Gonads Area (%)	
Fatty Acid	**Fatty Acid Derivation**	**Fatty Acid Synthesis**	**Control (n = 1)**	**Experimental (n = 2; average)**	**Control (n = 1)**	**Experimental (n = 2; average)**
Linoleic Acid (C18:2)	n-6 PUFA – LCFA	DS	7.693	36.510	10.492	35.125
Gamma-Linolenic Acid (C18:3n6)	n-6 PUFA – LCFA	DNS/DS	0.208	0.157	0.172	0.28
Alpha-Linolenic Acid (C18:3n3)	n-3 PUFA – LCFA	DS	12.488	11.178	32.514	9.24
Eicosadienoic Acid (C20:2)	n-6 PUFA – LCFA	DNS/DS	9.585	13.146	9.269	14.408
Dihomo-Gamma-Linolenic Acid (C20:3n6)	n-6 PUFA – LCFA	DNS/DS	0.702	0.634	0.666	0.726
Arachidonic Acid (C20:4)	n-6 PUFA – LCFA	DNS/DS	21.13	12.983	8.843	13.383
Eicosatrienoic Acid (C20:3n3)	n-3 PUFA – LCFA	DNS/DS	3.358	1.0767	3.84	1.191
Eicosapentaenoic Acid (C20:5)	n-3 PUFA – LCFA	DNS/DS	3.72	1.465	3.598	2.128
Docosadienoic Acid (C22:2)	n-6 PUFA – LCFA	DNS/DS	0.062	0.104	0.07	0.119
Adrenic Acid (C22:4)	n-6 PUFA – LCFA	DNS/DS	4.759	2.228	2.154	2.397
Docosapentaenoic Acid (C22:5n6)	n-6 PUFA – LCFA	DNS/DS	0.04	0.075	0.087	0.0825
Docosapentaenoic Acid (C22:5n3)	n-3 PUFA – LCFA	DNS/DS	1.44	1.116	3.102	1.737
Docosahexaenoic Acid (C22:6n3)	n-3 PUFA – LCFA	DNS/DS	0.064	0.063	0.106	0.093

*n refers to the number of samples

Thesis Conclusion

This thesis explored how an environmental (density) and a nutritional (dietary linoleic acid exposure) factor influence the chemicals of the free-swimming cercarial stage of a trematode parasite. I focused on characterizing the oxylipin profiles of one type of trematode, *Echinostoma trivolvis* lineage c. Across two chapters, I tested the influence of parasite density and host dietary exposure in shaping the chemical profiles of the cercariae, with the broader aim of exploring how parasites may be chemically regulated in freshwater systems.

In chapter 1, I tested the hypothesis that cercarial density influences the production and emission of oxylipins. Using HPLC-MS/MS, I successfully identified a diverse range of oxylipins released by the cercariae into water, even in the absence of host cues, including compounds derived from linoleic acid via LOX and CYP pathways. These findings show that *E. trivolvis* lineage c cercariae themselves actively produce and release oxylipins on their own, rather than these compounds being solely derived from host tissue. Notably, oxylipin concentrations did not show a linear trend with density as initially predicted. Instead, several oxylipins peaked at intermediate densities, suggesting other potential mechanisms at play. These results provide evidence that echinostome cercariae may modulate their chemical emissions in complex, content-dependent ways that need to be further explored.

In chapter 2, I shifted the focus to a host-centered perspective, testing whether dietary exposure to linoleic acid, a key omega-6 fatty acid precursor of oxylipins, would alter snail tissue composition and consequently affect cercarial oxylipin output. Gas chromatography confirmed successful incorporation of linoleic acid into snail tissues, and snails exposed to linoleic acid consistently shed more cercariae than the control groups. However, statistical analyses of the cercarial samples revealed no statistically significant differences in the diversity of types or

concentration of oxylipins between the treatment groups nor clustering according to treatment group. These findings suggest that dietary linoleic acid may affect cercarial emergence through indirect physiological effects on the host, but does not directly shape the oxylipins of the developing cercariae under the conditions tested.

All in all, these findings highlight the potential for cercariae of *E. trivolvis* lineage c to dynamically modulate their chemical emissions in response to biotic conditions, such as crowding, rather than passively reflect host biochemistry. This thesis establishes foundational protocols for quantifying oxylipins in low-yield parasite water-conditioned samples, expanding the analytical toolkit available for chemical ecology in parasitology. Furthermore, this work opens up several important avenues for future research. First, time-course experiments could be conducted to assess whether oxylipin production and emission varies over shorter or longer periods of time following dietary exposure or during different parasite developmental stages. Shorter time frames, in the scale of seconds to minutes, would be more relevant to understanding the role of oxylipins in penetration behaviour. Longer time frames, in the scale of minutes to hours, relates more to emissions during host-seeking behaviour. Considering that cercariae are released in groups from their first intermediate host, they may also emit oxylipins to communicate with each other especially with respect to the presence of hosts and non-hosts in the environment. As cercariae are generated through asexual reproduction, cercariae stemming from the same miracidia are clonal and thus, the role of chemical emissions in kin selection.

Second, isolating and analyzing whole cercarial tissues, rather than just their secretions through water-conditioned samples, would help provide insights into whether dietary fatty acids are directly incorporated into parasite oxylipin synthesis. Furthermore, comparative studies

across other trematode species and host taxa could reveal how generalizable these findings are, and whether oxylipin profiles are taxonomically conserved or dependent upon the host species.

Moreover, functional experiments could also be conducted to assess the ecological and behavioural relevance of specific oxylipins identified in this study. For instance, future work could examine how these compounds influence parasite dispersal, host-finding, and infection success. Future studies could examine whether these compounds act as signalling molecules to coordinate parasite development or emergence. Furthermore, integrating environmental stressors such as pollution, temperature change, or resource competition could help reveal how oxylipin production and emission of these cercariae is modulated under ecologically realistic conditions. These assays could help offer a better understanding of the pathways and gene networks that are involved in the chemical underlying mechanisms of the parasites. Furthermore, field-based validation of these lab findings could also help provide valuable insights for these findings, examining whether compounds are present in natural water bodies, and whether they could be assessed as molecular indicators of parasite presence, abundance, or transmission risk.

Future studies could also broaden the scope of dietary manipulations on the host by testing the effects of other PUFAs, including both omega-3 and omega-6 fatty acids. While my work focused on linoleic acid (LA), an omega-6 fatty acid, comparing its influence with that of omega-3 precursors such as alpha-linoleic acid (ALA) or eicosapentaenoic acid (EPA) could help reveal whether different groups of fatty acids distinctly shape parasite development, cercarial, or chemical emissions. As omega-3 fatty acids are often times associated with anti-inflammatory or immunomodulatory processes in certain mammals, studying its effect in comparison to omega-6 fatty acids could help determine how these specific fatty acids affect the oxylipin emissions of parasites. Thus, by examining both omega-3 and omega-6 fatty acids, these

findings could offer deeper insights into how diet composition potentially alters parasite physiology. In addition, future work could look into examining the genetic and enzymatic mechanisms underlying oxylipin production and emission in cercariae. By investigating the expression of genes associated with oxylipin pathways, including the LOX, COX, and CYP pathways, we could also gain insights into how factors such as parasite density and host diet modulate oxylipin emissions at the molecular level.

This thesis provides one of the first characterizations of oxylipin production and emission by *E. trivolvis* lineage c cercariae using HPLC-MS/MS. While other studies often required tens of thousands of cercariae to quantify lipids, this work demonstrates that oxylipins can be successfully quantified from a few hundred to low thousands of cercariae. This methodological advancement is significant as it allows researchers to work around issues regarding low infection prevalence within hosts and still generate sufficient samples for analysis. By showing that smaller pools of cercariae ranging from low hundreds to low thousands are sufficient for oxylipin detection, this approach enables more flexible experimental designs and replication, allowing scientists to overcome practical limitations commonly encountered in parasitology research. Furthermore, this study integrates environmental (density) and host-related (dietary linoleic acid) factors, offering a multifaceted perspective on chemical ecology in the realm of parasitology.

In this study, the relatively low infection prevalence (5.6%) in the dietary linoleic acid experiment, was a limitation that affected my ability to create additional or higher density samples. Another limitation in this study was that by analyzing only water-conditioned samples with cercariae rather than the entire cercarial tissues, which may be very difficult to achieve due to the small size of cercarial parasites, limits the ability to determine the extent to which host-derived fatty acids are incorporated within the biochemical pathways of parasites.

Despite these limitations, this thesis advances research on the role of chemicals in parasite-modified host behaviour and host specify. By characterizing the diversity of types and concentrations of oxylipins emitted by *E. trivolvis* lineage c cercariae, this work provides foundational evidence that these compounds are produced and released by these parasites, even if I did not find linear density-dependent effects on oxylipin emissions. Although direct links to host behaviour or specificity were not assessed, identifying this array of oxylipins establishes a baseline for future studies to explore how these compounds might influence host attraction, avoidance, or other host-specific interactions. Thus, this thesis provides both methodological approaches and foundational knowledge to investigate chemical ecology in host-parasite interactions.

Overall, this thesis provides insights into how trematode cercariae may be interacting with their chemical environment and regulating their own signalling outputs. In this way, this work lays the groundwork for broader investigations into the ecological and evolutionary roles of parasite-derived bioactive compounds, with potential applications in controlling either the transmission of parasites or identifying molecular biomarkers for freshwater parasite surveillance.

Appendix

Chapter 1 Figures and Tables

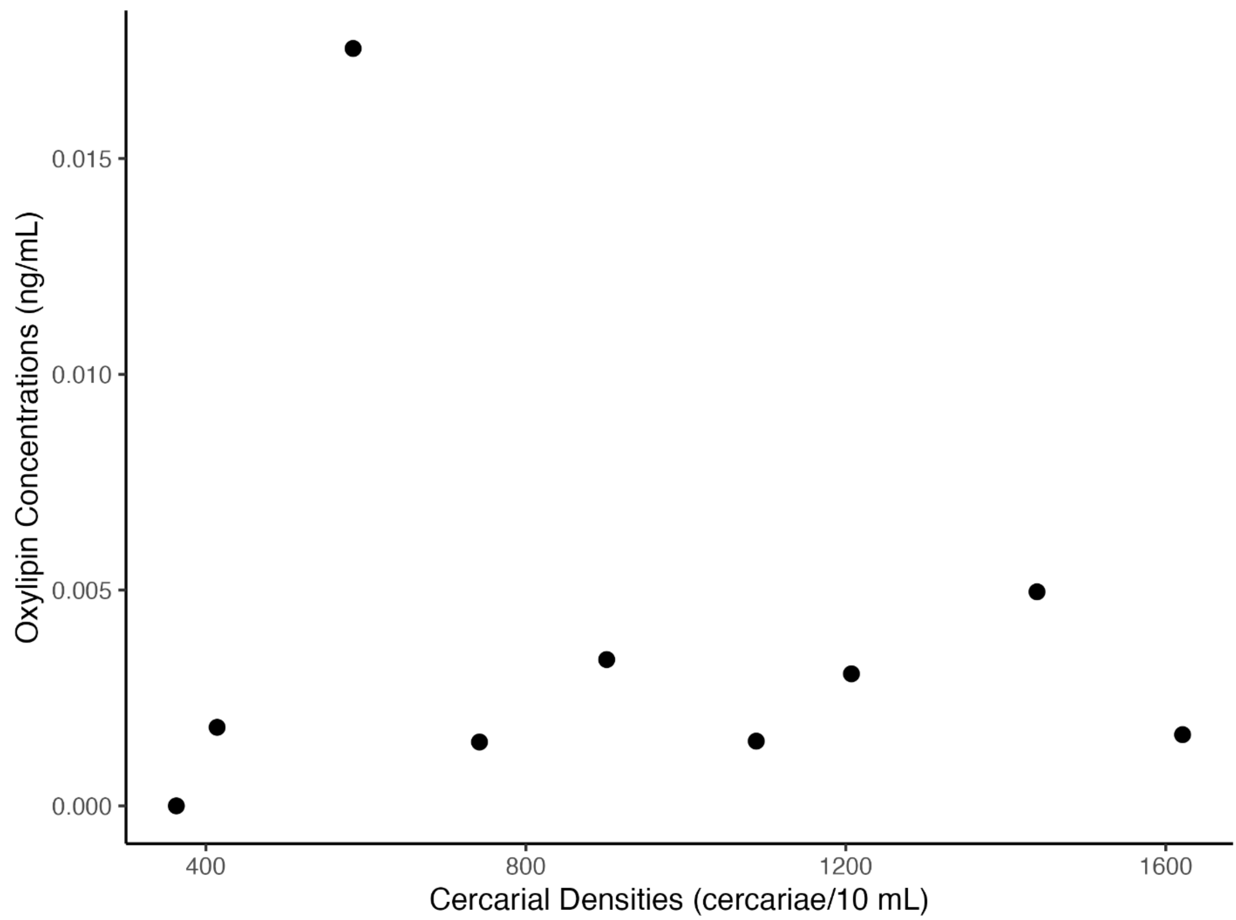

Figure 3.1. Concentration of PGE_2 (ng/mL) across cercarial densities (cercariae/10 mL).

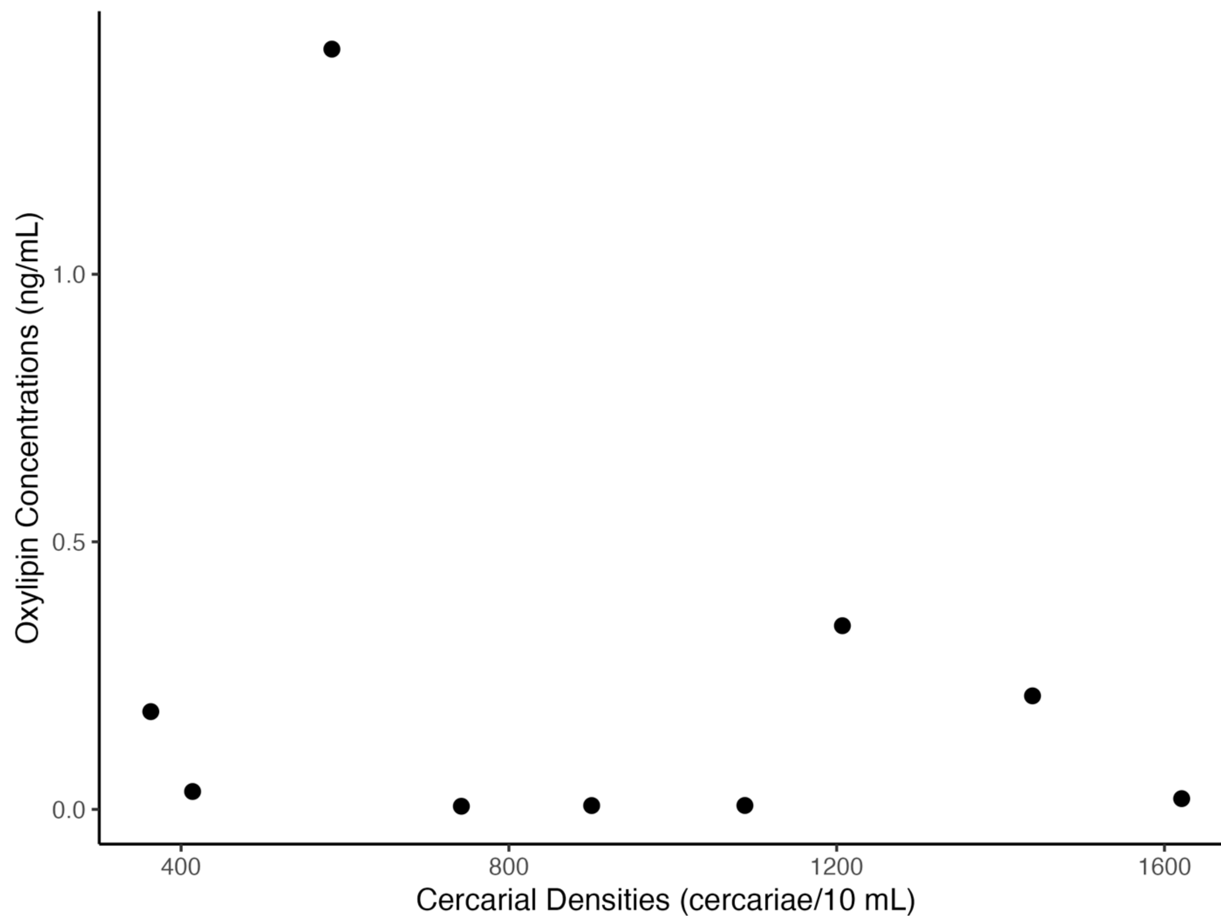

Figure 3.2. Concentration of 9-HODE (ng/mL) across cercarial densities (cercariae/10 mL).

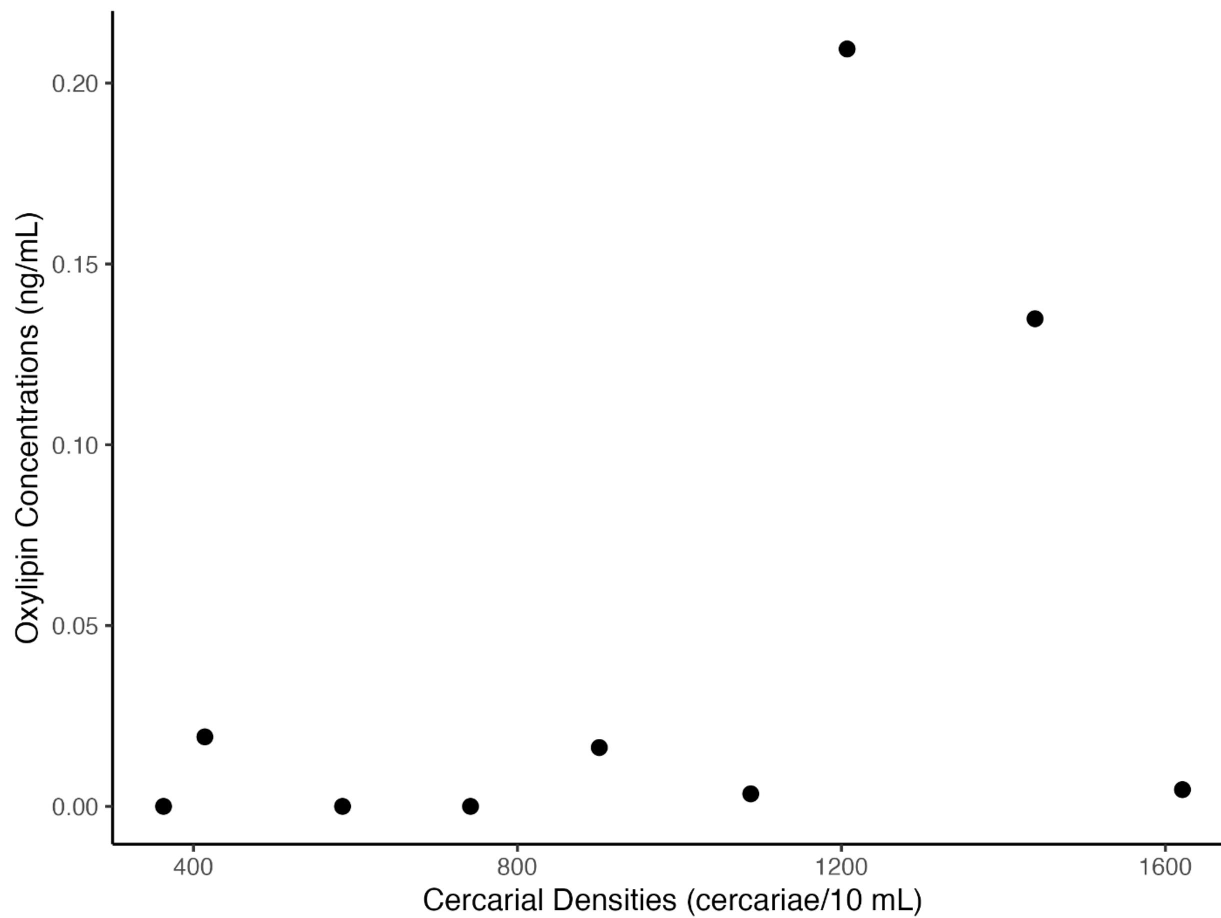

Figure 3.3. Concentration of 9-oxoODE (ng/mL) across cercarial densities (cercariae/10 mL).

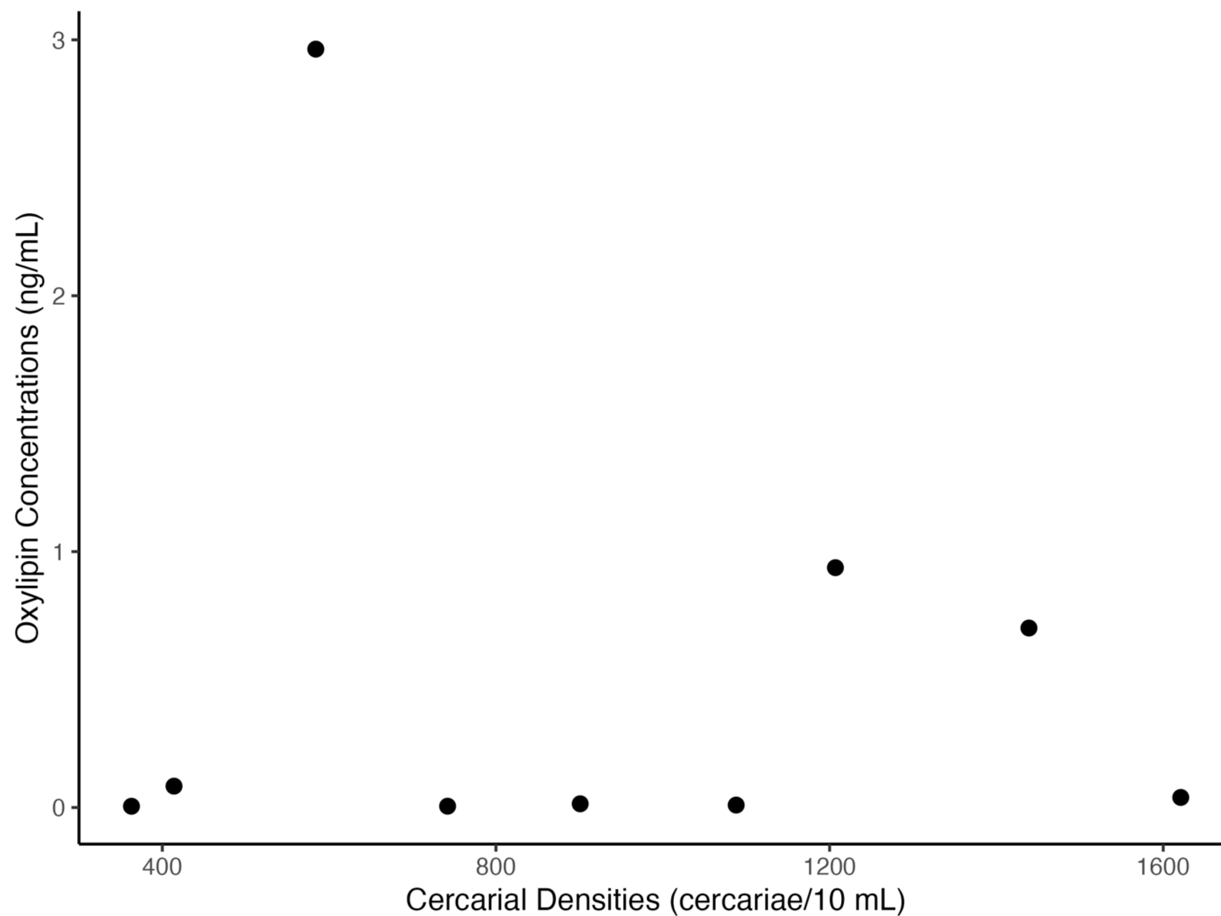

Figure 3.4. Concentration of 13-HODE (ng/mL) across cercarial densities (cercariae/10 mL).

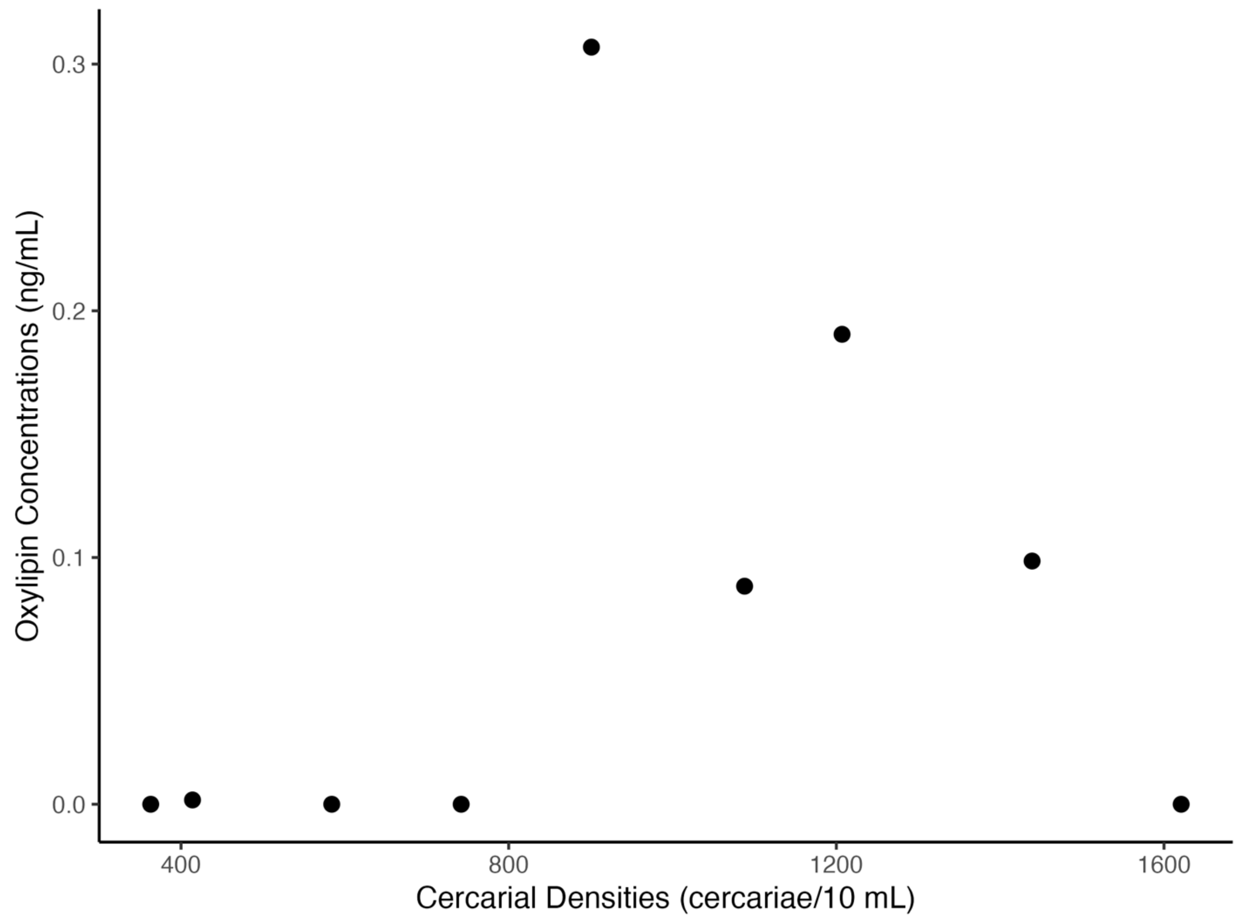

Figure 3.5. Concentration of 13-oxoODE (ng/mL) across cercarial densities (cercariae/10 mL).

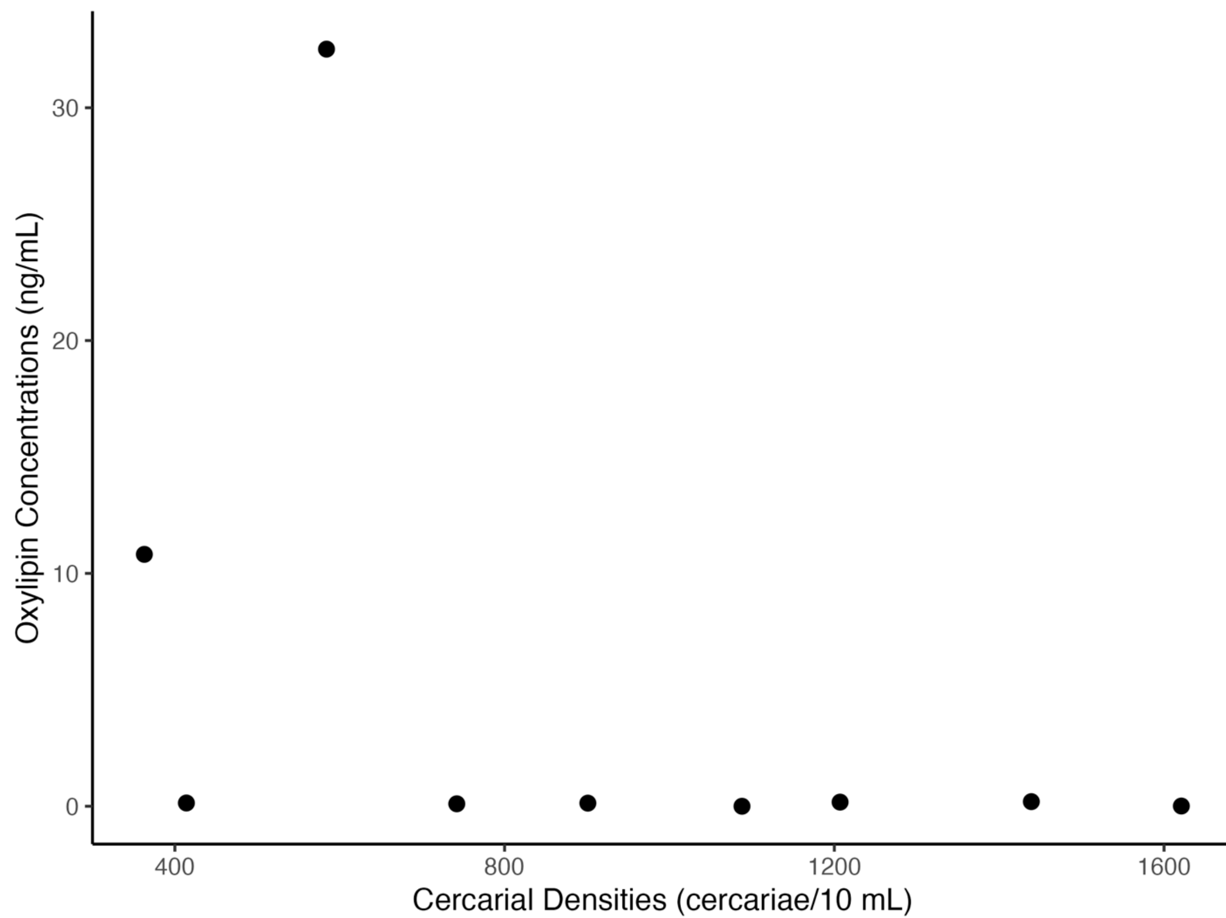

Figure 3.6. Concentration of 9,10,13-triHOME (ng/mL) across cercarial densities (cercariae/10 mL).

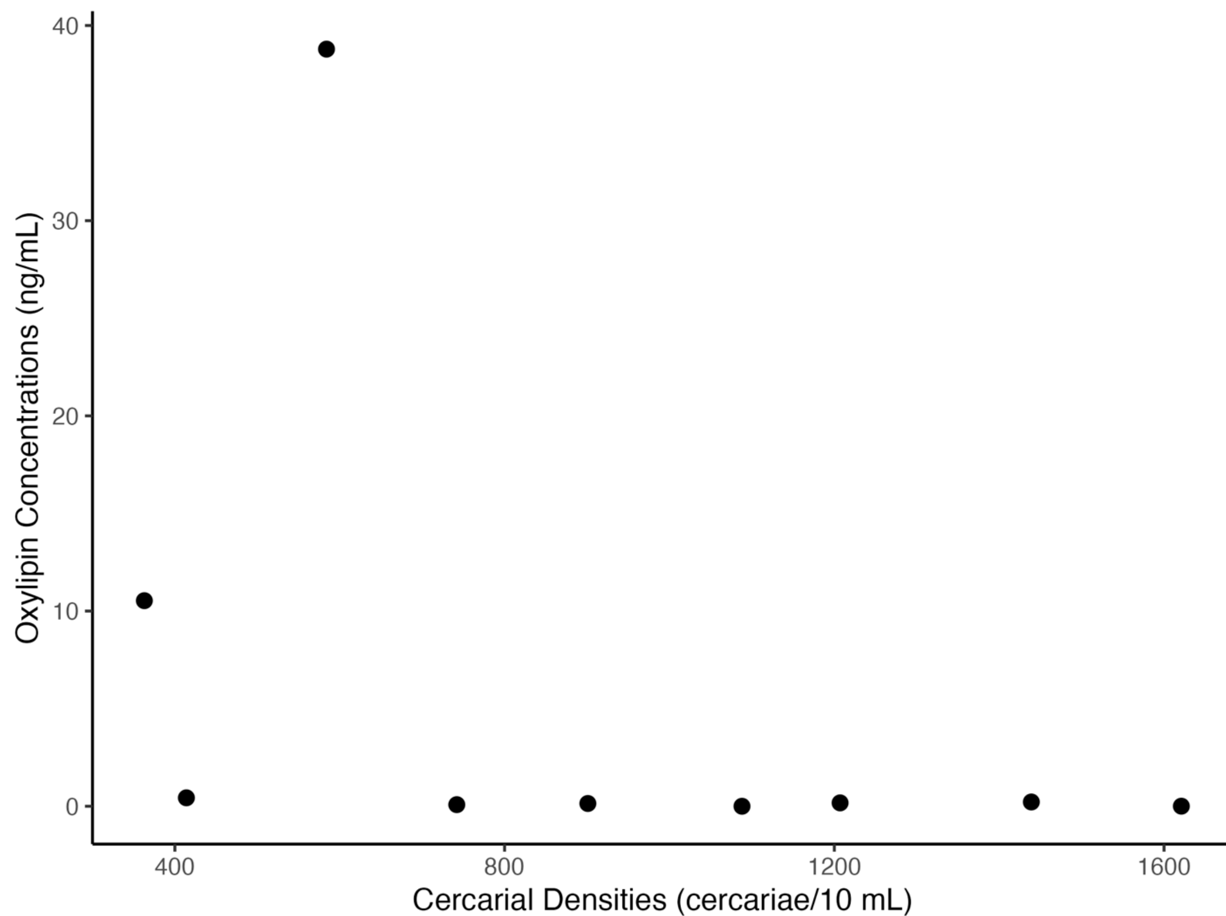

Figure 3.7. Concentration of 9,12,13-triHOME (ng/mL) across cercarial densities (cercariae/10 mL).

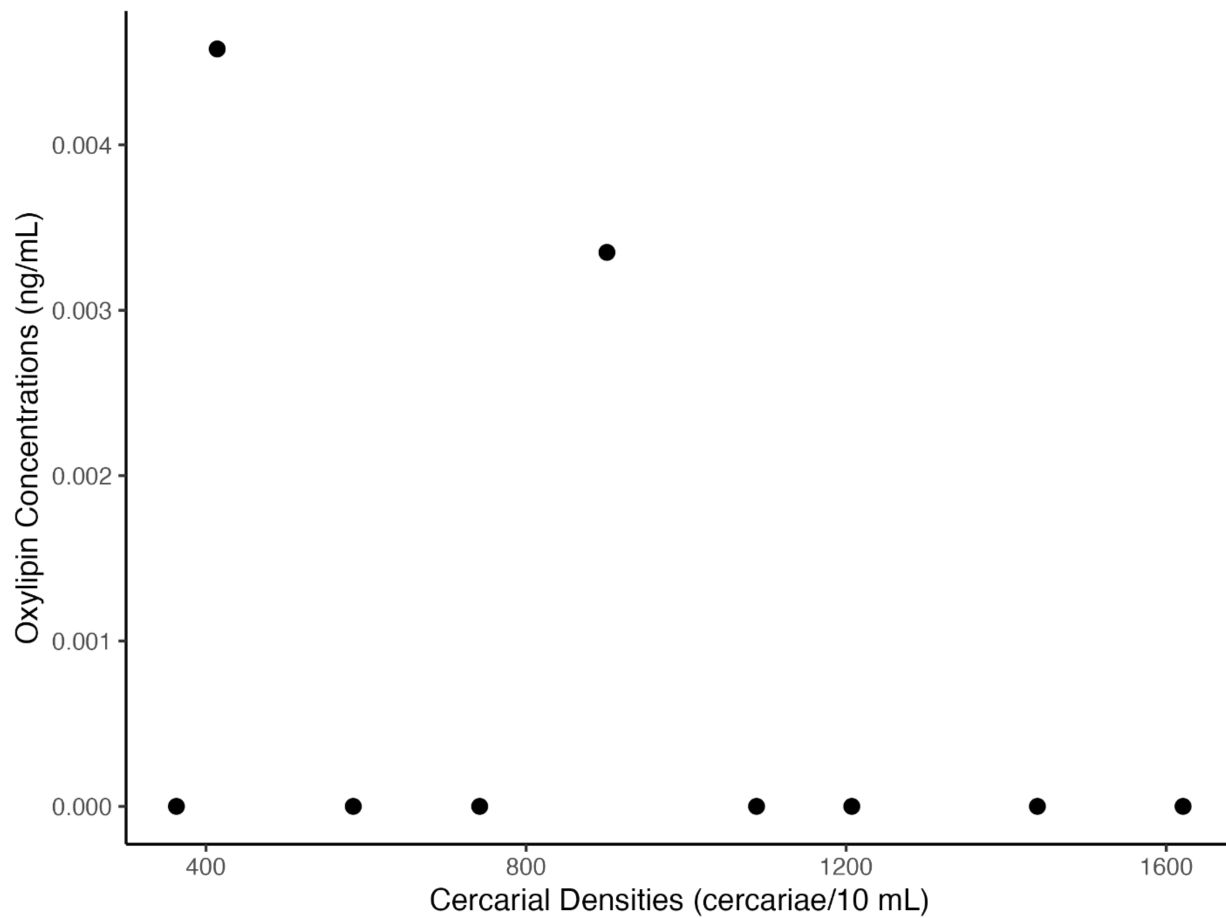

Figure 3.8. Concentration of 13-HOTrE-y (ng/mL) across cercarial densities (cercariae/10 mL).

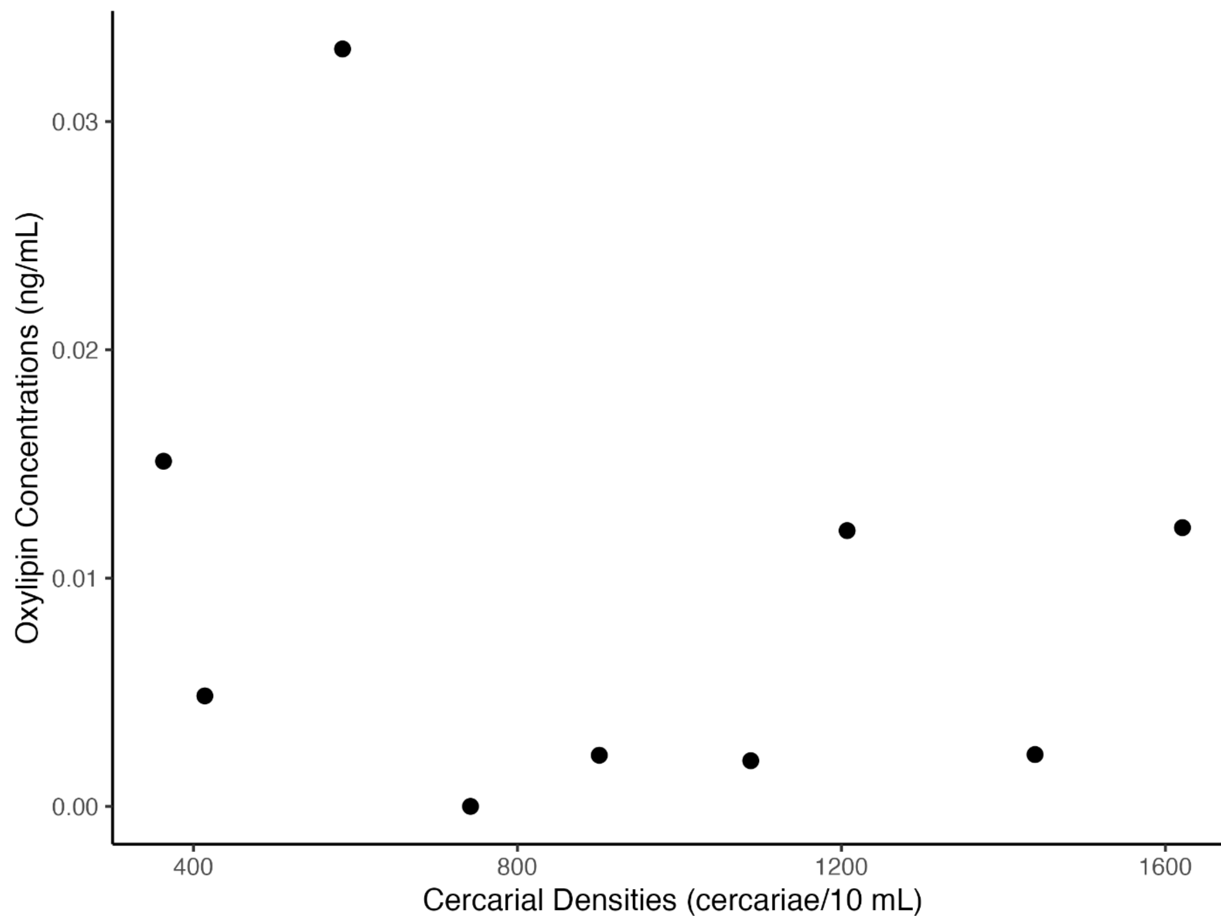

Figure 3.9. Concentration of 13-HOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

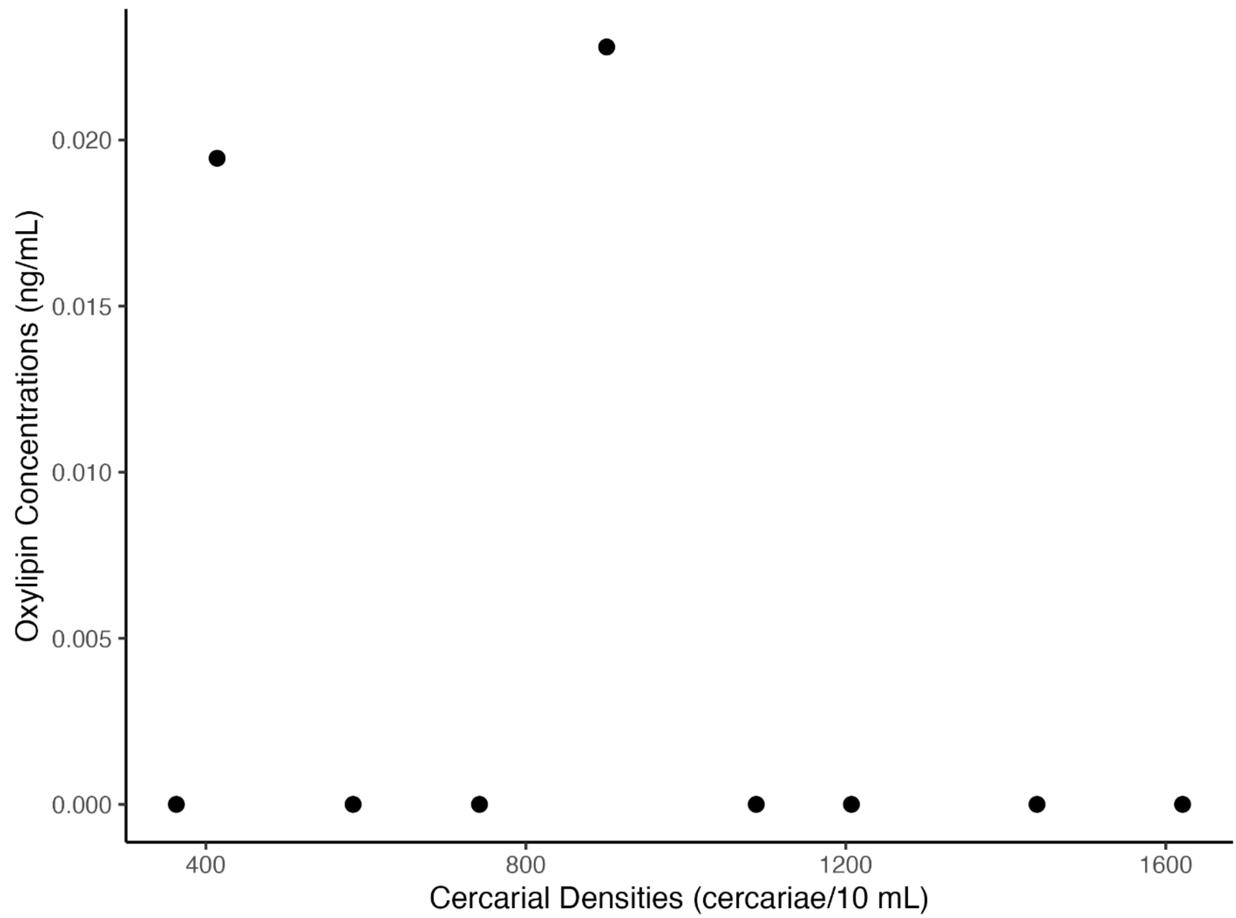

Figure 3.10. Concentration of 15-HEPE (ng/mL) across cercarial densities (cercariae/10 mL).

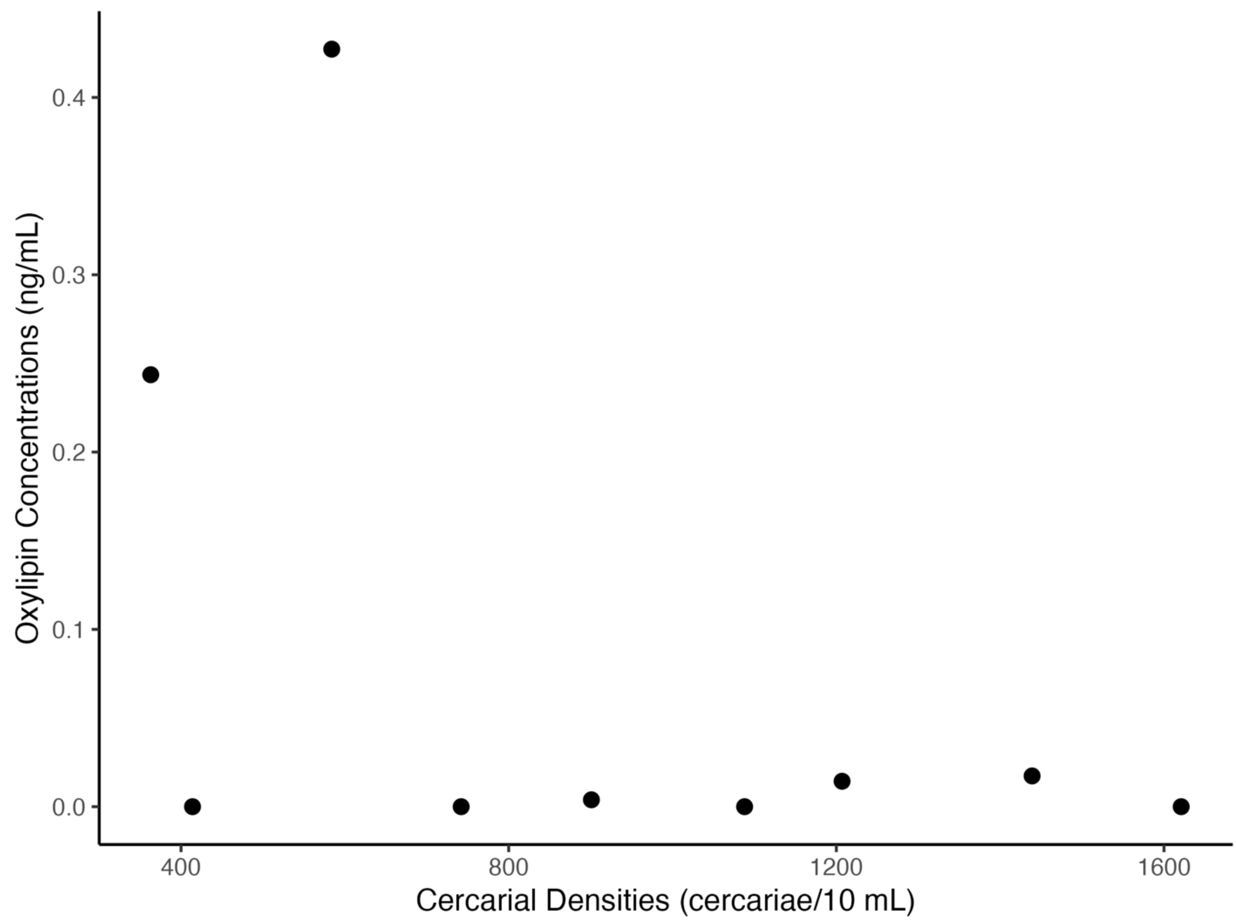

Figure 3.11. Concentration of 9,10-diHOME (ng/mL) across cercarial densities (cercariae/10 mL).

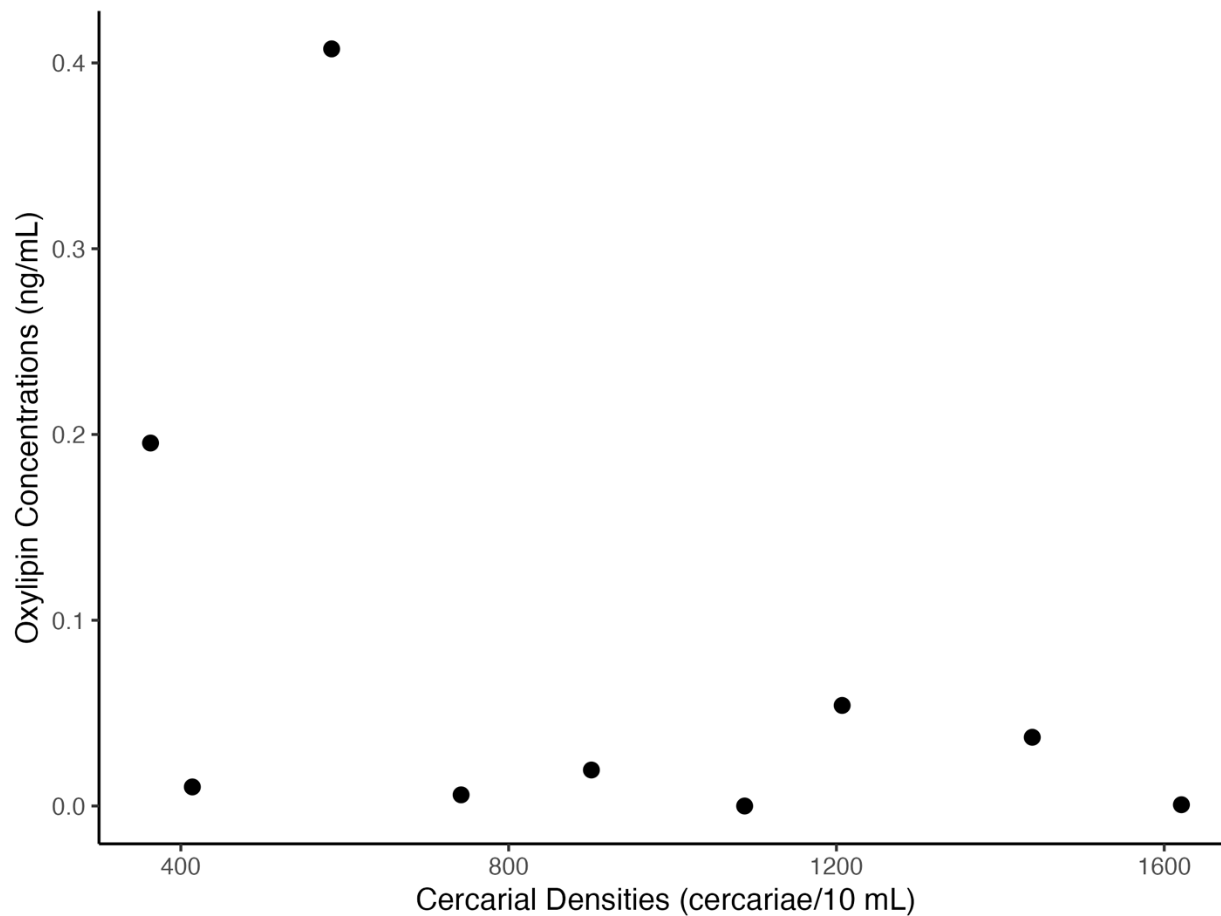

Figure 3.12. Concentration of 12,13-diHOME (ng/mL) across cercarial densities (cercariae/10 mL).

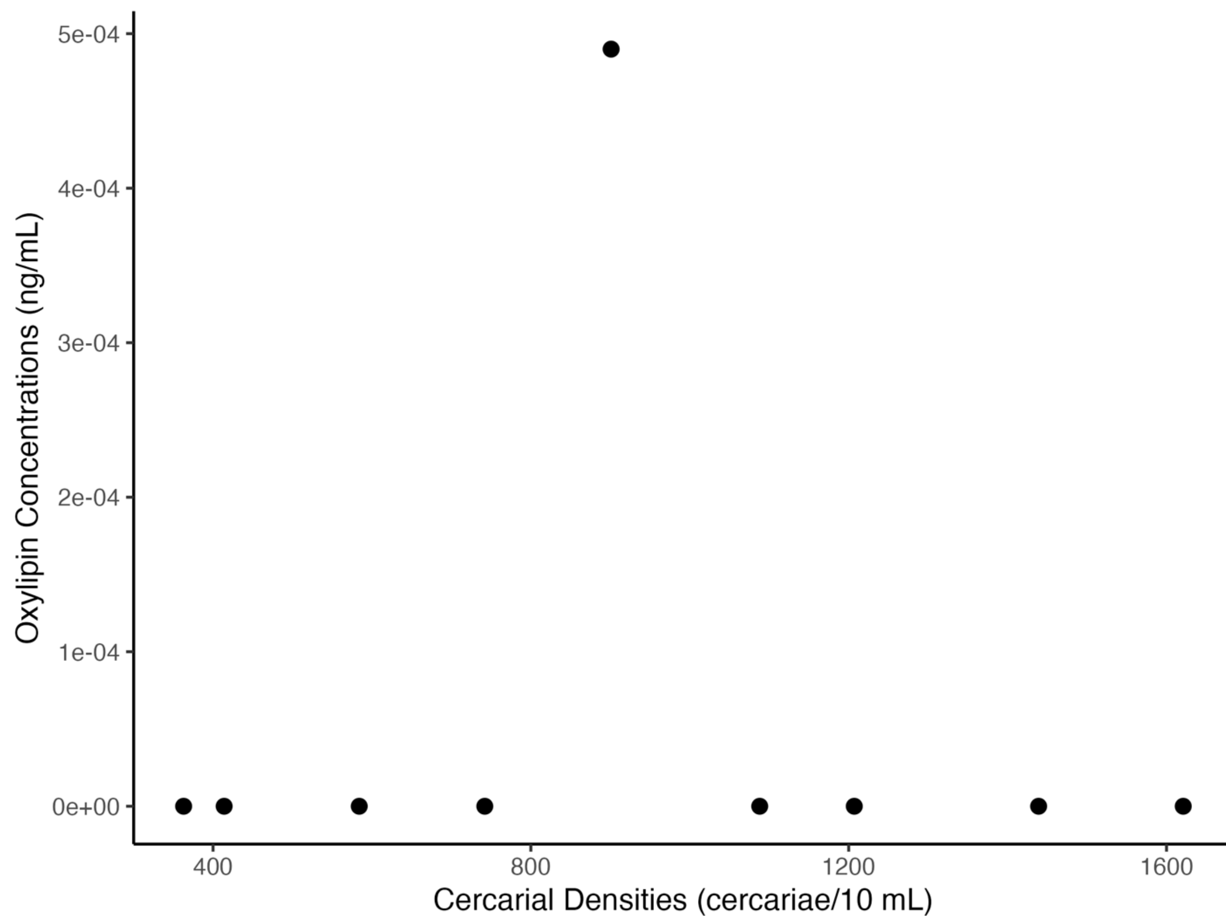

Figure 3.13. Concentration of 14,15-DiHETrE (ng/mL) across cercarial densities (cercariae/10 mL).

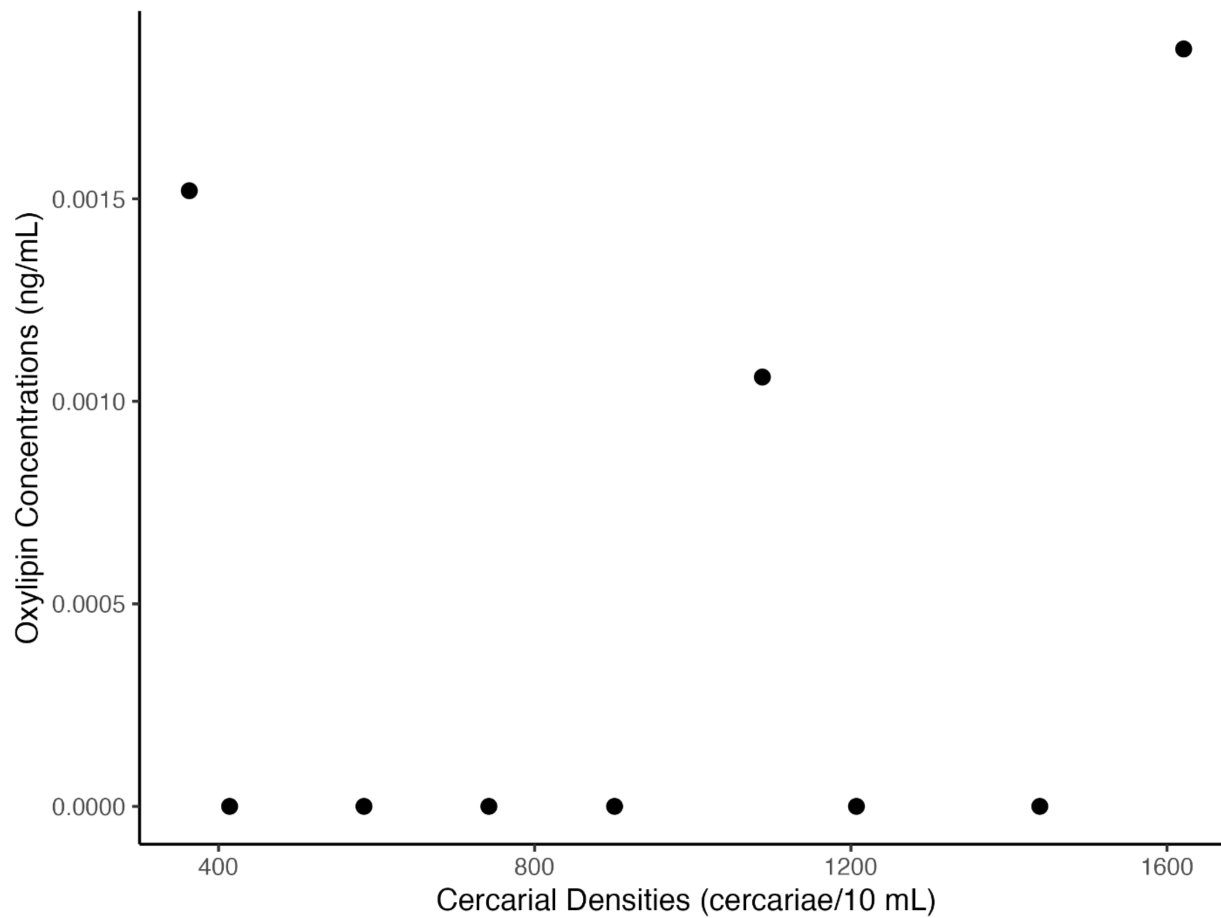

Figure 3.14. Concentration of 8-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

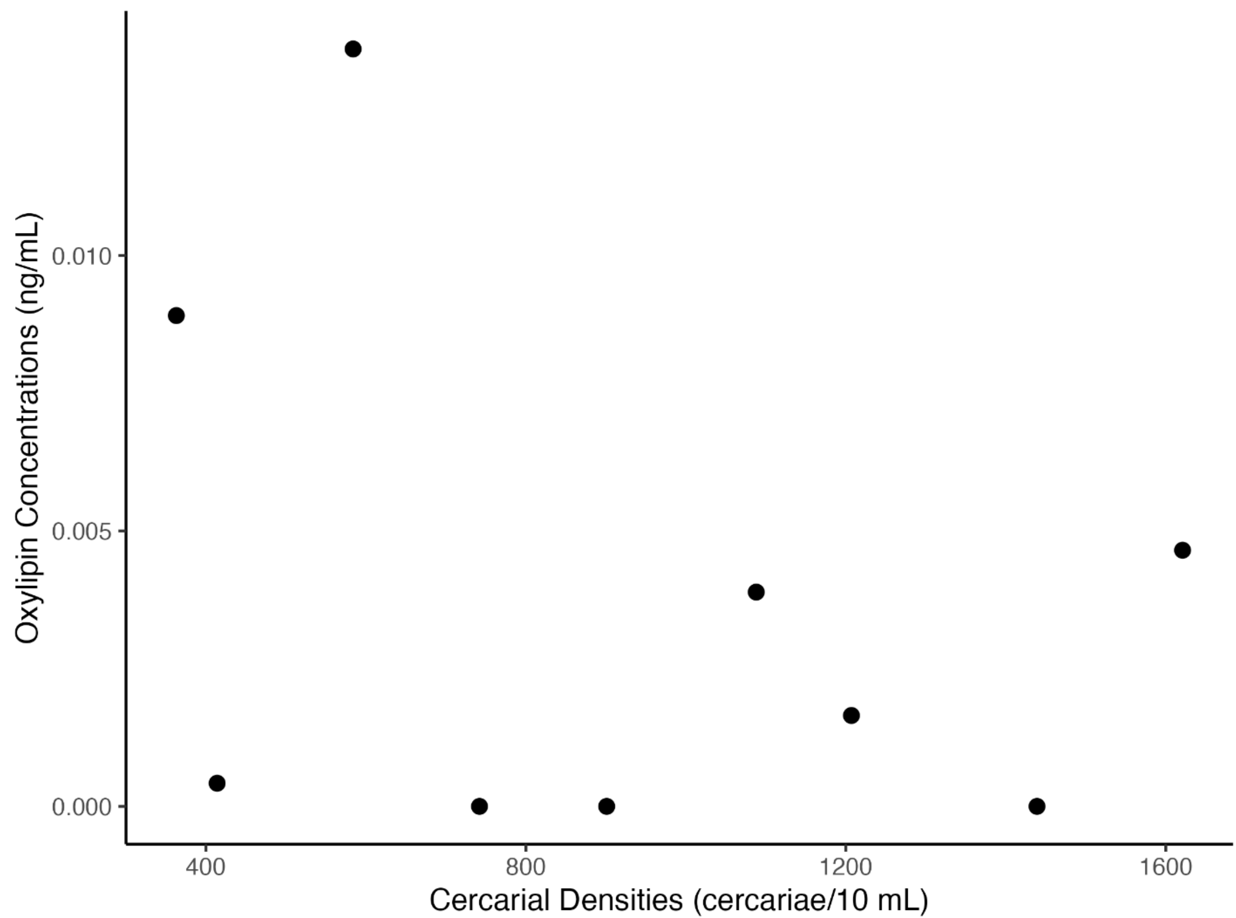

Figure 3.15. Concentration of 9-HOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

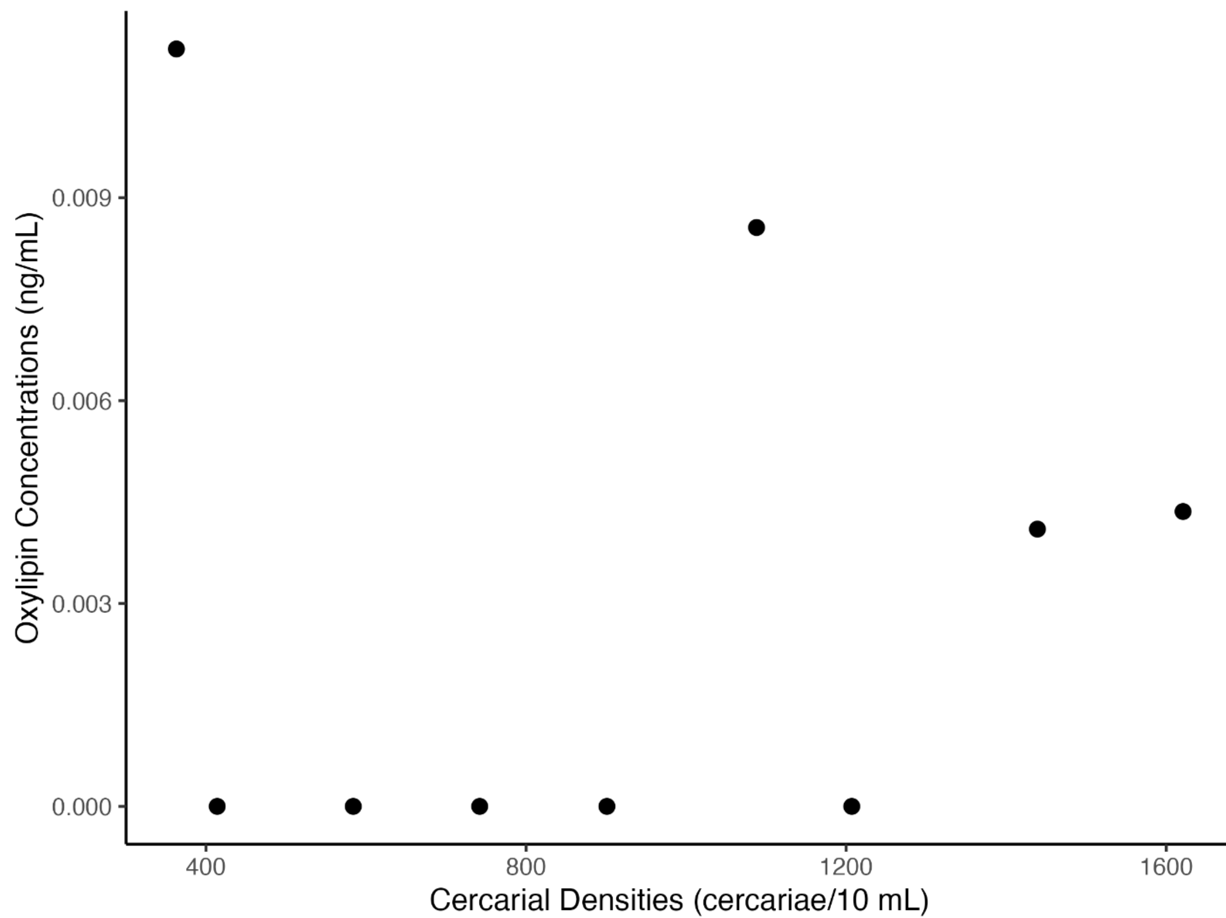

Figure 3.16. Concentration of 9-oxoOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

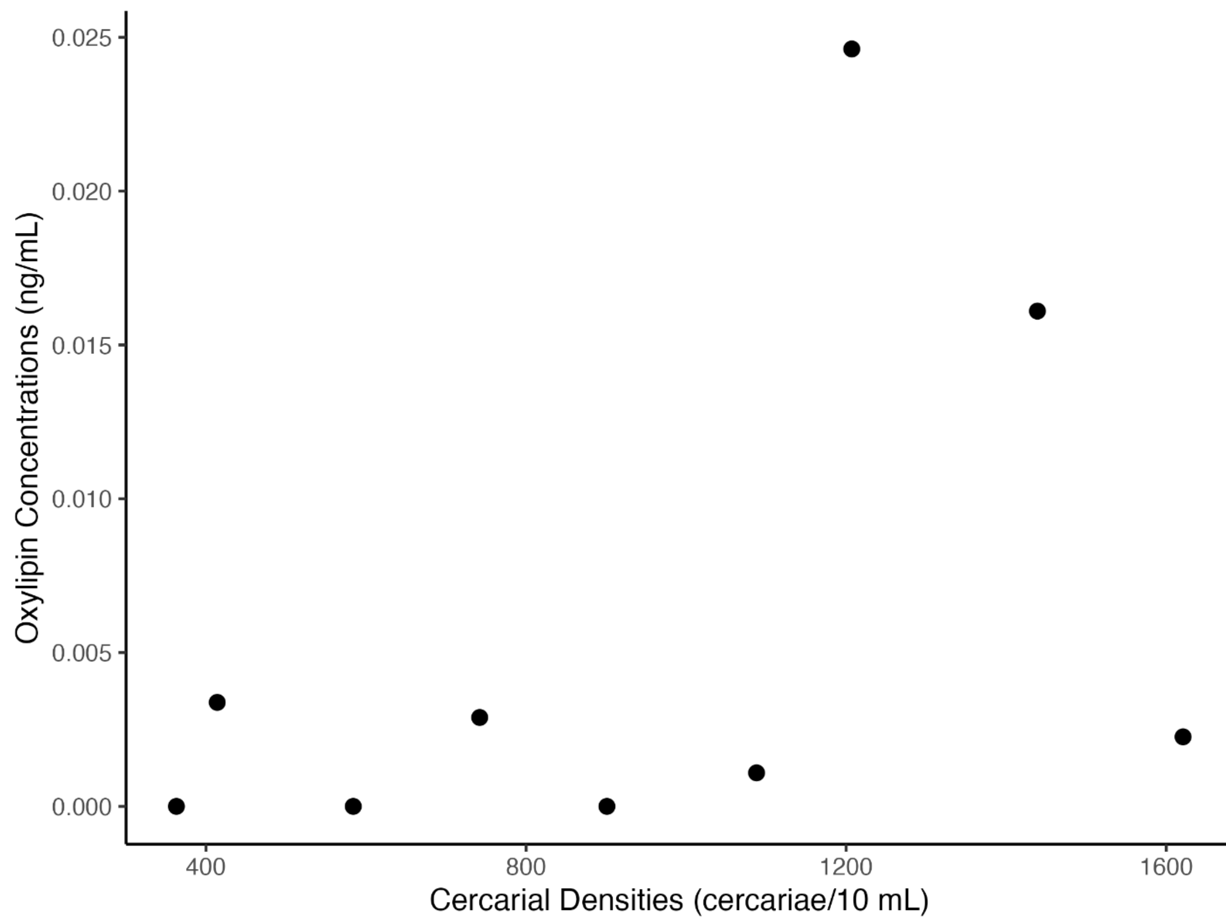

Figure 3.17. Concentration of 9,10-EpOME (ng/mL) across cercarial densities (cercariae/10 mL).

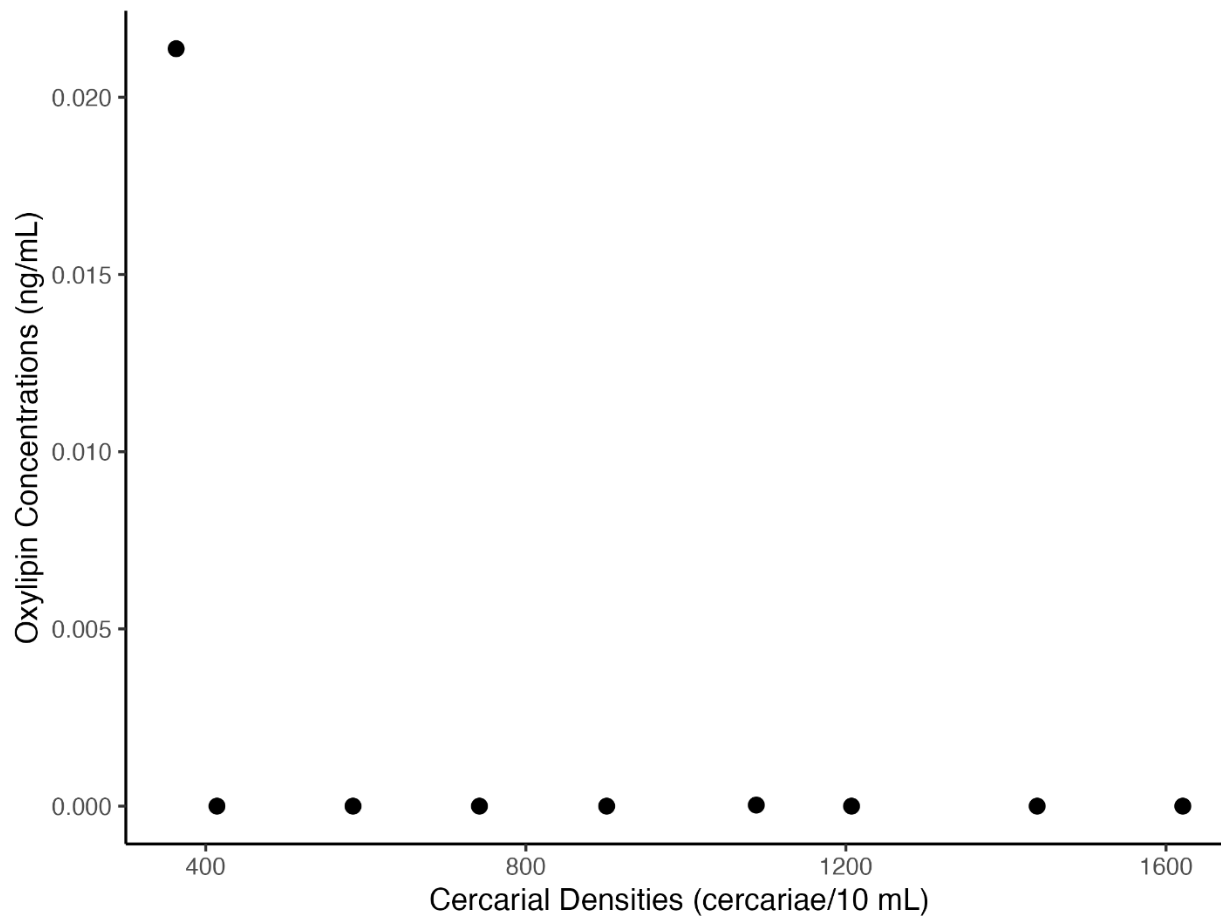

Figure 3.18. Concentration of 16-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

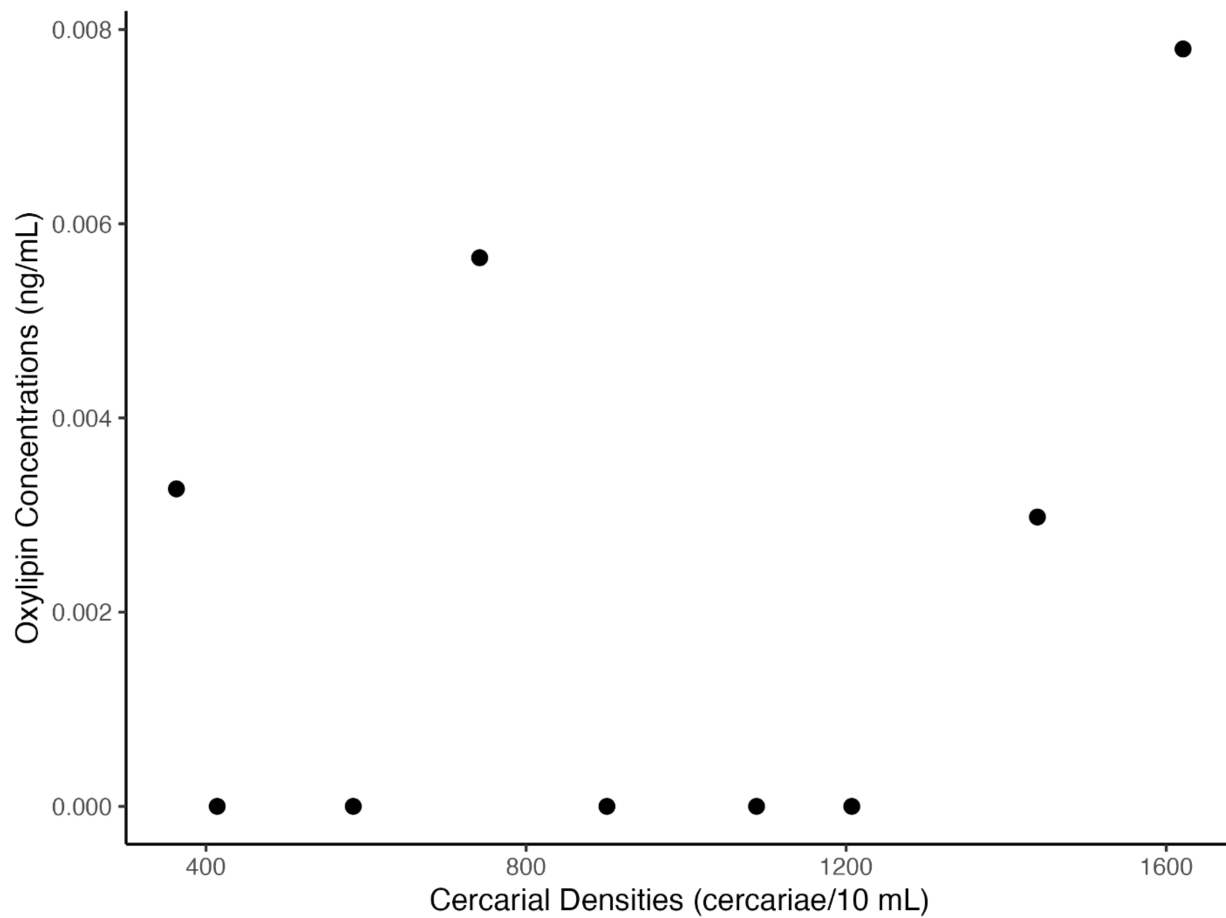

Figure 3.19. Concentration of 5-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

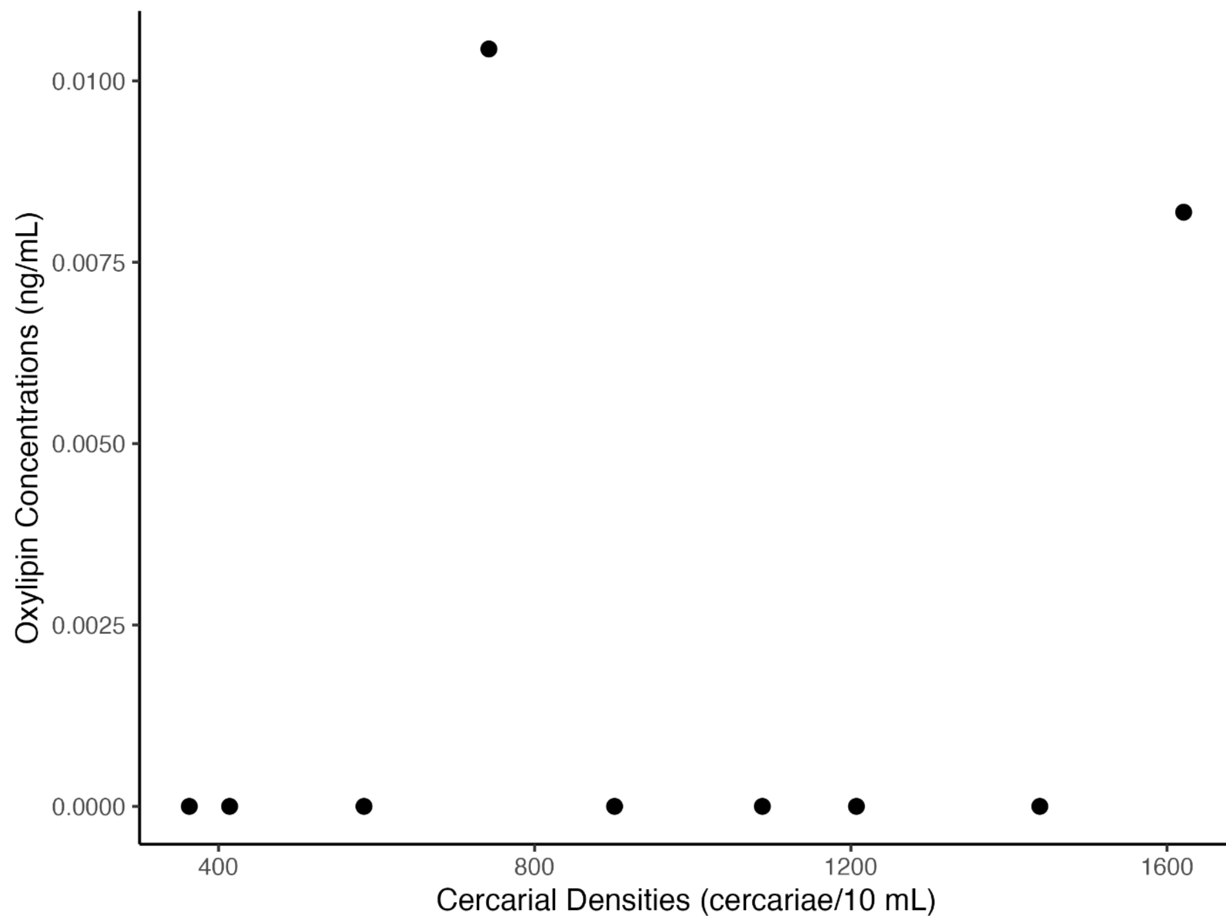

Figure 3.20. Concentration of 12-oxoETE (ng/mL) across cercarial densities (cercariae/10 mL).

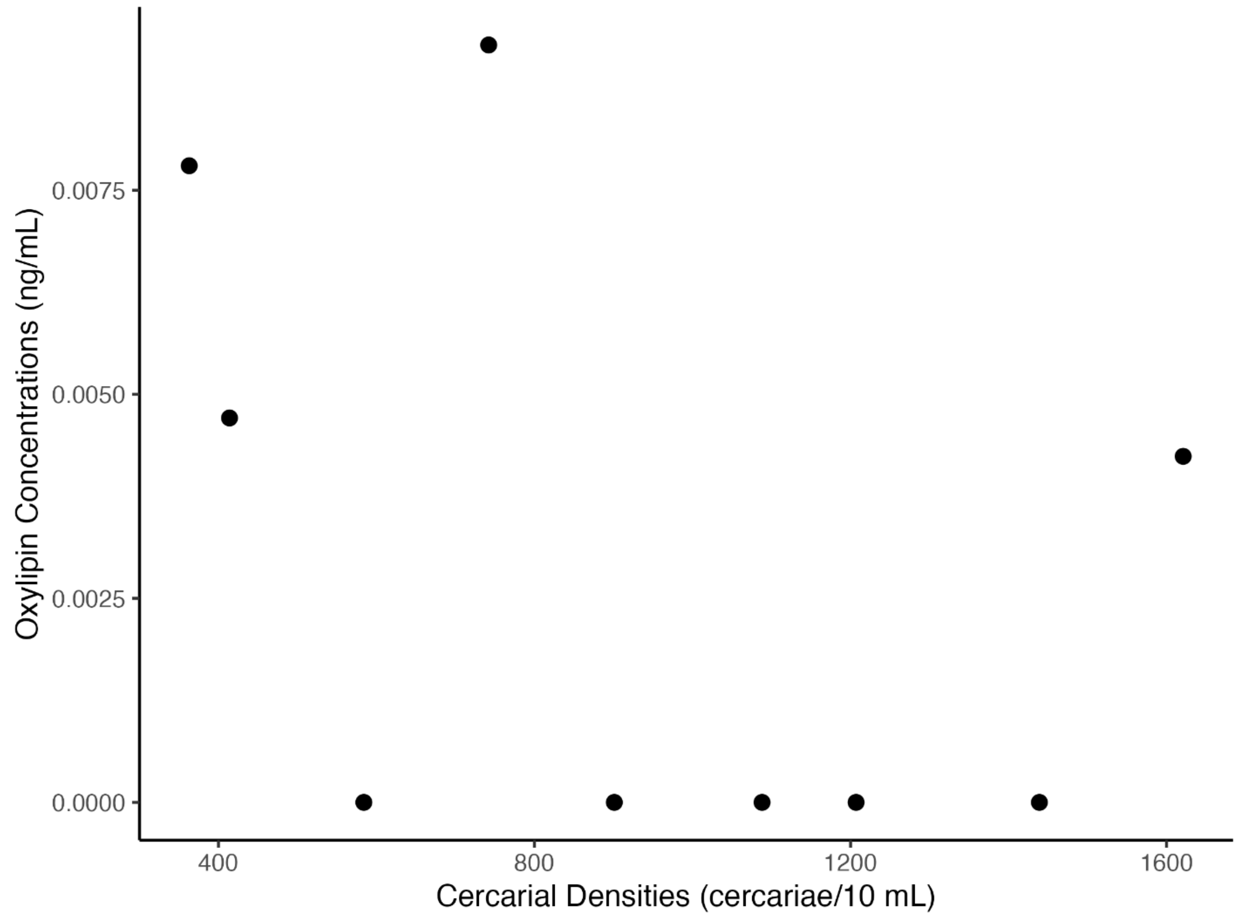

Figure 3.21. Concentration of 15-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

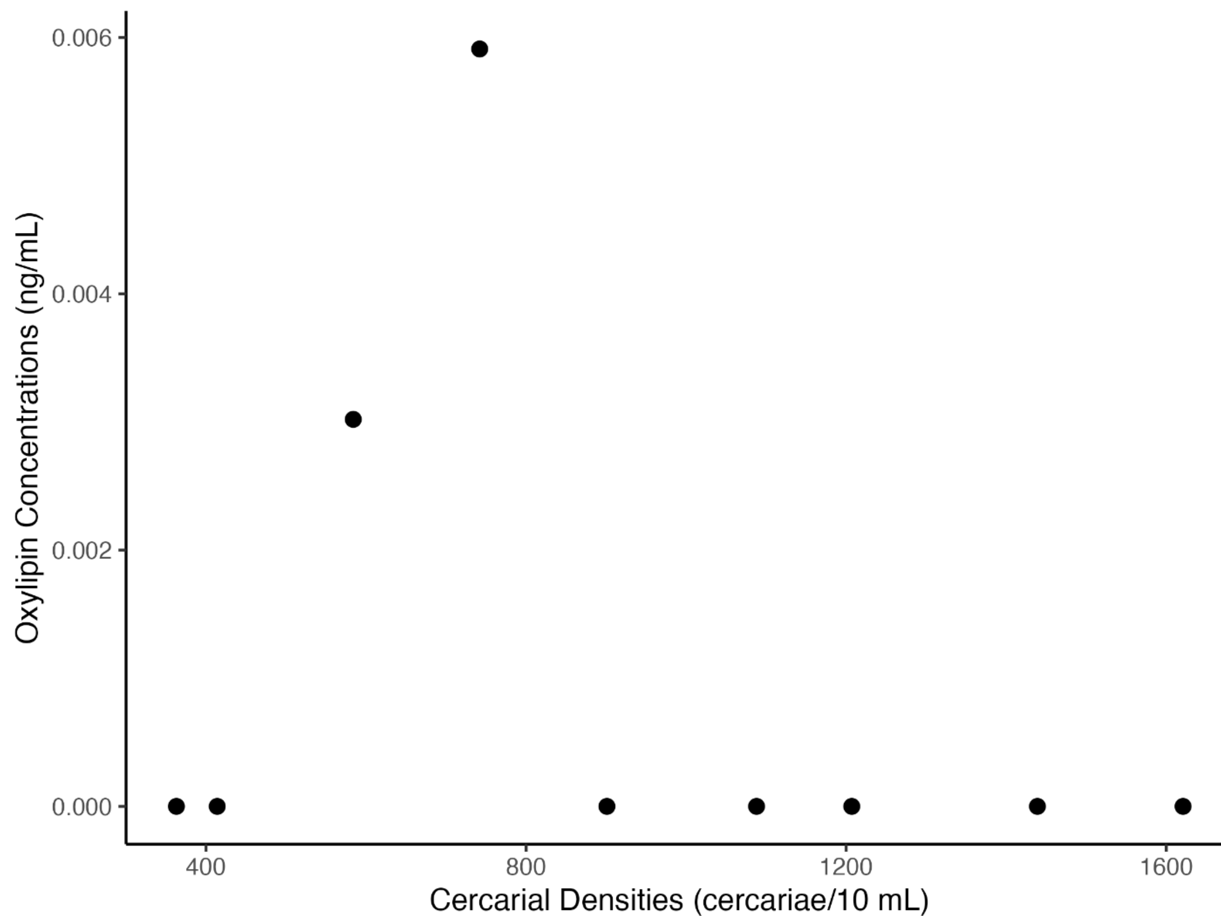

Figure 3.22. Concentration of 15-oxoETE (ng/mL) across cercarial densities (cercariae/10 mL).

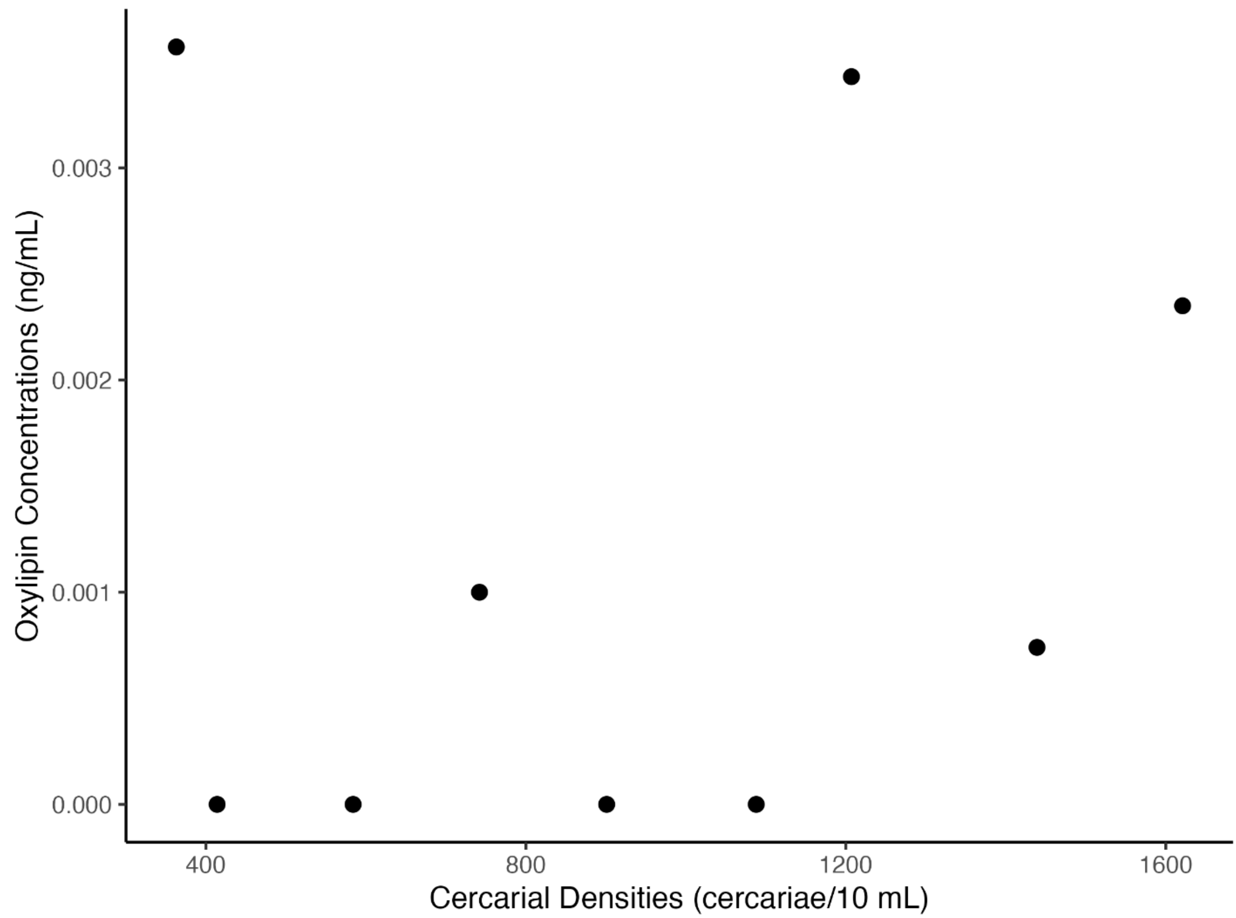

Figure 3.23. Concentration of 12-HEPE (ng/mL) across cercarial densities (cercariae/10 mL).

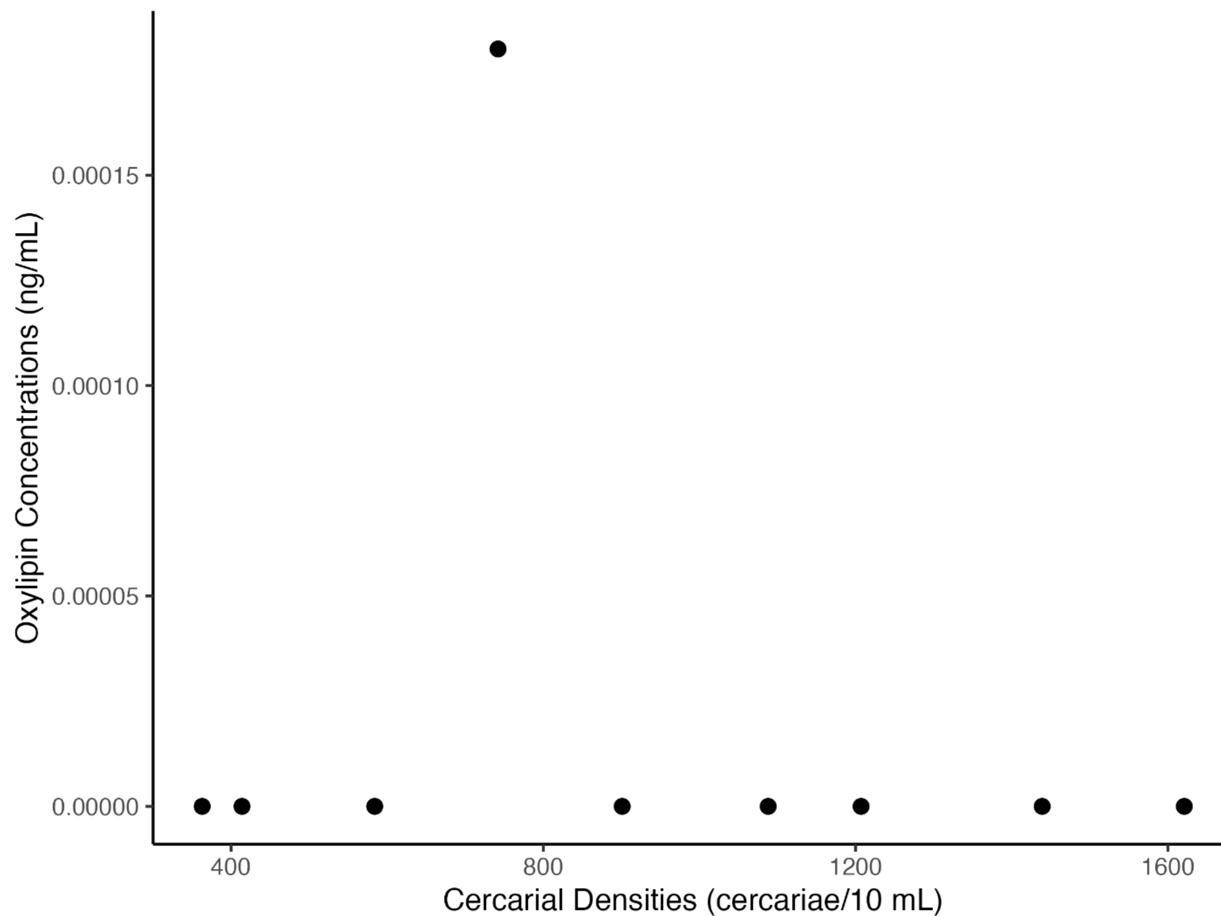

Figure 3.24. Concentration of 8,9-DiHETrE (ng/mL) across cercarial densities (cercariae/10 mL).

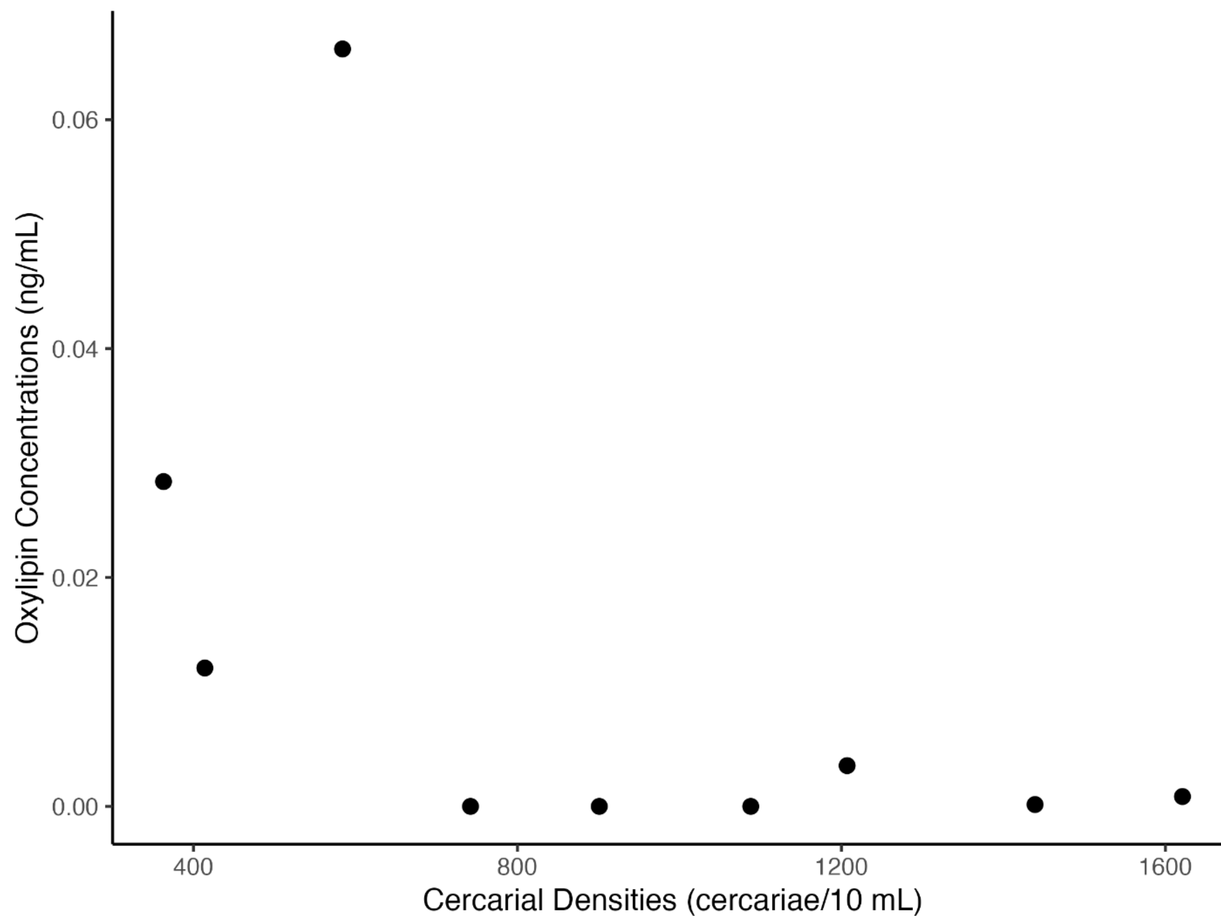

Figure 3.25. Concentration of 20-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

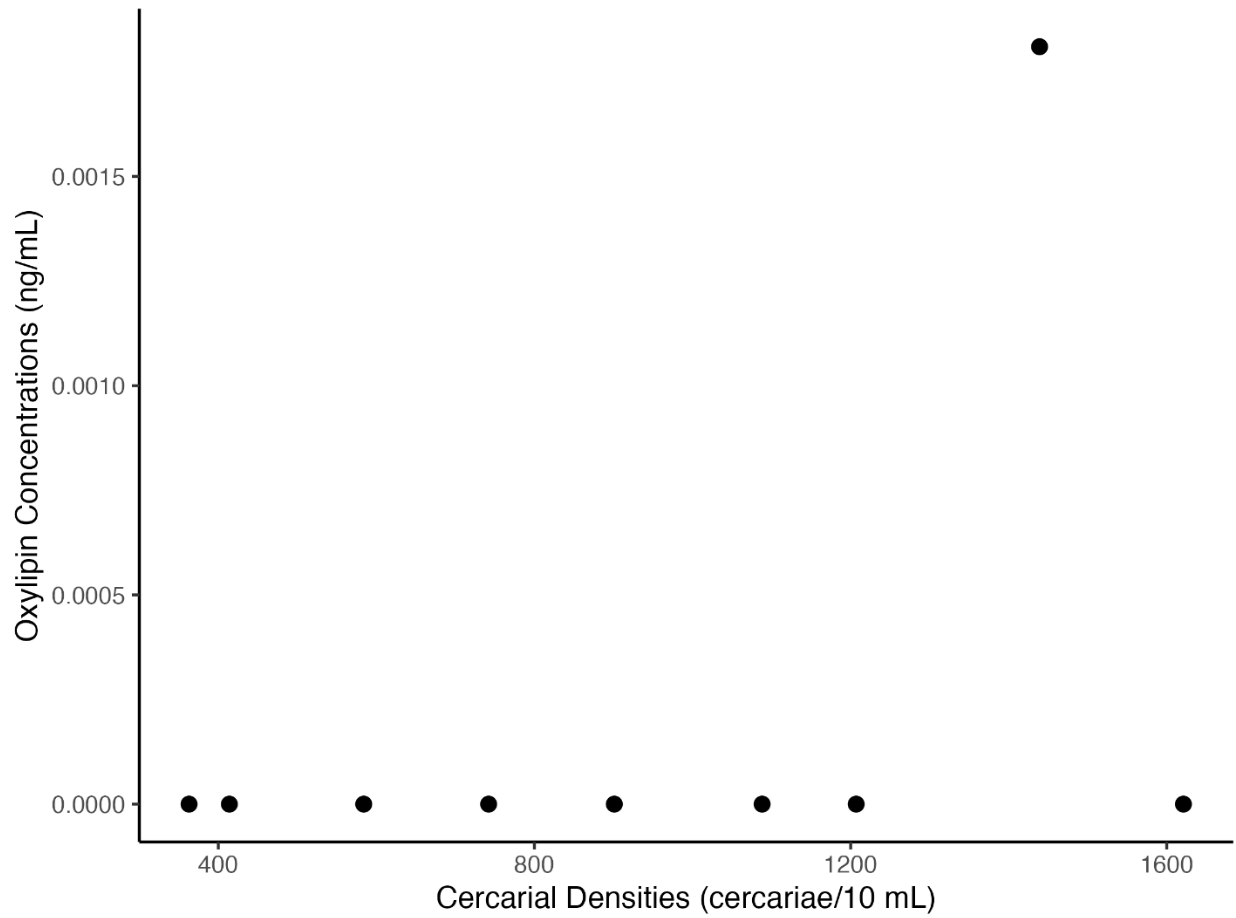

Figure 3.26. Concentration of 15-keto-PGE_2 (ng/mL) across cercarial densities (cercariae/10 mL).

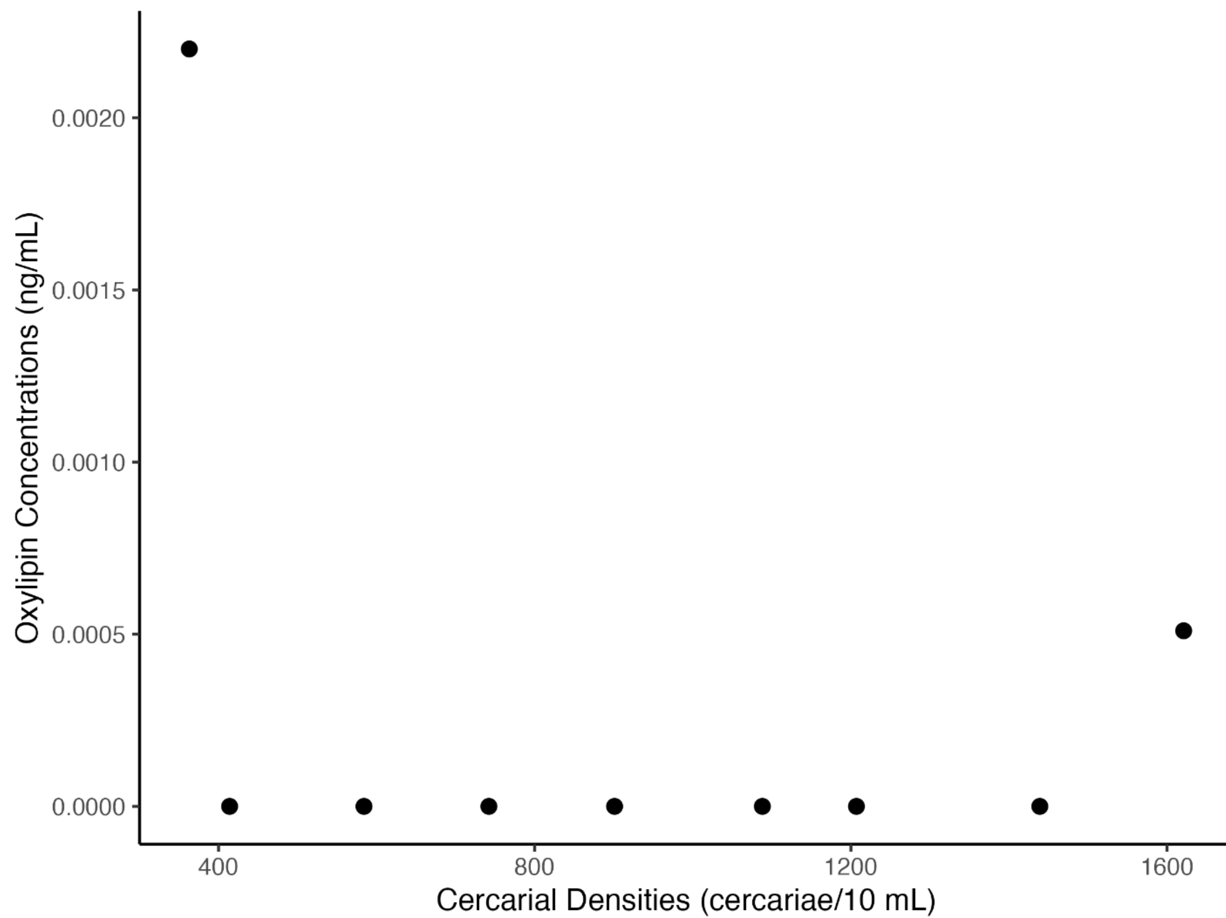

Figure 3.27. Concentration of 11-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

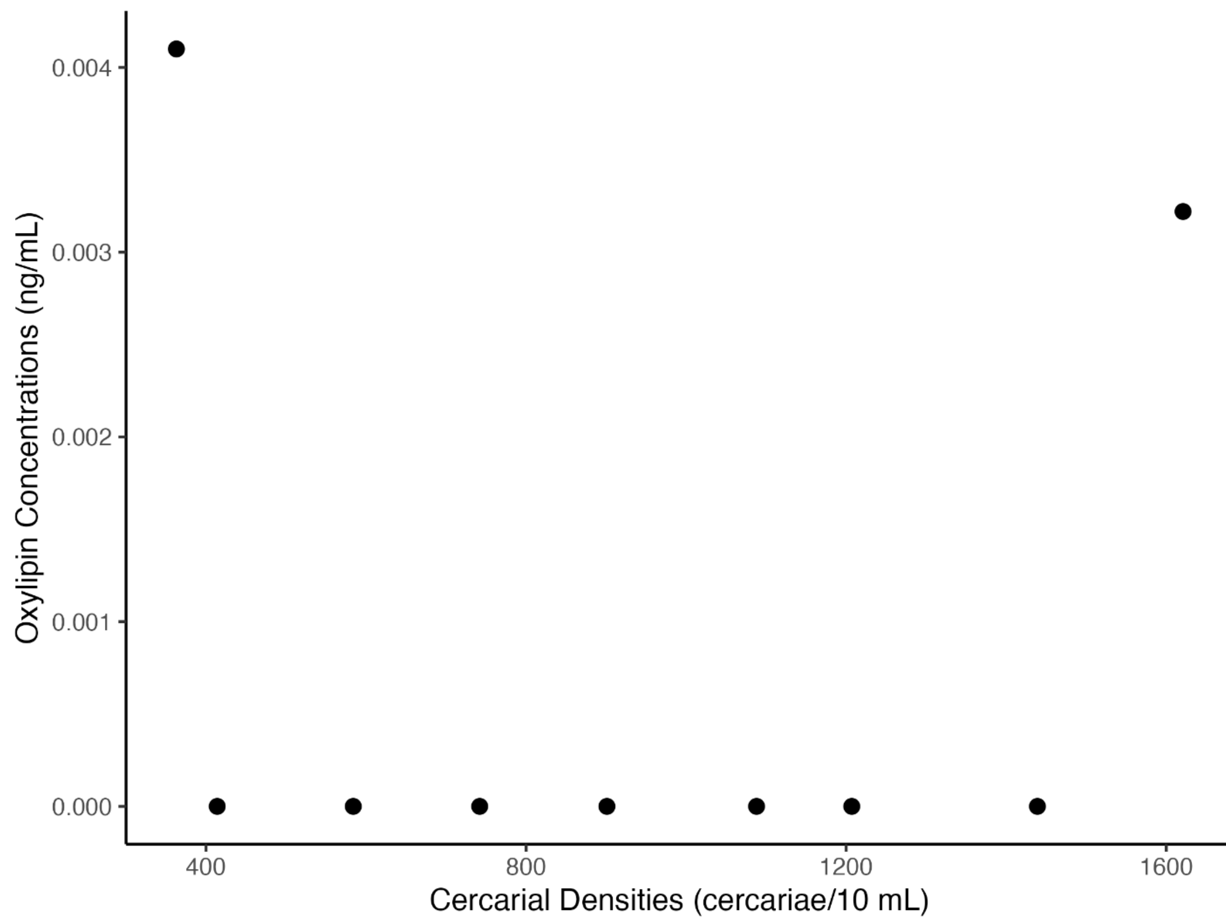

Figure 3.28. Concentration of 12,13-EpODE (ng/mL) across cercarial densities (cercariae/10 mL).

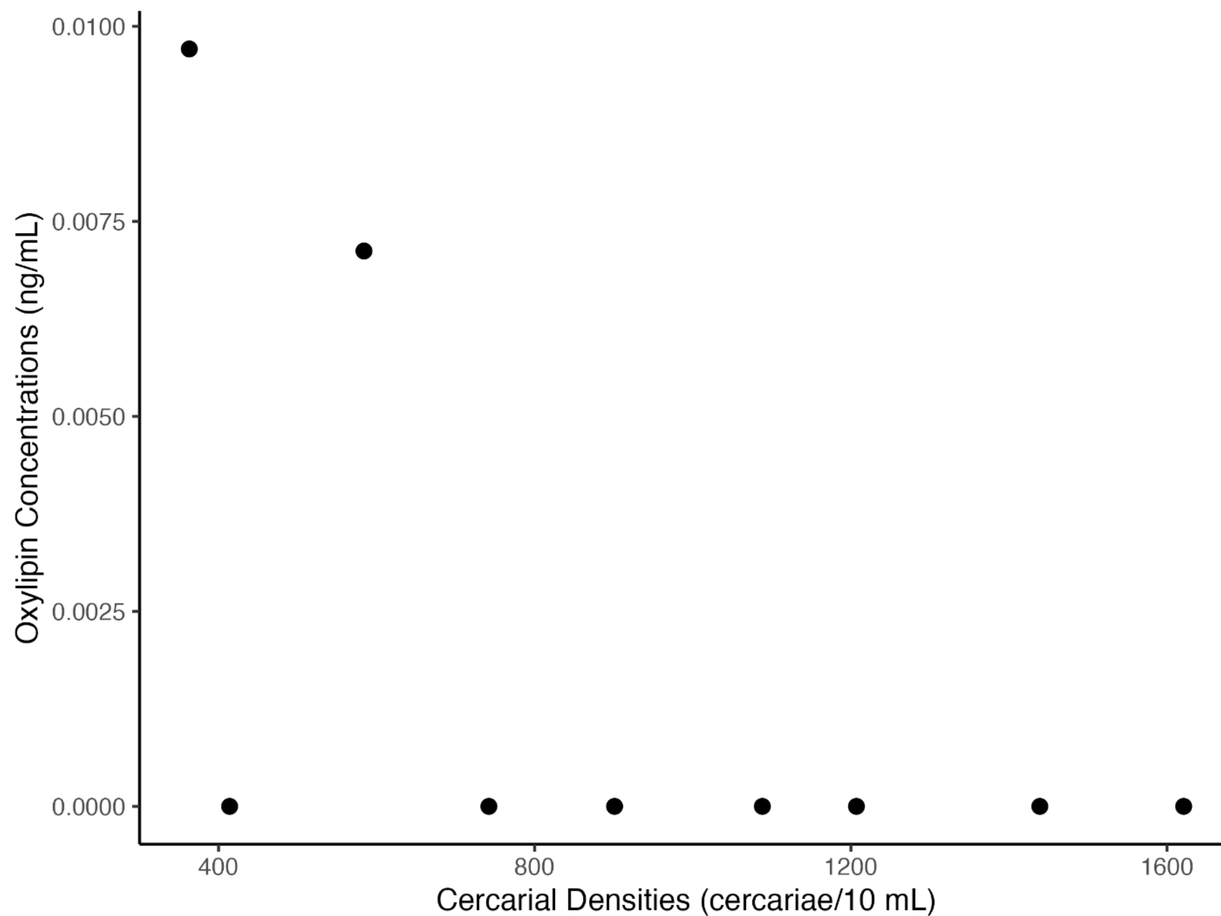

Figure 3.29. Concentration of 2,3-dinor-TXB_2 (ng/mL) across cercarial densities (cercariae/10 mL).

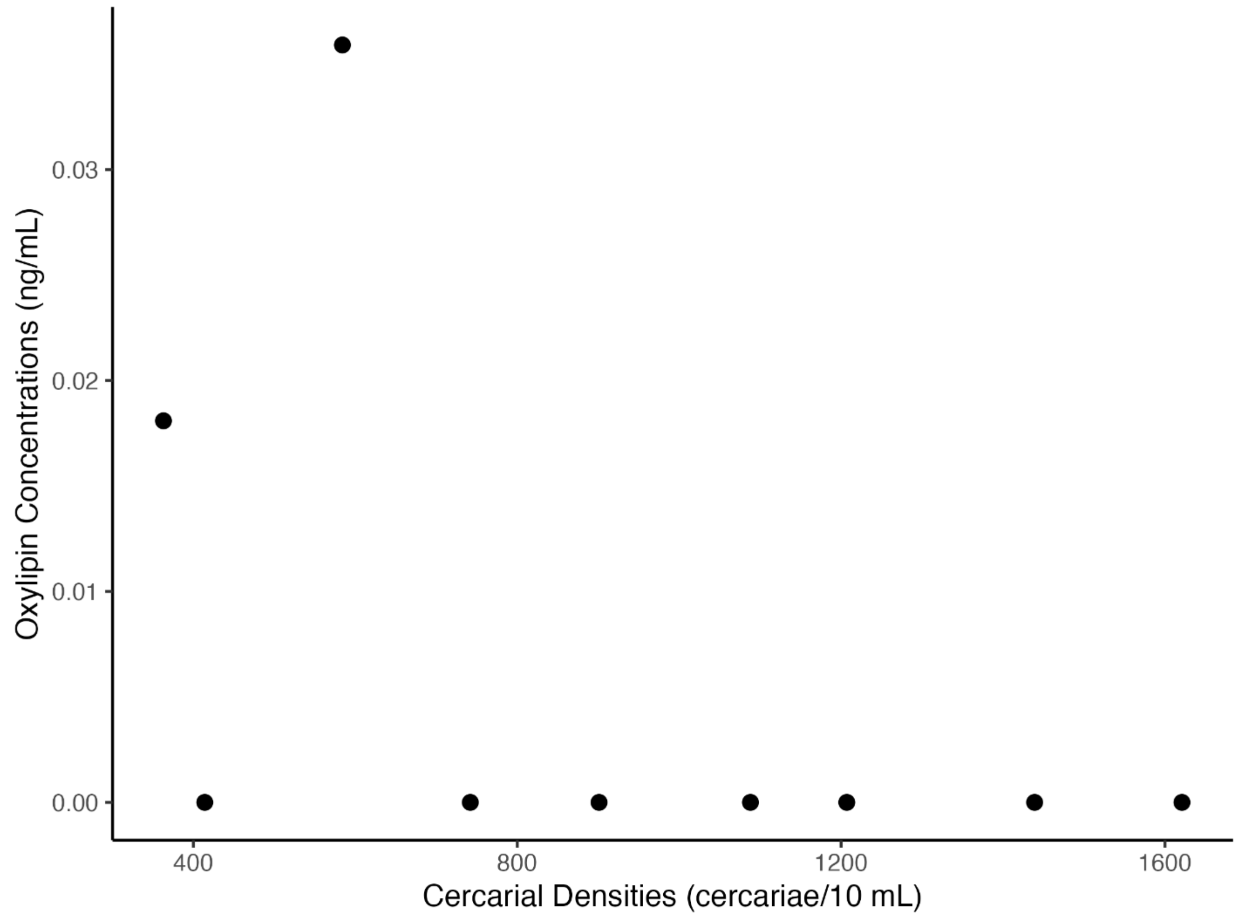

Figure 3.30. Concentration of PGD_1 (ng/mL) across cercarial densities (cercariae/10 mL).

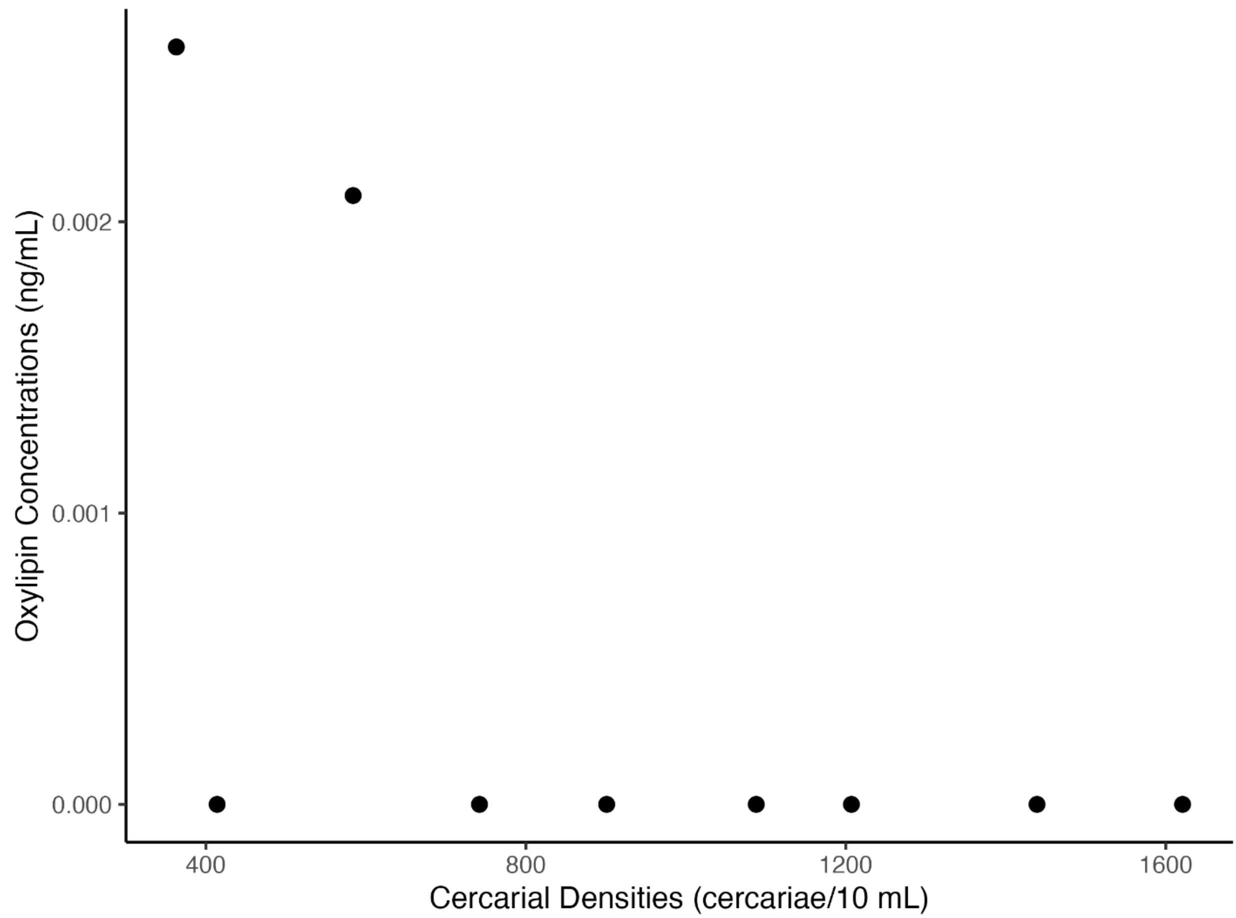

Figure 3.31. Concentration of TXB_1 (ng/mL) across cercarial densities (cercariae/10 mL).

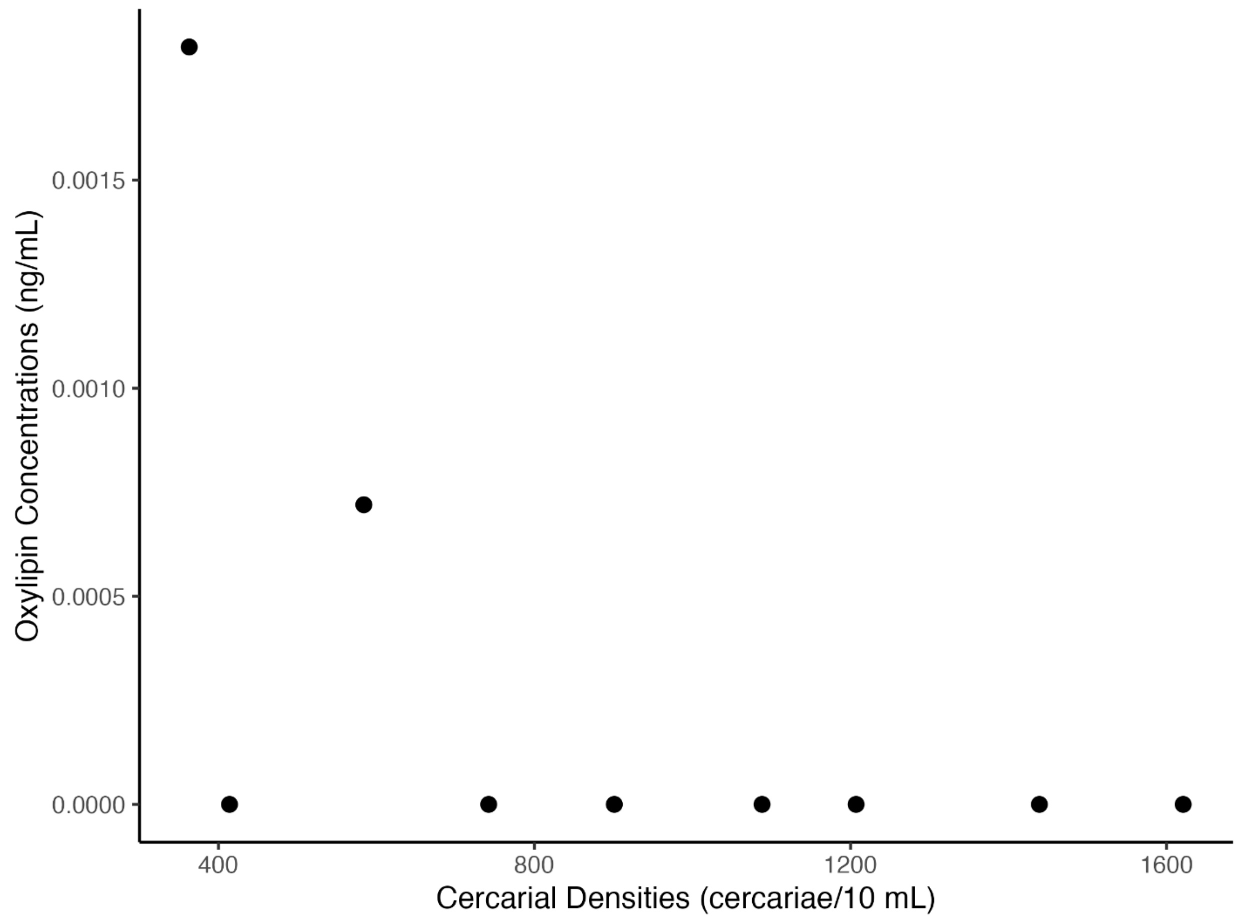

Figure 3.32. Concentration of TXB_3 (ng/mL) across cercarial densities (cercariae/10 mL).

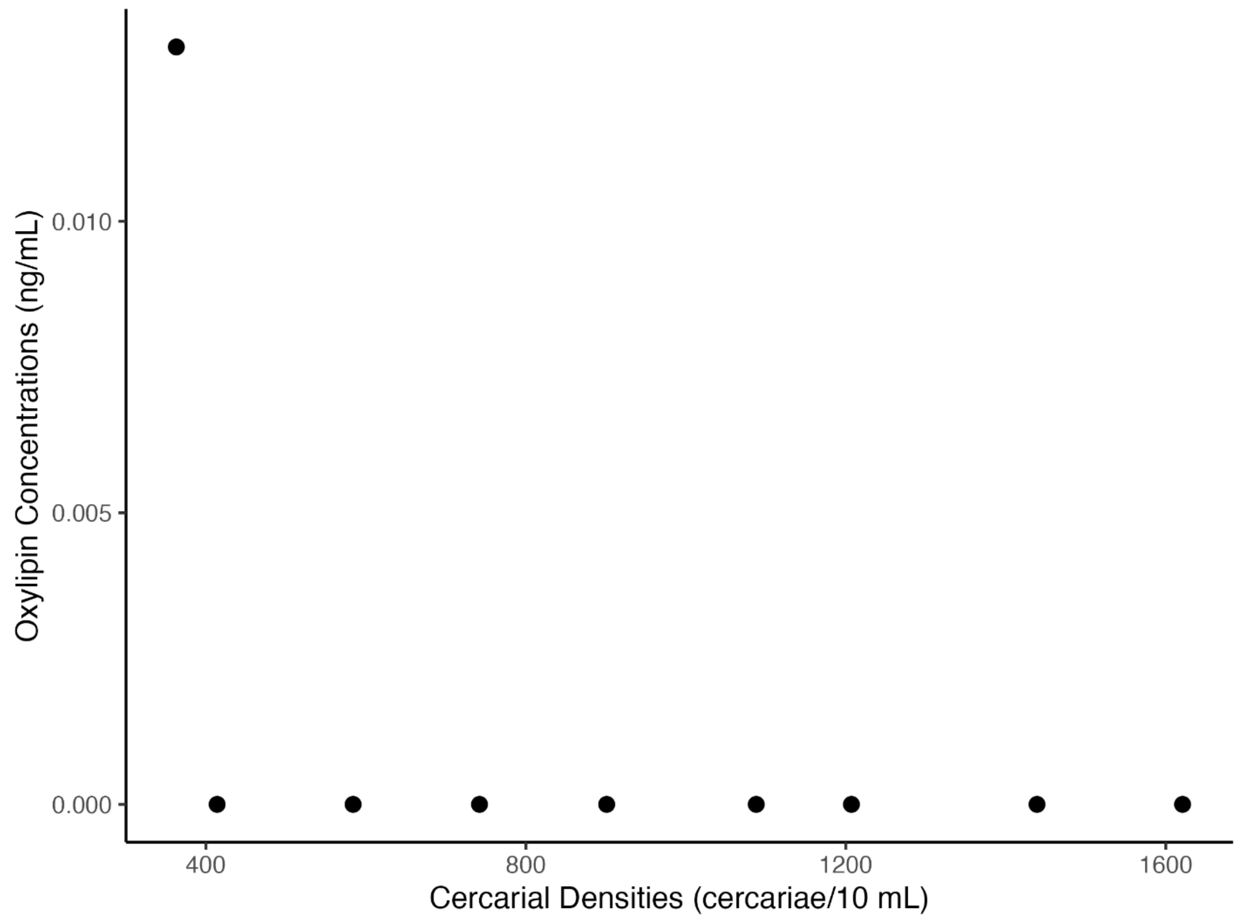

Figure 3.33. Concentration of RvD_2 (ng/mL) across cercarial densities (cercariae/10 mL).

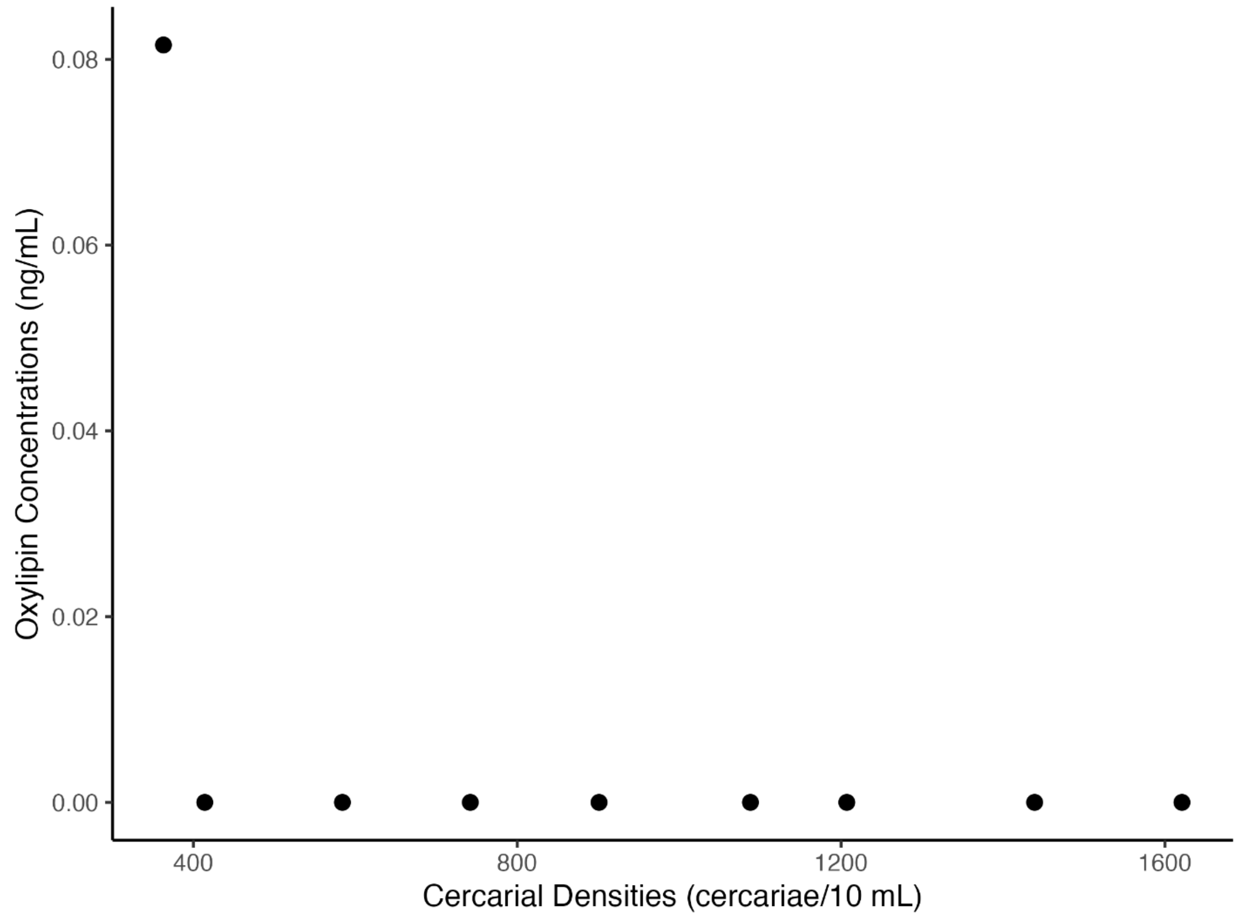

Figure 3.34. Concentration of RvD_5 (ng/mL) across cercarial densities (cercariae/10 mL).

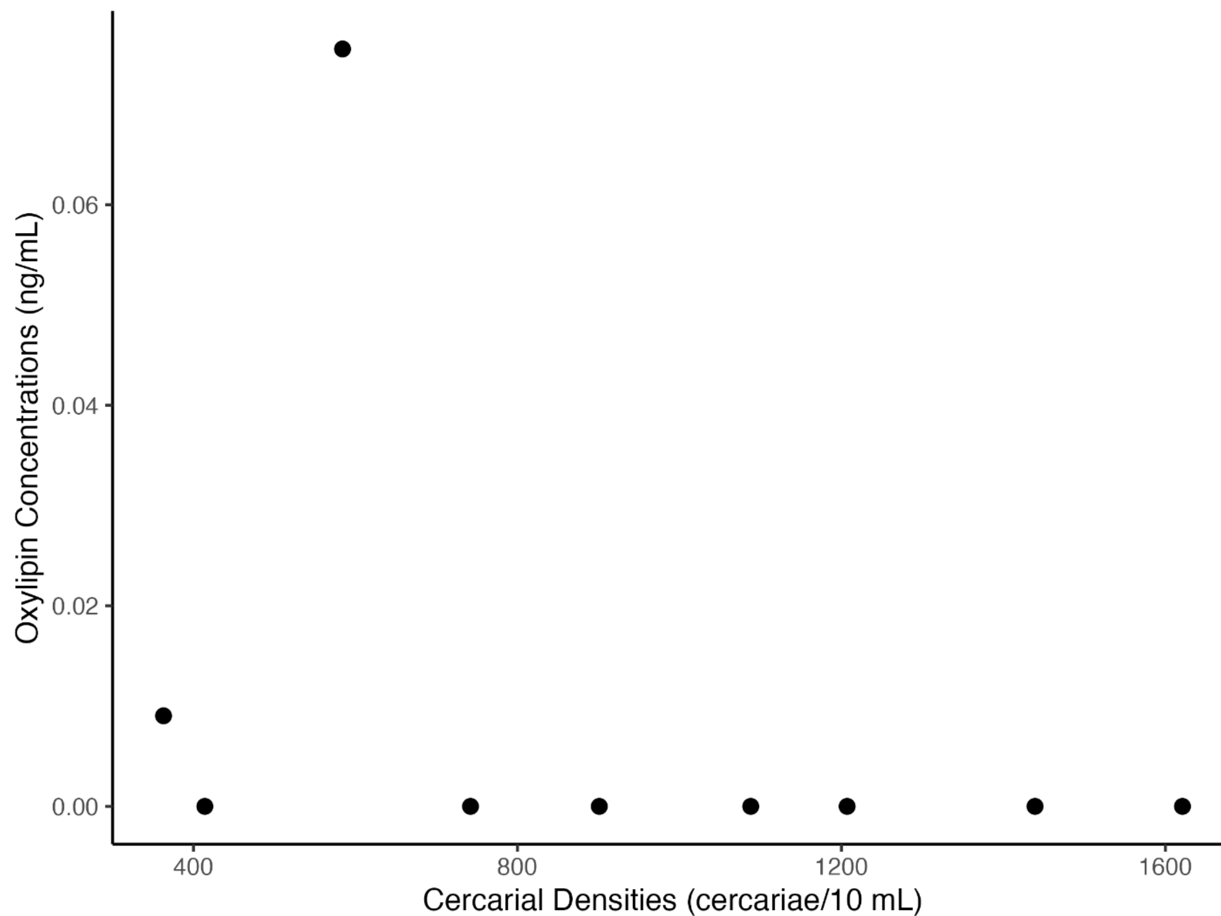

Figure 3.35. Concentration of 10S,17S-DiHDoHE (PDX) (ng/mL) across cercarial densities (cercariae/10 mL).

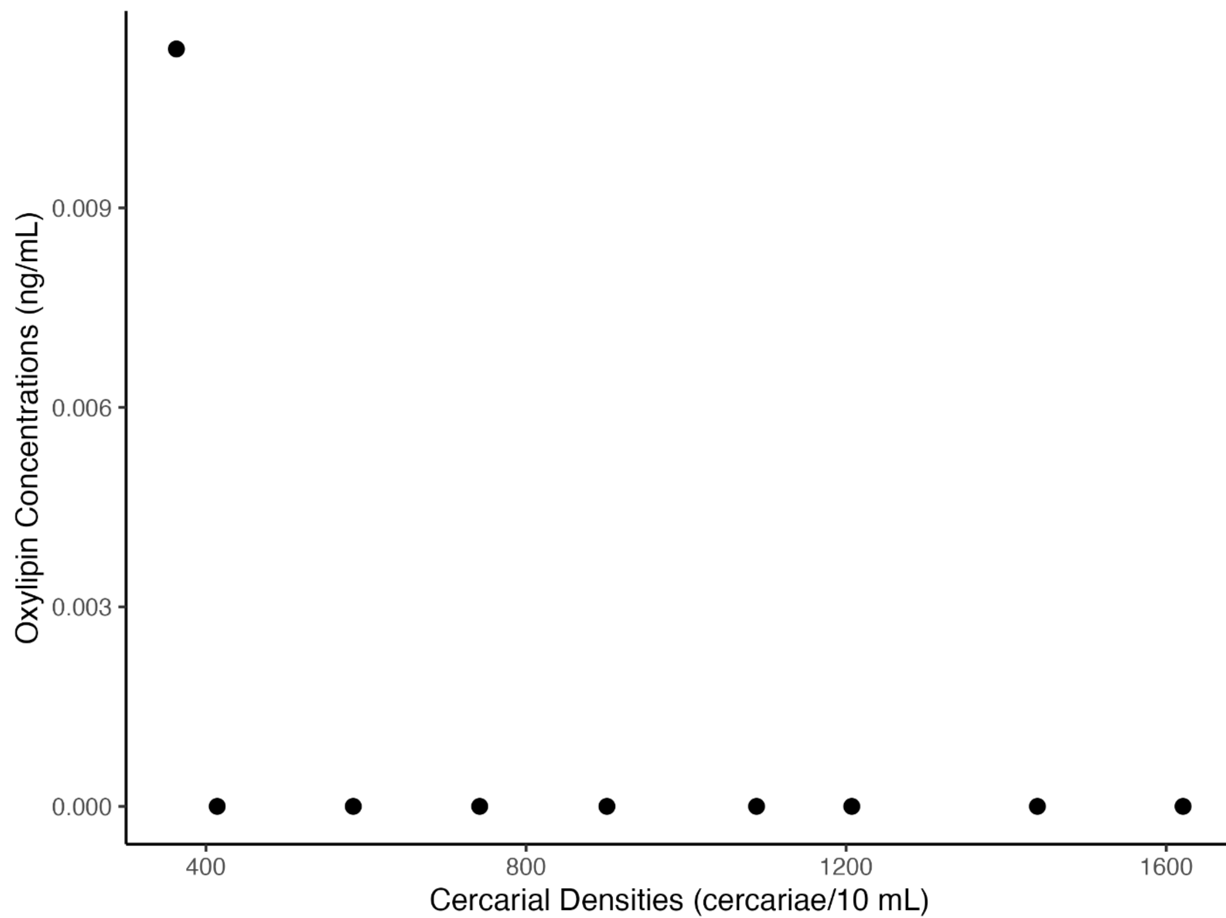

Figure 3.36. Concentration of 15,16-diHODE (ng/mL) across cercarial densities (cercariae/10 mL).

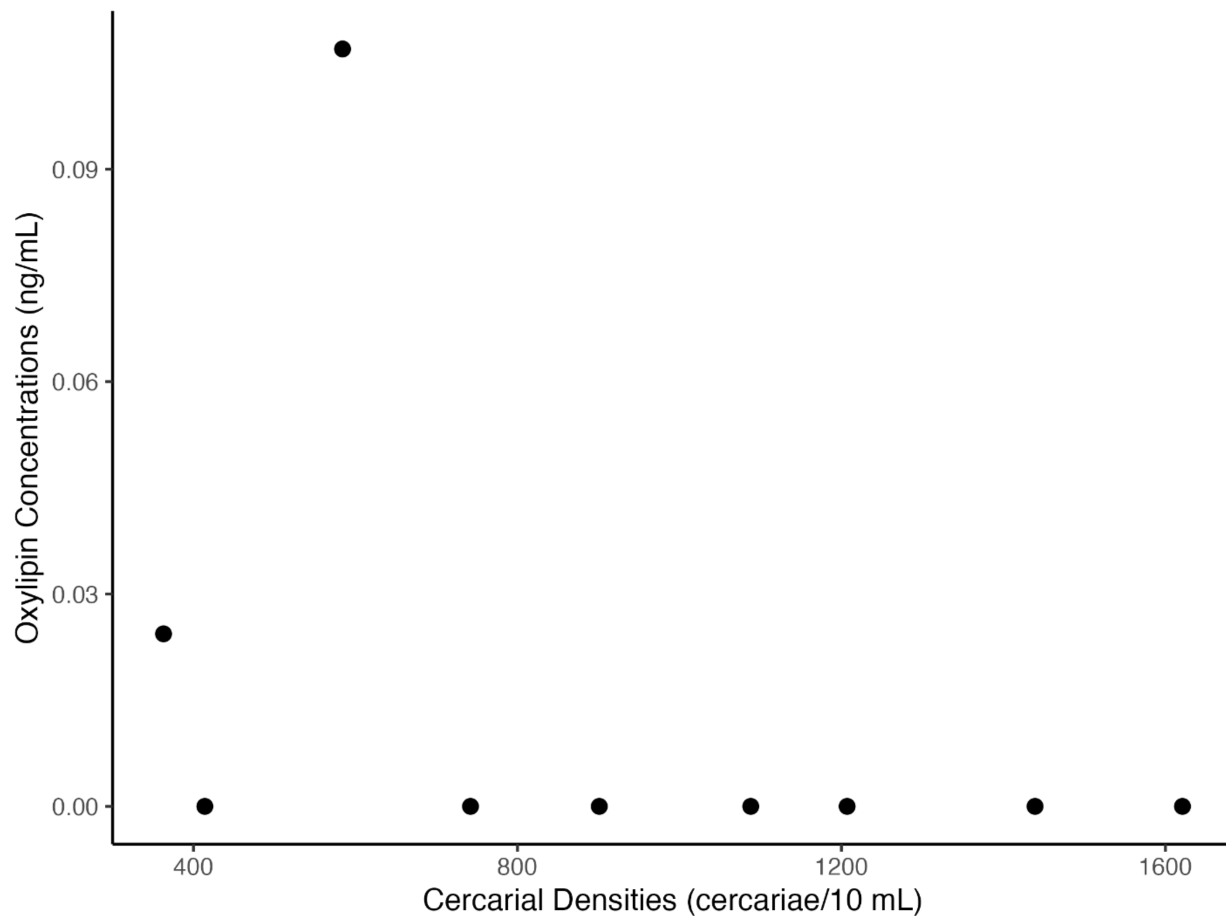

Figure 3.37. Concentration of 2,3-dinor 8-iso $PGF_{2\alpha}$ (ng/mL) across cercarial densities (cercariae/10 mL).

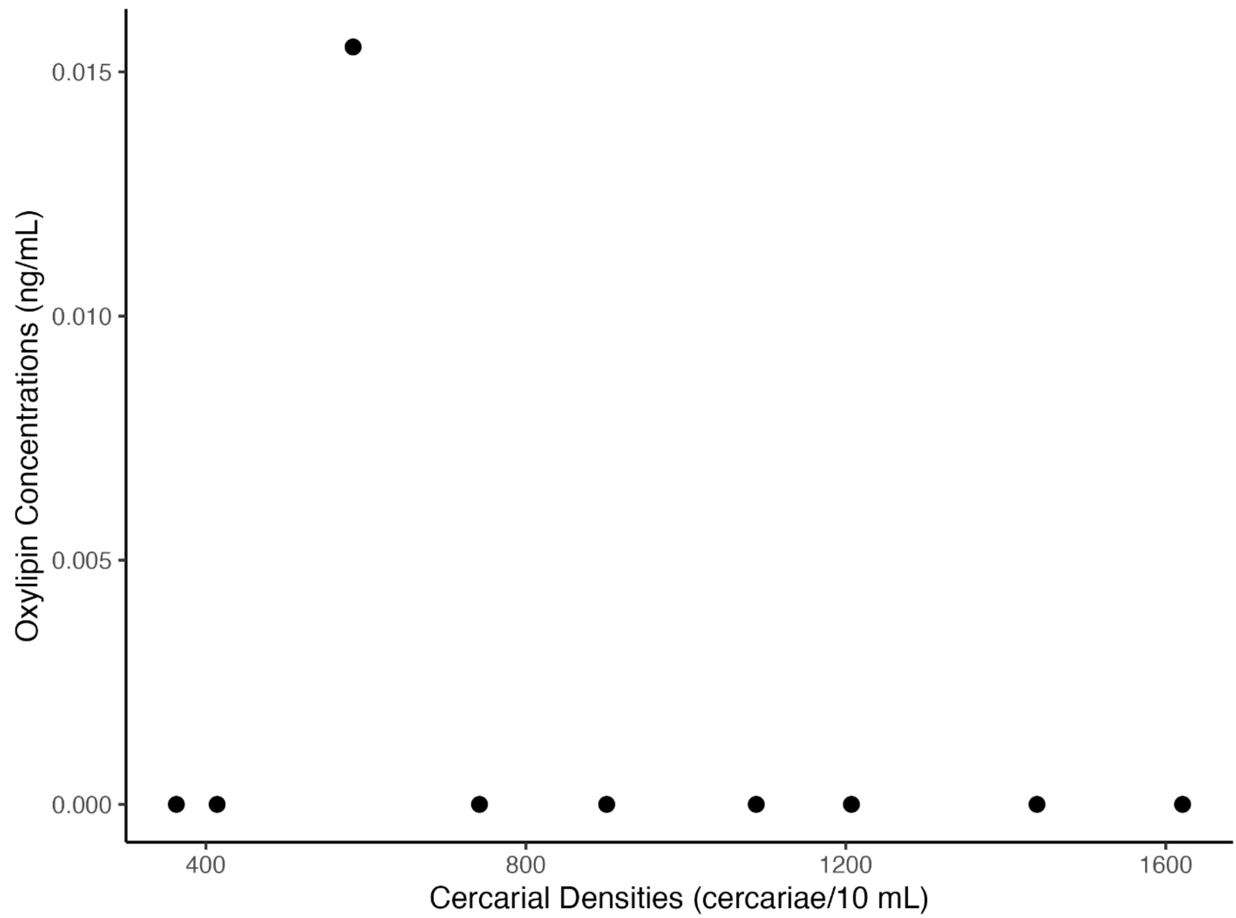

Figure 3.38. Concentration of dhk $PGF_{2\alpha}$ (ng/mL) across cercarial densities (cercariae/10 mL).

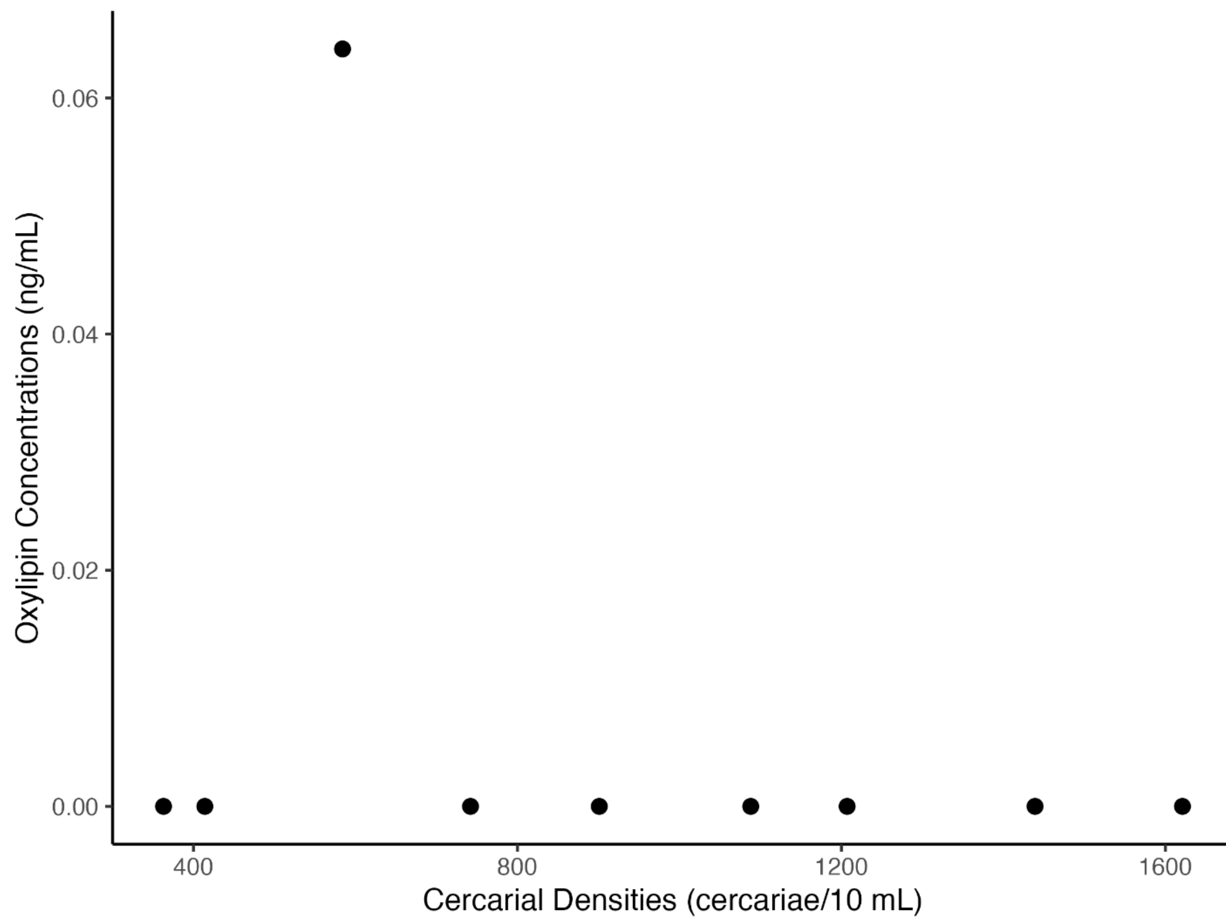

Figure 3.39. Concentration of 8-HETrE (ng/mL) across cercarial densities (cercariae/10 mL).

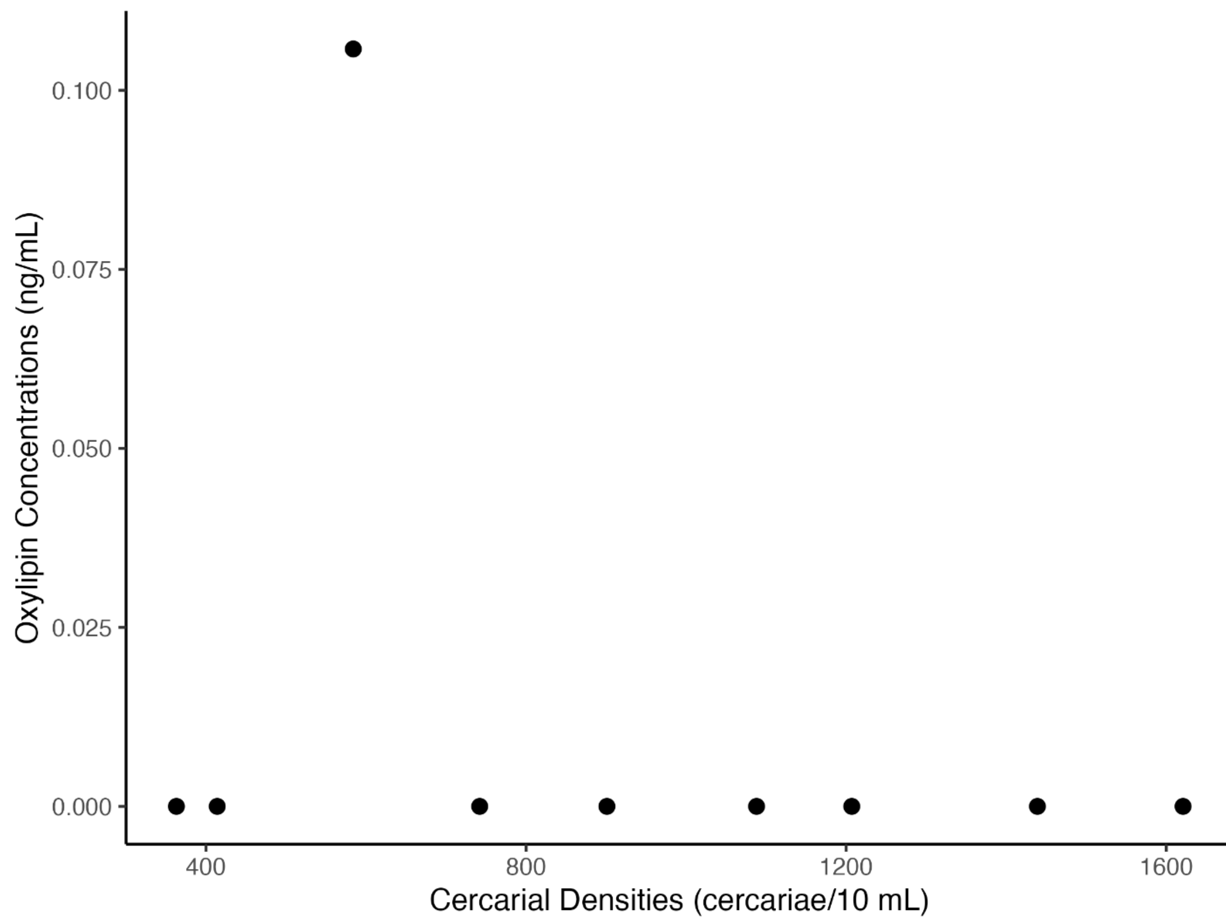

Figure 3.40. Concentration of 19,20-EpDPE (ng/mL) across cercarial densities (cercariae/10 mL).

Chapter 1 R Code

```
#Load and install libraries
library(dplyr)
library(ggplot2)
library(tidyr)
library(car)
library(broom)
library(tidyverse)
library(vegan)
library(viridis)
library(ggforce)
library(lmtest)
library(MASS)
library(patchwork)
library(tibble)

#Set working directory
setwd("/Users/joshitasehgal/Desktop")

#Set output directory
output_dir <- "/Users/joshitasehgal/Desktop/Chapter1Figures"
if (!dir.exists(output_dir)) dir.create(output_dir)

#Load data file
library(readr)
JS_Chapter1CercarialOxylipins <- read_csv("JS_Chapter1CercarialOxylipins.csv")
View(JS_Chapter1CercarialOxylipins)

#Notes on dataset
#1) Scannned total of 169 chemicals originally using HPLC with Tanja - see Joshita Results
March142025 file
#2) From 169 chemicals, omitted 7 precursors (ARA, ADA, EPA, DHA, ALA, LA, and gLA)
AND omitted 4 oxylipins (15,16 EpODE; 9,10 diHODE; 9,10 EpODE; HXB3 - these oxylipins
are ratios according to Tanja)
#3) Omit oxylipins quantified in only 1 sample (may need to omit oxylipins quantified in only 2
samples, but discuss with Jillian and Harold)
#4) Omit sample of cercarial density 544 (discussed replacing this sample and running another
sample instead of this sample as this sample detected fewer oxylipins than all other samples)
#Use Viridis for consistent colour palette (purples, blues, greens, and yellows)

#Select densities to loop through, omitting sample 544
densities <- c(901, 1088, 742, 1207, 1439, 1621, 414, 363, 584)

#List of precursors and oxylipins to omit
precursors_to_omit <- c("ARA", "ADA", "EPA", "DHA", "ALA", "LA", "gLA")
oxylipins_to_omit <- c("15,16 EpODE", "9,10 diHODE", "9,10 EpODE", "HXB3")
```

```
#Loop through each density, filter data, create plot, save plot
for (density in densities) {

#Filter data
filtered_data <- JS_Chapter1CercarialOxylipins %>%
filter(CercarialDensity == density,
`OxylipinConcentration(ng)` > 0,
!OxylipinName %in% c(precursors_to_omit, oxylipins_to_omit))

#Create plots
p <- ggplot(filtered_data, aes(x = OxylipinName, y = `OxylipinConcentration(ng)`)) +
geom_point(size = 2, color = "grey30") +
labs(x = "Oxylipins",
y = "Oxylipin Concentration (ng)") +
theme_classic() +
theme(panel.grid = element_blank(),
axis.text.x = element_text(angle = 90, hjust = 1),
plot.title = element_blank())

#Filter dataset for relevant samples, nonzero oxylipin concentrations, and omitting specified
precursors and oxylipins
filtered_data <- JS_Chapter1CercarialOxylipins %>%
filter(CercarialDensity %in% c(901, 1088, 742, 1207, 1439, 1621, 414, 363, 584) &
`OxylipinConcentration(ng)` > 0 &
!OxylipinName %in% c(precursors_to_omit, oxylipins_to_omit))

#Create plot, showing oxylipin diversity at different cercarial densities
plot1 <- ggplot(filtered_data, aes(x = OxylipinName,
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
labs(x = "Oxylipins",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
theme_minimal(base_size = 12) +
theme(axis.text.x = element_text(angle = 45, hjust = 1)) +
scale_color_viridis_d(option = "D") +
theme(plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
title = element_blank())

#Create plot, showing oxylipin diversity at different cercarial densities
plot2 <- ggplot(filtered_data, aes(x = as.factor(CercarialDensity),
y = `OxylipinConcentration(ng)`,
color = OxylipinName)) +
```

```
geom_point(size = 3) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)",
color = "Oxylipins") +
theme_minimal(base_size = 12) +
theme(axis.text.x = element_text(angle = 45, hjust = 1)) +
theme(plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
title = element_blank())

#Create plot, showing oxylipin diversity at different cercarial densities
plot3 <- ggplot(filtered_data, aes(x = as.factor(CercarialDensity),
y = OxylipinName,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
scale_color_viridis_d(option = "D") +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins",
color = "Cercarial Density") +
theme_minimal(base_size = 12) +
theme(axis.text.x = element_text(angle = 45, hjust = 1)) +
theme(plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
title = element_blank())

#Calculate tallies
oxylipin_levels <- levels(as.factor(filtered_data$OxylipinName))
density_levels <- levels(as.factor(filtered_data$CercarialDensity))

#Tally of oxylipins per cercarial density
tally_oxylipin_per_cercdensitysample <- filtered_data %>%
group_by(CercarialDensity) %>%
summarise(tally_oxylipin_per_cercdensitysample = n_distinct(OxylipinName)) %>%
ungroup()

#Tally of detections per oxylipin
tally_oxylipin_detected <- filtered_data %>%
group_by(OxylipinName) %>%
summarise(tally_oxylipin_detected = n_distinct(CercarialDensity)) %>%
ungroup()

tally_oxylipin_per_cercdensitysample <- tally_oxylipin_per_cercdensitysample %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = density_levels),
CercarialDensity_numeric = as.numeric(CercarialDensity_factor))

tally_oxylipin_detected <- tally_oxylipin_detected %>%
```

```
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels),
OxylipinName_numeric = as.numeric(OxylipinName_factor))

#View tallies (view all rows for tibble)
print(tally_oxylipin_per_cercdensitysample, n = Inf)
print(tally_oxylipin_detected, n = Inf)

#Create plot, showing oxylipin diversity at different cercarial densities
plot4 <- ggplot(filtered_data, aes(x = factor(CercarialDensity, levels = density_levels),
y = factor(OxylipinName, levels = oxylipin_levels),
fill = factor(CercarialDensity))) +
geom_tile(color = "grey", width = 0.9, height = 0.9) +
scale_fill_viridis_d(option = "D") +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
legend.position = "none",
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +

#Add tallies at top showing tally of oxylipins per density
geom_text(data = tally_oxylipin_per_cercdensitysample,
aes(x = CercarialDensity_factor,
y = length(oxylipin_levels) + 0.5,
label = tally_oxylipin_per_cercdensitysample),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +

#Add tallies at right showing tally of detections per oxylipin
geom_text(data = tally_oxylipin_detected,
aes(x = length(density_levels) + 0.5,
y = OxylipinName_factor,
label = tally_oxylipin_detected),
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
expand_limits(y = length(oxylipin_levels) + 1,
x = length(density_levels) + 1) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins", fill = "Cercarial Density")

#Filter oxylipins quantified at least 7 times across samples
oxylipins_to_keep <- tally_oxylipin_detected %>%
filter(tally_oxylipin_detected >= 7) %>%
```

```
pull(OxylipinName)

#Filter original data for specific oxylipins quantified at least 7 times across samples
filtered_data_plot5 <- filtered_data %>%
filter(OxylipinName %in% oxylipins_to_keep)

#Update oxylipin and density levels
oxylipin_levels_7 <- levels(as.factor(filtered_data$OxylipinName))
density_levels_7 <- levels(as.factor(filtered_data$CercarialDensity))

#Calculate tallies based on filtered data
tally_oxylipin_per_cercdensitysample_7 <- filtered_data_plot5 %>%
group_by(CercarialDensity) %>%
summarise(tally_oxylipin_per_cercdensitysample = n_distinct(OxylipinName)) %>%
ungroup() %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = density_levels_7))

tally_oxylipin_detected_7 <- filtered_data_plot5 %>%
group_by(OxylipinName) %>%
summarise(tally_oxylipin_detected = n_distinct(CercarialDensity)) %>%
ungroup() %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels_7))

#Create plot, showing oxylipin diversity at different cercarial densities
plot5 <- ggplot(filtered_data_plot5, aes(x = factor(CercarialDensity, levels = density_levels_7),
y = factor(OxylipinName, levels = oxylipin_levels_7),
fill = factor(CercarialDensity))) +
geom_tile(color = "grey", width = 0.9, height = 0.9) +
scale_fill_viridis_d(option = "D") +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
legend.position = "none",
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +
geom_text(data = tally_oxylipin_per_cercdensitysample_7,
aes(x = CercarialDensity_factor,
y = length(oxylipins_to_keep) + 0.5,
label = tally_oxylipin_per_cercdensitysample),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
geom_text(data = tally_oxylipin_detected_7,
```

```
aes(x = length(density_levels_7) + 0.5,
y = OxylipinName_factor,
label = tally_oxylipin_detected),
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
expand_limits(y = length(oxylipins_to_keep) + 1, x = length(density_levels_7) + 1) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins",
fill = "Cercarial Density")

#Update factor levels for densities
filtered_data_plot4 <- filtered_data_plot4 %>%
mutate(CercarialDensity = factor(CercarialDensity, levels = density_levels))

#Create combined scatter and line plot
plot6 <- ggplot(filtered_data_plot4,
aes(x = CercarialDensity,
y = `OxylipinConcentration(ng)`,
color = OxylipinName, group = OxylipinName)) +
geom_point(size = 2, alpha = 0.7) +
geom_line(size = 0.8, alpha = 0.7) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)",
color = "Oxylipins") +
theme_minimal(base_size = 14) +
theme(panel.grid = element_blank(),
panel.background = element_rect(fill = "white"),
plot.background = element_rect(fill = "white"),
panel.border = element_rect(fill = NA, color = "white"),
axis.text.x = element_text(angle = 45, hjust = 1),
legend.position = "right") +
scale_color_viridis_d(option = "D")

#Loop through oxylipins
for (ox_name in oxylipins_plot4){

#Filter data for each oxylipin
oxylipin_data <- filtered_data_plot4 %>%
filter(OxylipinName == ox_name)

#Create plot
p <- ggplot(oxylipin_data,
aes(x = factor(CercarialDensity, levels = density_levels),
y = `OxylipinConcentration(ng)`)) +
geom_point(size = 2, alpha = 0.7, color = "black") +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)",
```

```
title = NULL) +
theme_minimal(base_size = 14) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
panel.grid = element_blank(),
panel.background = element_rect(fill = "white"),
plot.background = element_rect(fill = "white"),
panel.border = element_rect(fill = NA, color = "white"))

#Clean filename
file_name <- paste0("Oxylipin_", gsub("[^[:alnum:]_]", "", ox_name), ".png")

#Create data frame
regression_data <- data.frame(CercarialDensity = c(363, 414, 584, 742, 901, 1088, 1207, 1439,
1621),
OxylipinsDetected = c(26, 15, 20, 13, 13, 11, 14, 16, 19))

#View data
print(regression_data)

#Run linear regression
model <- lm(OxylipinsDetected ~ CercarialDensity, data = regression_data)

#View model summary
summary(model)

#Create plot
library(ggplot2)
ggplot(regression_data, aes(x = CercarialDensity, y = OxylipinsDetected)) +
geom_point(size = 3) +
geom_smooth(method = "lm", se = TRUE, color = "black") +
theme_minimal(base_size = 14) +
theme(panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
panel.background = element_rect(fill = "white", color = "white")) +
labs(x = "Cercarial Density (cercariae/10 mL)",
y = "Number of Oxylipins quantified")

#Create data frame
regression_data <- data.frame(CercarialDensity = c(363, 414, 584, 742, 901, 1088, 1207, 1439,
1621),
OxylipinsDetected = c(10, 12, 10, 8, 10, 8, 14, 13, 12))

#View data
print(regression_data)

#Run linear regression
```

```
model <- lm(OxylipinsDetected ~ CercarialDensity, data = regression_data)

#View model summary
summary(model)

#Create plot
library(ggplot2)
ggplot(regression_data, aes(x = CercarialDensity, y = OxylipinsDetected)) +
geom_point(size = 3) +
geom_smooth(method = "lm", se = TRUE, color = "black") +
theme_minimal(base_size = 14) +
theme(panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
panel.background = element_rect(fill = "white", color = "white")) +
labs(x = "Cercarial Density (cercariae/10 mL)",
y = "Number of Oxylipins quantified")

#Clean column names
colnames(JS_Chapter1CercarialOxylipins) <-
make.names(colnames(JS_Chapter1CercarialOxylipins))

#List of precursors and oxylipins to omit and filter them out
JS_Chapter1CercarialOxylipins <- JS_Chapter1CercarialOxylipins %>%
filter(!(OxylipinName %in% c(precursors_to_omit, oxylipins_to_omit)))

#Filter oxylipins with at least 3 detections
oxylipin_counts <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinConcentration.ng. > 0, CercarialDensity != 544) %>%
group_by(OxylipinName) %>%
summarise(DetectionCount = n()) %>%
filter(DetectionCount >= 7)
oxylipins_for_regression <- oxylipin_counts$OxylipinName

#Initialize empty result list
results_list <- list()

#Loop through each oxylipin
for (ox in oxylipins_for_regression) {
subset_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName == ox,
OxylipinConcentration.ng. > 0,
CercarialDensity != 544)

#Skip if not enough data or no variation in predictor
if (nrow(subset_data) < 3 || length(unique(subset_data$CercarialDensity)) < 2) {
results_list[[ox]] <- data.frame(Oxylipin = ox,
```

```
Shapiro_p = NA,
Breusch_Pagan_p = NA,
Regression_p = NA,
R_squared = NA,
Assumptions_Met = FALSE)
next}

#Fit model
model <- lm(OxylipinConcentration.ng. ~ CercarialDensity, data = subset_data)
residuals_model <- residuals(model)

#Run Shapiro-Wilk test (normality)
if (length(residuals_model) >= 3 && length(unique(residuals_model)) > 1) {
shapiro_result <- shapiro.test(residuals_model)
shapiro_p <- shapiro_result$p.value
} else {
shapiro_p <- NA}

#Run Breusch-Pagan test (homoscedasticity)
bp_result <- tryCatch({
bptest(model)
}, error = function(e) NULL)
bp_p <- if (!is.null(bp_result)) bp_result$p.value else NA

#Run regression only if assumptions are met
if (!is.na(shapiro_p) && !is.na(bp_p) && shapiro_p > 0.05 && bp_p > 0.05) {
reg_summary <- summary(model)
reg_p <- coef(reg_summary)[2, 4]
r2 <- reg_summary$r.squared
assumptions_met <- TRUE
} else {
reg_p <- NA
r2 <- NA
assumptions_met <- FALSE}

#Store results with oxylipin names
results_list[[ox]] <- data.frame(
Oxylipin = gsub("[^[:alnum:][:space:]]", "", ox),
Shapiro_p = shapiro_p,
Breusch_Pagan_p = bp_p,
Regression_p = reg_p,
R_squared = r2,
Assumptions_Met = assumptions_met)}

#Combine into a single dataframe
results_df <- bind_rows(results_list)
```

```
#View/export data
print(results_df)

#Loop only through oxylipins where assumptions were met and create plots for those oxylipins
for (i in seq_along(results_list)) {
res <- results_list[[i]]
if (!is.null(res) && res$Assumptions_Met) {
ox_name <- res$Oxylipin

#Subset original data
subset_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName == names(results_list)[i],
OxylipinConcentration.ng. > 0,
CercarialDensity != 544)

#Fit model
model <- lm(OxylipinConcentration.ng. ~ CercarialDensity, data = subset_data)

#Clean filename
file_name <- paste0("Regression_Plots/", gsub("[^[:alnum:]_]", "_", ox_name), ".png")

#Create plots
png(filename = file_name, width = 800, height = 600)
plot(subset_data$CercarialDensity,
subset_data$OxylipinConcentration.ng.,
main = paste("Regression for", ox_name),
xlab = "Cercarial Densities (cercariae/10 mL)",
ylab = "Oxylipin Concentration (ng)",
pch = 19,
col = "black")
abline(model, col = "red", lwd = 2)

#Add r-squared and p-values to plots
legend("topright",
legend = c(paste0("R² = ", round(res$R_squared, 3)),
paste0("p = ", signif(res$Regression_p, 3))),
bty = "n")
dev.off()}}

#List of oxylipins to plot with similar trend
oxylipins_to_plot <- c("12,13 diHOME", "9-HODE", "9-oxoODE", "13-HODE", "9,10
EpOME")

#Filter data for specific oxylipins
filtered_data <- JS_Chapter1CercarialOxylipins %>%
```

```
filter(OxylipinName %in% oxylipins_to_plot, OxylipinConcentration.ng. > 0, CercarialDensity
!= 544)

#Create plot
ggplot(filtered_data, aes(x = CercarialDensity, y = OxylipinConcentration.ng., color =
OxylipinName)) +
geom_point(size = 3, alpha = 0.6) +
geom_smooth(method = "lm", se = FALSE, aes(group = OxylipinName), linetype = "solid") +
labs(x = "Cercarial Density (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)",
color = "Oxylipins") +
theme_minimal() +
theme(legend.position = "right",
panel.grid = element_blank())

#List of oxylipins to plot with similar trend
oxylipins_to_plot <- c("12,13 diHOME", "9-HODE", "9-oxoODE", "13-HODE", "9,10
EpOME")

#Filter data for specific oxylipins
filtered_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName %in% oxylipins_to_plot, OxylipinConcentration.ng. > 0, CercarialDensity
!= 544)

#Create plot
ggplot(filtered_data, aes(x = CercarialDensity, y = OxylipinConcentration.ng., color =
OxylipinName)) +
geom_point(size = 3, alpha = 0.6) +
geom_smooth(method = "loess", se = FALSE, aes(group = OxylipinName), linetype = "solid") +
labs(x = "Cercarial Density (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)",
color = "Oxylipins") +
theme_minimal() +
theme(legend.position = "right",
panel.grid = element_blank())

#List of oxylipins to analyze
oxylipins_to_analyze <- c("12,13 diHOME", "9-HODE", "9-oxoODE", "13-HODE", "9,10
EpOME")

#Create function to run GLM for each oxylipin
run_glm_gamma <- function(oxylipin_name) {
# Filter data for the specific oxylipin
filtered_oxylipin_data <- filtered_data %>%
filter(OxylipinName == oxylipin_name)
```

```
#Run GLM with gamma distribution
glm_model <- glm(OxylipinConcentration.ng. ~ CercarialDensity,
family = Gamma(link = "log"),
data = filtered_oxylipin_data)

#Return summary of model
return(summary(glm_model))}

#Run GLM for each oxylipin
glm_results <- lapply(oxylipins_to_analyze, run_glm_gamma)

#Print results
names(glm_results) <- oxylipins_to_analyze
glm_results

#Determine unique oxylipin names that were detected (i.e. with nonzero concentrations)
oxylipins_detected <- JS_Chapter1CercarialOxylipins %>%
filter(`OxylipinConcentration(ng)` > 0 &
CercarialDensity != 544 &
!OxylipinName %in% c("ARA", "EPA", "ALA", "DHA")) %>%
distinct(OxylipinName) %>%
pull(OxylipinName)

#Print list of detected oxylipins
print(oxylipins_detected)

#Count number of unique oxylipins quantified at each cercarial density level
oxylipin_counts <- JS_Chapter1CercarialOxylipins %>%
filter(`OxylipinConcentration(ng)` > 0 &
CercarialDensity != 544 &
!OxylipinName %in% c("ARA", "EPA", "ALA", "DHA")) %>%
group_by(CercarialDensity) %>%
summarize(Num_Oxylipins = n_distinct(OxylipinName))

#Create bar plot
ggplot(oxylipin_counts, aes(x = factor(CercarialDensity), y = Num_Oxylipins, fill =
factor(CercarialDensity))) +
geom_bar(stat = "identity", width = 0.6, alpha = 0.8) +
geom_text(aes(label = Num_Oxylipins), vjust = -0.5, size = 4) +
scale_fill_viridis_d(guide = "none") +
theme_minimal() +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Number of Oxylipins quantified") +
ylim(0, 25) +
theme(axis.title = element_text(size = 12),
axis.text = element_text(size = 10),
```

```
axis.text.x = element_text(angle = 45, hjust = 1),
legend.position = "none",
panel.grid.major = element_blank(),
panel.grid.minor = element_blank())

#Filter data: Keep nonzero oxylipins, omit certain oxylipins, remove cercarial density 544
filtered_data <- JS_Chapter1CercarialOxylipins %>%
filter(`OxylipinConcentration(ng)` > 0 &
CercarialDensity != 544 &
!OxylipinName %in% c("ARA", "EPA", "ALA", "DHA"))

#Count number of detections per oxylipin at each cercarial density
oxylipin_breakdown <- filtered_data %>%
group_by(CercarialDensity, OxylipinName) %>%
summarise(DetectionCount = n(), .groups = "drop")

#Summarize total detections per cercarial density
total_counts <- oxylipin_breakdown %>%
group_by(CercarialDensity) %>%
summarise(TotalDetectionCount = sum(DetectionCount), .groups = "drop")

#Create stacked bar plot
ggplot(oxylipin_breakdown, aes(x = factor(CercarialDensity), y = DetectionCount, fill =
OxylipinName)) +
geom_bar(stat = "identity", width = 0.6, alpha = 0.9) +
geom_text(data = total_counts, aes(x = factor(CercarialDensity), y = TotalDetectionCount,
label = TotalDetectionCount), vjust = -0.5, size = 4, inherit.aes = FALSE) +
scale_fill_viridis_d(name = "Oxylipins", option = "D") +
theme_minimal() +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipin Detection Count") +
theme(axis.title = element_text(size = 12),
axis.text = element_text(size = 10),
axis.text.x = element_text(angle = 45, hjust = 1),
legend.position = "right",
legend.title = element_text(size = 12),
legend.text = element_text(size = 10),
panel.grid.major = element_blank(),
panel.grid.minor = element_blank())

#Define list of oxylipins to check
oxylipins_list <- c("PGE2", "9-HODE", "9-oxoODE", "13-HODE", "13-oxoODE", "9,10,13
triHOME",
"9,12,13 triHOME", "13-HOTrE-y", "13-HOTrE", "15-HEPE", "9,10 diHOME",
"12,13 diHOME", "14,15 DiHETrE", "9,10 diHODE", "8-HETE", "9-HOTrE",
"9 oxoOTrE", "9,10 EpOME", "16-HETE", "5-HETE", "12-oxoETE", "15-HETE",
```

```
"15-oxoETE", "12-HEPE", "8,9 DiHETrE", "HXB3", "9,10 EpODE", "20-HETE",
"15k PGE2", "11-HETE", "12,13 EpODE", "15,16 EpODE")

#Rename column to remove special characters
colnames(JS_Chapter1CercarialOxylipins) <-
make.names(colnames(JS_Chapter1CercarialOxylipins))

#Check new column names
print(colnames(JS_Chapter1CercarialOxylipins))

#Filter dataset for cercarial densities
filtered_data <- JS_Chapter1CercarialOxylipins %>%
filter(CercarialDensity %in% c(901, 1088, 742, 1207, 1439, 1621, 414))

#Create empty data frame to store counts
oxylipin_counts <- data.frame(Oxylipin = oxylipins_list, DetectionCount =
integer(length(oxylipins_list)))

#Loop over each oxylipin and count number of times each oxylipin was quantified (where
concentration > 0)
for (i in 1:length(oxylipins_list)) {
oxylipin <- oxylipins_list[i]

#Subset data for current oxylipin and filter for detections (concentration > 0)
oxylipin_data <- filtered_data %>%
filter(OxylipinName == oxylipin) %>%
filter(OxylipinConcentration.ng. > 0) %>%
pull(OxylipinConcentration.ng.)

#Count number of non-NA and non-zero detections
oxylipin_counts$DetectionCount[i] <- length(oxylipin_data)}

#Print counts
print(oxylipin_counts)

#Create bar plot for oxylipin detection counts with oxylipin names on x-axis and detection count
on y-axis
ggplot(oxylipin_counts, aes(x = Oxylipin, y = DetectionCount)) +
geom_bar(stat = "identity", fill = "gray") +
geom_text(aes(label = DetectionCount), vjust = -0.3, color = "black", size = 3.5) +
labs(x = "Oxylipins",
y = "Oxylipin Detection Count") +
theme_minimal() +
theme(axis.text.x = element_text(angle = 90, hjust = 1),
axis.title = element_text(size = 12),
plot.title = element_blank(),
```

```
panel.grid = element_blank())

#Clean column names
colnames(JS_Chapter1CercarialOxylipins) <-
make.names(colnames(JS_Chapter1CercarialOxylipins))

#Filter for non-zero detections, remove unwanted oxylipins and cercarial density 544
detected_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinConcentration.ng. > 0,
!OxylipinName %in% c("ARA", "EPA", "ALA", "DHA"),
CercarialDensity != 544)

#Count detections by oxylipin and cercarial density
detection_breakdown <- detected_data %>%
group_by(OxylipinName, CercarialDensity) %>%
summarise(DetectionCount = n(), .groups = "drop")

#Calculate total detection count per oxylipin
total_counts <- detection_breakdown %>%
group_by(OxylipinName) %>%
summarise(TotalCount = sum(DetectionCount), .groups = "drop")

#Create stacked bar chart
ggplot(detection_breakdown, aes(x = OxylipinName, y = DetectionCount, fill =
as.factor(CercarialDensity))) +
geom_bar(stat = "identity") +
geom_text(data = total_counts,
aes(x = OxylipinName, y = TotalCount, label = TotalCount),
inherit.aes = FALSE,
vjust = -0.5, size = 3.5, color = "black") +
scale_fill_viridis_d(option = "D") +
labs(x = "Oxylipins",
y = "Oxylipin Detection Count",
fill = "Cercarial Density") +
theme_minimal() +
theme(axis.text.x = element_text(angle = 90, hjust = 1),
panel.grid = element_blank())

#Loop through each oxylipin
for (oxylipin in setdiff(unique(filtered_data$OxylipinName), c("ARA", "EPA", "ALA",
"DHA"))) {

#Filter data for specific oxylipin and omit 544
oxylipin_data <- filtered_data %>%
filter(OxylipinName == oxylipin & CercarialDensity != 544)
```

```
#Create plot
p <- ggplot(oxylipin_data, aes(x = as.factor(CercarialDensity),
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
labs(title = paste("Oxylipin:", oxylipin),
x = "Cercarial Density",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
theme_classic() +
theme(axis.text.x = element_text(angle = 45, hjust = 1))

#Loop through each oxylipin
for (oxylipin in setdiff(unique(filtered_data$OxylipinName), c("ARA", "EPA", "ALA",
"DHA"))) {

#Filter data for specific oxylipin and omit 544
oxylipin_data <- filtered_data %>%
filter(OxylipinName == oxylipin & CercarialDensity != 544)

#Create plot
p <- ggplot(oxylipin_data, aes(x = as.factor(CercarialDensity),
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
geom_line(aes(group = 1), color = "grey", alpha = 0.5) +
labs(title = paste("Oxylipin:", oxylipin),
x = "Cercarial Density",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
theme_classic() +
theme(axis.text.x = element_text(angle = 45, hjust = 1))

#Load data file
library(readr)
JS_Chapter1CercarialOxylipinsNMDS <-
read_csv("JS_Chapter1CercarialOxylipinsNMDS.csv")
View(JS_Chapter1CercarialOxylipinsNMDS)

#Read raw data with no headers (first column is variable names)
raw <- read_csv("JS_Chapter1CercarialOxylipinsNMDS.csv", col_names = FALSE)

#Transpose data
transposed <- as_tibble(t(raw))

#Set column names using first row of transposed data
```

```
colnames(transposed) <- transposed[1, ]

#Remove first row (now used as column names)
transposed <- transposed[-1, ]

#View column names
colnames(transposed)

#Clean numeric values
transposed_clean <- transposed %>%
mutate(`OxylipinConcentration(ng)` = as.numeric(`OxylipinConcentration(ng)`),
CercarialDensity = as.numeric(CercarialDensity))

#View column names
colnames(transposed_clean)

#Run NDMS
ndms_result <- metaMDS(oxylipin_matrix, distance = "bray", k = 2, trymax = 100)
nmds_scores <- as.data.frame(scores(ndms_result, display = "sites"))
nmds_scores$CercarialDensity <- filtered_data$CercarialDensity
centroids <- nmds_scores %>%
group_by(CercarialDensity) %>%
summarize(NMDS1 = mean(NMDS1, na.rm = TRUE), NMDS2 = mean(NMDS2, na.rm =
TRUE))

#Create NDMS plot according to cercarial density
ggplot(centroids, aes(x = NMDS1, y = NMDS2, label = CercarialDensity)) +
geom_point(size = 5, color = "grey") +
geom_text(vjust = -0.5, size = 4) +
theme_minimal() +
labs(title = "NMDS According to Cercarial Density",
x = "NMDS1",
y = "NMDS2")

#Create NDMS plot showing oxylipin concentrations according to cercarial density
hull_data <- nmds_scores %>%
group_by(CercarialDensity) %>%
slice(chull(NMDS1, NMDS2))
ggplot(nmds_scores, aes(x = NMDS1, y = NMDS2, color = CercarialDensity)) +
geom_polygon(data = hull_data, aes(fill = CercarialDensity, group = CercarialDensity),
alpha = 0.2, color = NA) +
geom_point(size = 3) +
scale_color_viridis_d(name = "Cercarial Density") +
scale_fill_viridis_d(name = "Cercarial Density") +
theme_minimal() +
labs(title = "NMDS According to Cercarial Density",
```

```
x = "NMDS1",
y = "NMDS2") +
theme(legend.position = "right")

#Filter dataset for COX pathway oxylipins and non-zero concentrations
filtered_data_cox <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName %in% c("PGE2", "15k PGE2") &
`OxylipinConcentration(ng)` > 0)

#Create COX pathway plot
ggplot(filtered_data_cox, aes(x = OxylipinName,
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
labs(title = "COX Pathway Oxylipins",
x = "Oxylipin",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
scale_color_manual(values = c("901" = "blue", "1088" = "green", "742" = "red",
"1207" = "purple", "1439" = "orange", "1621" = "pink",
"414" = "yellow", "544" = "cyan")) +
theme_minimal() +
theme(axis.text.x = element_text(angle = 45, hjust = 1))

#Filter dataset for LOX pathway oxylipins and non-zero concentrations
filtered_data_lox <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName %in% c("11-HETE", "13-HODE", "15-HETE", "16-HETE", "5-HETE",
"9-HETE", "8-HETE", "12-HEPE", "9-HEPE", "12-oXOETE",
"13-HOTrE", "13-HOTrE-y", "9 ox00TrE", "9-OXOODE",
"12,13 diHOME", "14,15 DiHETrE", "8,9 DiHETrE", "9,10 diHODE",
"9,10 diHOME", "12,13 EpODE", "15,16 EpODE", "9,10 EpODE") &
`OxylipinConcentration(ng)` > 0)

#Create LOX pathway plot
ggplot(filtered_data_lox, aes(x = OxylipinName,
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
labs(title = "LOX Pathway Oxylipins",
x = "Oxylipins",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
scale_color_manual(values = c("901" = "blue", "1088" = "green", "742" = "red",
"1207" = "purple", "1439" = "orange", "1621" = "pink",
"414" = "yellow", "544" = "cyan")) +
theme_minimal() +
```

```
theme(axis.text.x = element_text(angle = 45, hjust = 1))

#Filter dataset for CYP450 pathway oxylipins and non-zero concentrations
filtered_data_cyp450 <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName %in% c("9,12,13 triHOME", "9,10,13 triHOME", "DHA", "EPA", "ALA",
"ARA") &
`OxylipinConcentration(ng)` > 0)

#Create CYP450 pathway plot
ggplot(filtered_data_cyp450, aes(x = OxylipinName,
y = `OxylipinConcentration(ng)`,
color = as.factor(CercarialDensity))) +
geom_point(size = 3) +
labs(title = "CYP450 Pathway Oxylipins",
x = "Oxylipins",
y = "Oxylipin Concentration (ng)",
color = "Cercarial Density") +
scale_color_manual(values = c("901" = "blue", "1088" = "green", "742" = "red",
"1207" = "purple", "1439" = "orange", "1621" = "pink",
"414" = "yellow", "544" = "cyan")) +
theme_minimal() +
theme(axis.text.x = element_text(angle = 45, hjust = 1))

#Matrix should contain numeric data
numeric_cols <- sapply(filtered_data, is.numeric)
oxylipin_matrix <- as.matrix(filtered_data[, numeric_cols])
rownames(oxylipin_matrix) <- filtered_data$SampleName

#Run NDMS
ndms_result <- metaMDS(oxylipin_matrix, distance = "bray", k = 2, maxit = 999, trymax = 500,
wascores = TRUE)
nmds_scores <- as.data.frame(scores(ndms_result, display = "sites"))
nmds_scores$CercarialDensity <- filtered_data$CercarialDensity
centroids <- nmds_scores %>%
group_by(CercarialDensity) %>%
summarize(NMDS1 = mean(NMDS1, na.rm = TRUE), NMDS2 = mean(NMDS2, na.rm =
TRUE))

#Create NDMS plot according to cercarial density
ggplot(centroids, aes(x = NMDS1, y = NMDS2, label = CercarialDensity)) +
geom_point(size = 5, color = "grey") +
geom_text(vjust = -0.5, size = 4) +
theme_minimal() +
labs(title = "NMDS According to Cercarial Density",
x = "NMDS1",
y = "NMDS2")
```

```
#Create NDMS plot showing oxylipin concentrations according to cercarial density
hull_data <- nmds_scores %>%
group_by(CercarialDensity) %>%
slice(chull(NMDS1, NMDS2))
ggplot(nmds_scores, aes(x = NMDS1, y = NMDS2, color = CercarialDensity)) +
geom_polygon(data = hull_data, aes(fill = CercarialDensity, group = CercarialDensity),
alpha = 0.2, color = NA) +
geom_point(size = 3) +
scale_color_viridis_d(name = "Cercarial Density") +
scale_fill_viridis_d(name = "Cercarial Density") +
theme_minimal() +
labs(title = "NMDS According to Cercarial Density",
x = "NMDS1",
y = "NMDS2") +
theme(legend.position = "right")

#Clean column names to be syntactically valid
colnames(JS_Chapter1CercarialOxylipins) <-
make.names(colnames(JS_Chapter1CercarialOxylipins))

#Define precursors and ratios to omit
precursors_to_omit <- c("ARA", "ADA", "EPA", "DHA", "ALA", "LA", "gLA")
ratios_to_omit <- c("15,16 EpODE", "9,10 diHODE", "9,10 EpODE", "HXB3")

#Filter oxylipins with at least 3 detections out of 9 samples
oxylipin_counts <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinConcentration.ng. > 0, CercarialDensity != 544) %>%
group_by(OxylipinName) %>%
summarise(DetectionCount = n()) %>%
filter(DetectionCount >= 3)
oxylipins_for_regression <- oxylipin_counts$OxylipinName

#Initialize empty result list
results_list <- list()

#Loop through each oxylipin
for (ox in oxylipins_for_regression) {

#Subset data for current oxylipin
subset_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName == ox,
OxylipinConcentration.ng. > 0,
CercarialDensity != 544)

#Skip if not enough samples or no variation in predictor
```

```
if (nrow(subset_data) < 3 || length(unique(subset_data$CercarialDensity)) < 2) {
results_list[[ox]] <- data.frame(Oxylipin = ox,
Shapiro_p = NA,
Breusch_Pagan_p = NA,
Regression_p = NA,
R_squared = NA,
Assumptions_Met = FALSE)
next}

#Fit model
model <- lm(OxylipinConcentration.ng. ~ CercarialDensity, data = subset_data)
residuals_model <- residuals(model)

#Run Shapiro-Wilk test (normality)
if (length(residuals_model) >= 3 && length(unique(residuals_model)) > 1) {
shapiro_result <- shapiro.test(residuals_model)
shapiro_p <- shapiro_result$p.value
} else {
shapiro_p <- NA}

#Run Breusch-Pagan test (homoscedasticity)
bp_result <- tryCatch({
bptest(model)
}, error = function(e) NULL)
bp_p <- if (!is.null(bp_result)) bp_result$p.value else NA

#Run regression only if assumptions are met
if (!is.na(shapiro_p) && !is.na(bp_p) && shapiro_p > 0.05 && bp_p > 0.05) {
reg_summary <- summary(model)
reg_p <- coef(reg_summary)[2, 4]
r2 <- reg_summary$r.squared
assumptions_met <- TRUE
} else {
reg_p <- NA
r2 <- NA
assumptions_met <- FALSE}

#Store regression results
results_list[[ox]] <- data.frame(Oxylipin = gsub("[^[:alnum:][:space:]]", "", ox),
Shapiro_p = shapiro_p,
Breusch_Pagan_p = bp_p,
Regression_p = reg_p,
R_squared = r2,
Assumptions_Met = assumptions_met)}

#Combine results into single dataframe
```

```
results_df <- bind_rows(results_list)

#Export results
print(results_df)

#Set output directory
output_dir <- "/Users/joshitasehgal/Desktop/Chapter1Figures"
if (!dir.exists(output_dir)) dir.create(output_dir)

#Loop through results and create plots where assumptions were met
for (i in seq_along(results_list)) {
res <- results_list[[i]]

if (!is.null(res) && res$Assumptions_Met) {
ox_name <- res$Oxylipin

#Subset original data
subset_data <- JS_Chapter1CercarialOxylipins %>%
filter(OxylipinName == names(results_list)[i],
OxylipinConcentration.ng. > 0,
CercarialDensity != 544)

#Fit model
model <- lm(OxylipinConcentration.ng. ~ CercarialDensity, data = subset_data)

#Create filename
file_name <- file.path(output_dir, paste0("Chapter1Regression_", gsub("[^[:alnum:]]", "",
ox_name), ".png"))

#Create and save plot
png(filename = file_name, width = 800, height = 600)
plot(subset_data$CercarialDensity, subset_data$OxylipinConcentration.ng.,
main = paste("Regression for", ox_name),
xlab = "Cercarial Densities (cercariae/10 mL)",
ylab = "Oxylipin Concentration (ng)",
pch = 19, col = "black")
abline(model, col = "red", lwd = 2)

#Add legend with R-squared and p-value
legend("topright", legend = c(paste0("R² = ", round(res$R_squared, 3)),
paste0("p = ", signif(res$Regression_p, 3))), bty = "n")
dev.off()}}

#Save plots
ggsave(filename = paste(output_dir, "/plot1.png", sep = ""), plot = plot1, width = 8, height = 8,
dpi = 300)
```

```
ggsave(filename = paste(output_dir, "/plot2.png", sep = ""), plot = plot2, width = 8, height = 8,
dpi = 300)
ggsave(filename = paste(output_dir, "/plot3.png", sep = ""), plot = plot3, width = 8, height = 8,
dpi = 300)
ggsave(filename = paste(output_dir, "/plot4.png", sep = ""), plot = plot4, width = 10, height =
12, dpi = 300)
ggsave(filename = paste(output_dir, "/plot5.png", sep = ""), plot = plot5, width = 8, height = 8,
dpi = 300)
ggsave(filename = file.path(output_dir, "plot6_combined_oxylipins.png"), plot = plot6, width =
10, height = 6, dpi = 300)
ggsave(filename = file.path(output_dir, file_name), plot = p, width = 8, height = 6, dpi = 300)
ggsave(filename = paste0("Oxylipin_Figures_without_Lines/", oxylipin, ".png"), plot = p, width
= 6, height = 4, dpi = 300)}
dir.create("Oxylipin_Figures_with_Lines", showWarnings = FALSE)
ggsave(filename = paste0("Oxylipin_Figures_with_Lines/", oxylipin, ".png"), plot = p, width =
6, height = 4, dpi = 300)}
file_name <- file.path(output_dir, paste0("Regression_", gsub("[^[:alnum:]]", "", ox_name),
".png"))
filename <- paste0("Oxylipin_Concentration_Density_Sample_", density, ".png")
ggsave(filename = file.path(output_dir, filename), plot = p, width = 8, height = 6, dpi = 300)}
```

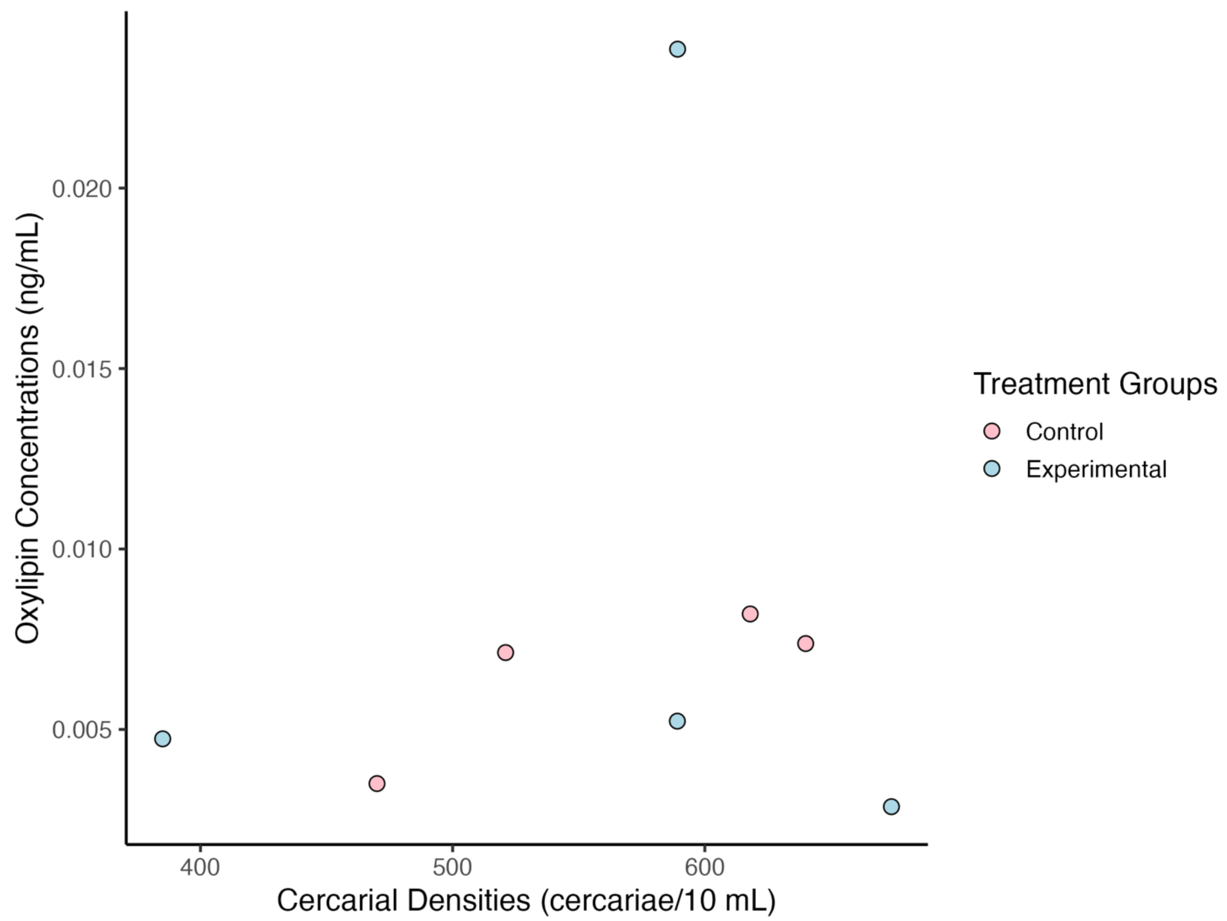

Figure 3.41. Concentration of 11-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

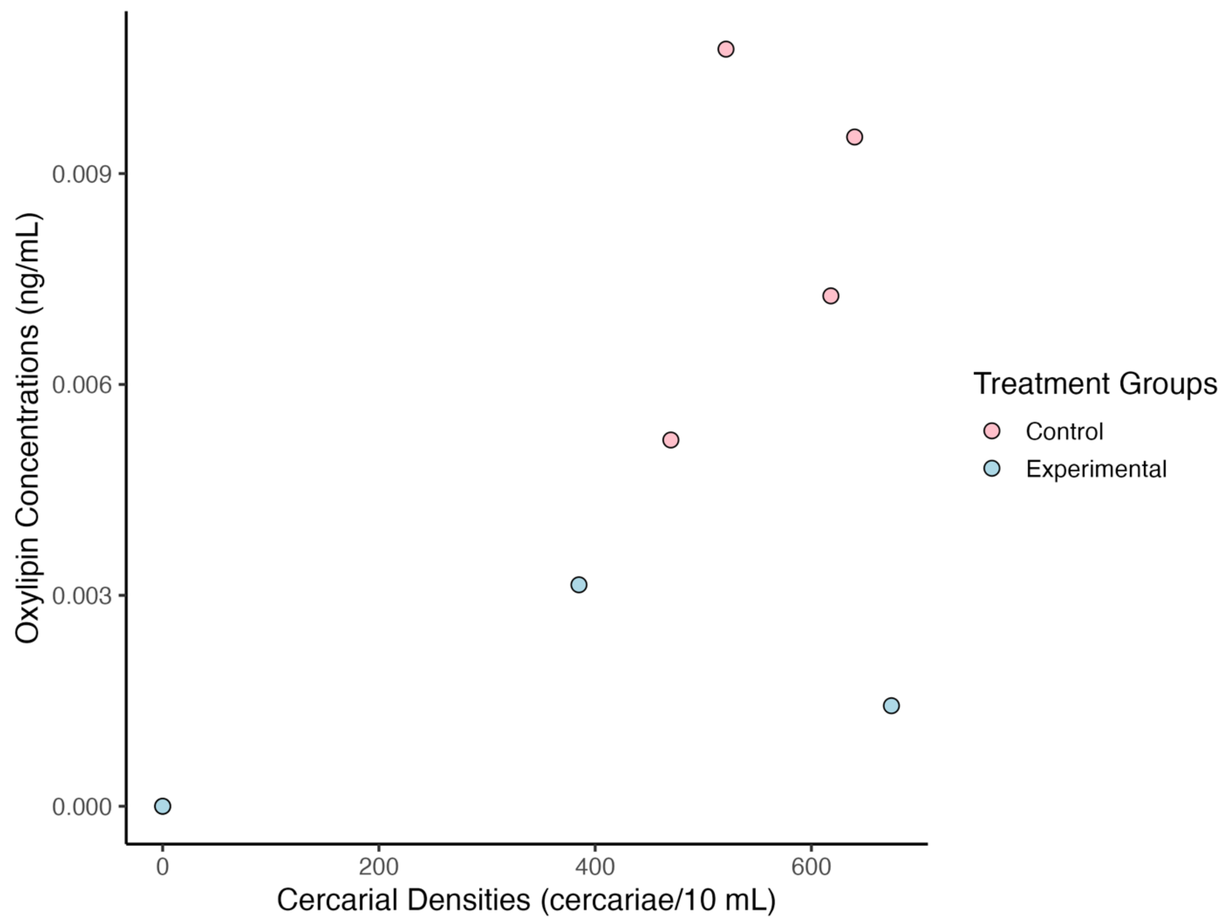

Figure 3.42. Concentration of 12-HEPE (ng/mL) across cercarial densities (cercariae/10 mL).

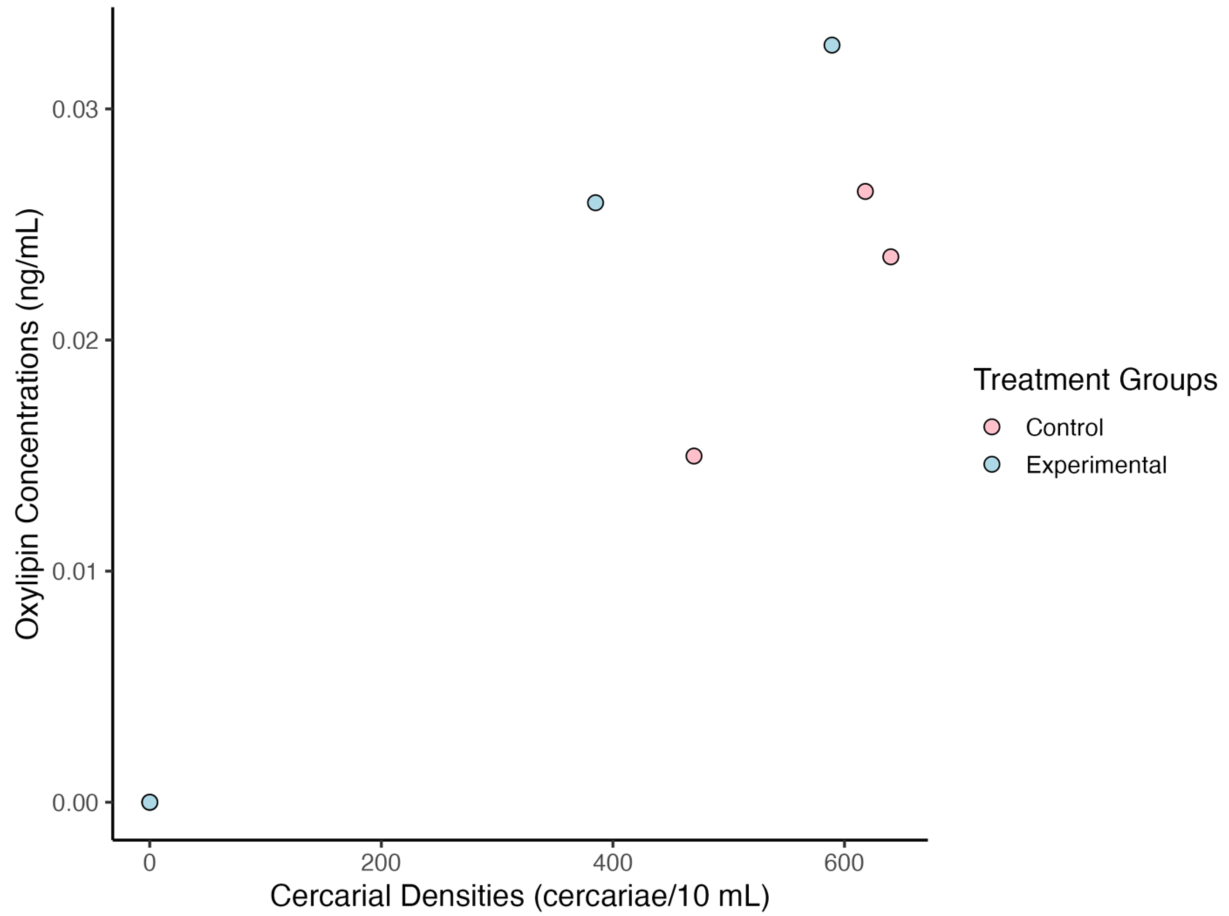

Figure 3.43. Concentration of 12-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

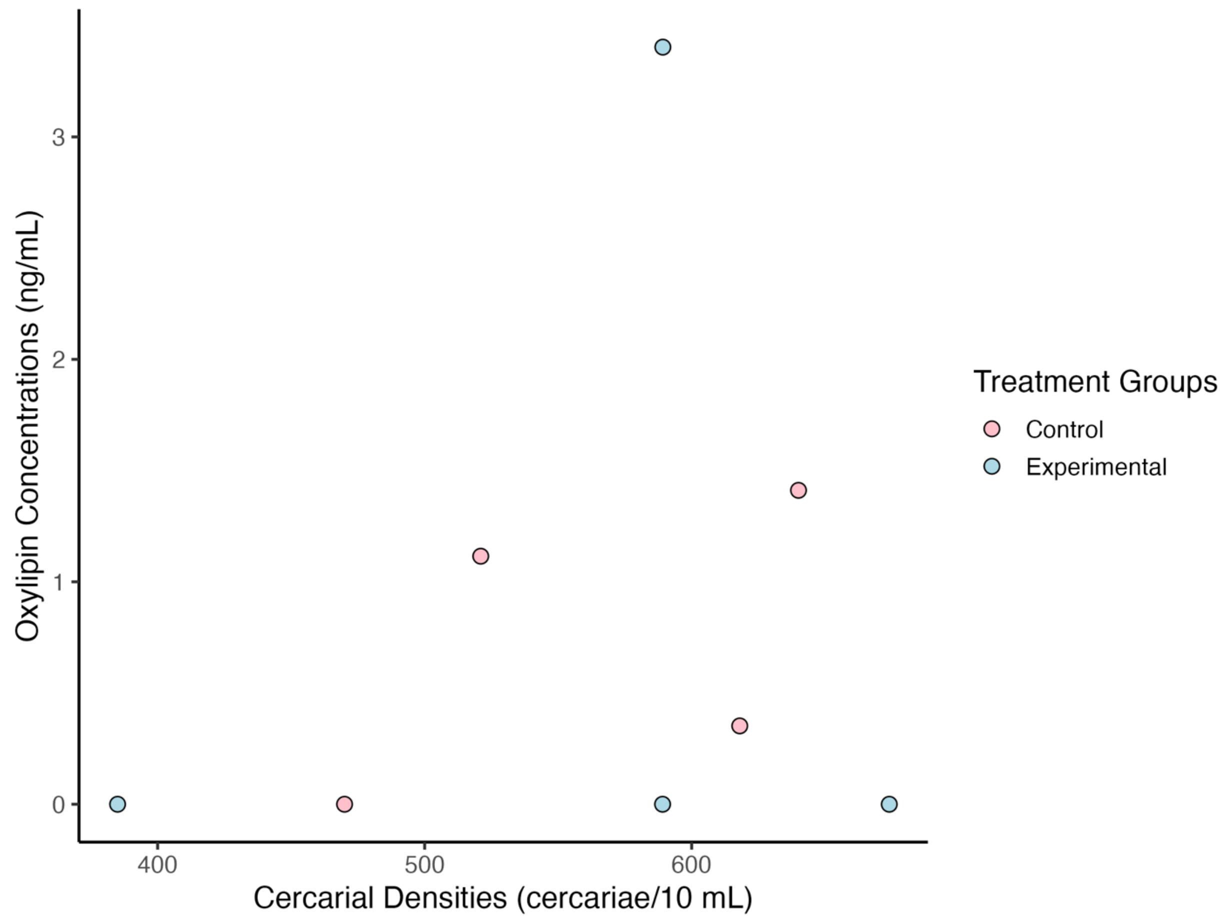

Figure 3.44. Concentration of 13-HODE (ng/mL) across cercarial densities (cercariae/10 mL).

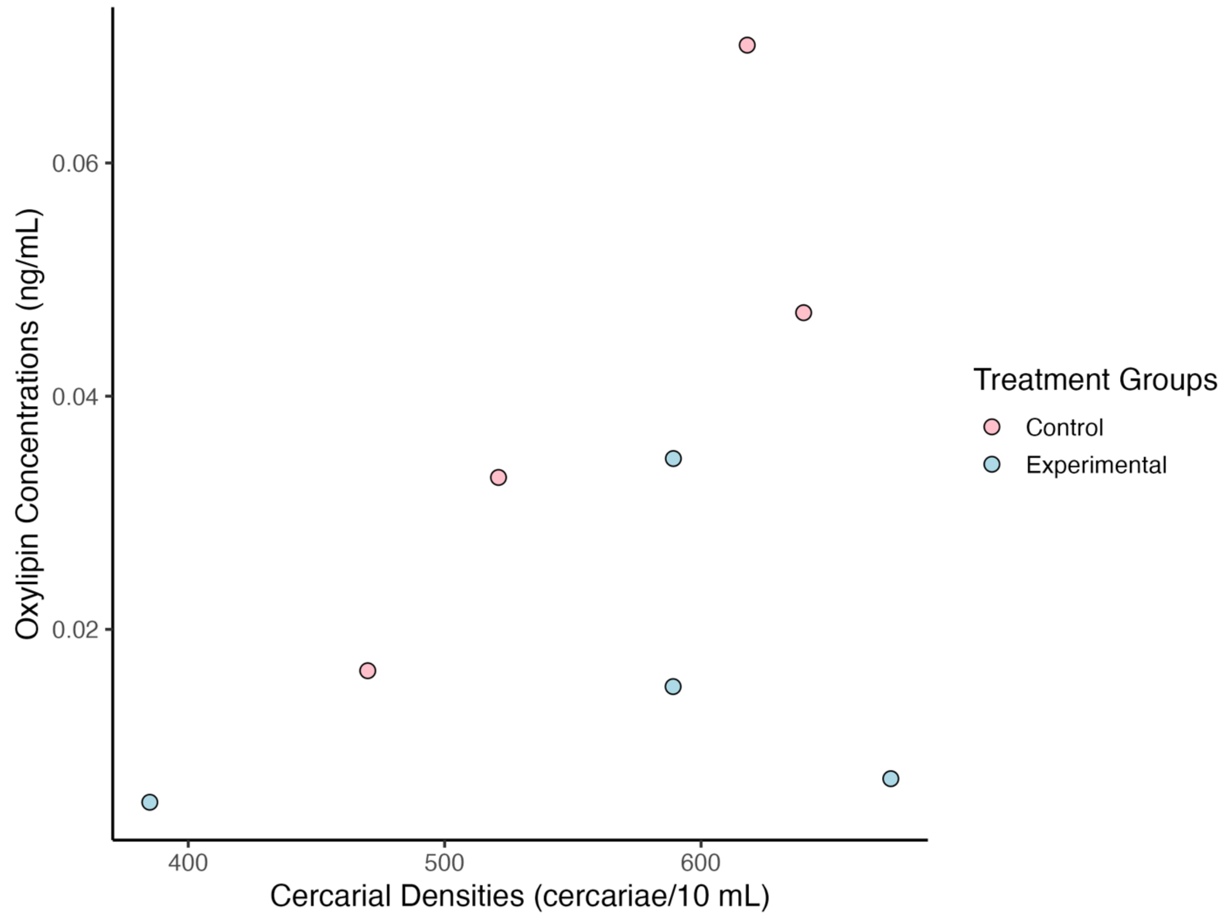

Figure 3.45. Concentration of 13-HOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

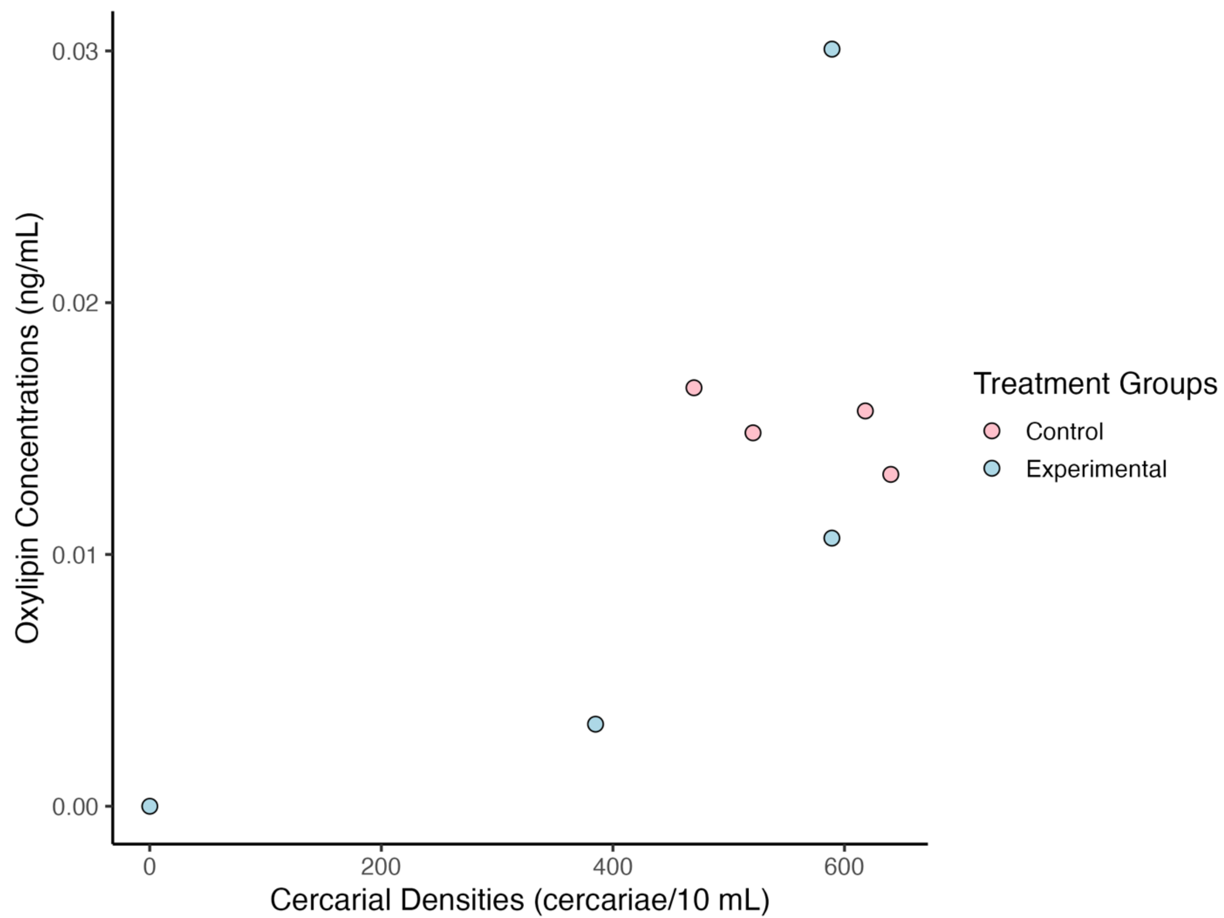

Figure 3.46. Concentration of 15-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

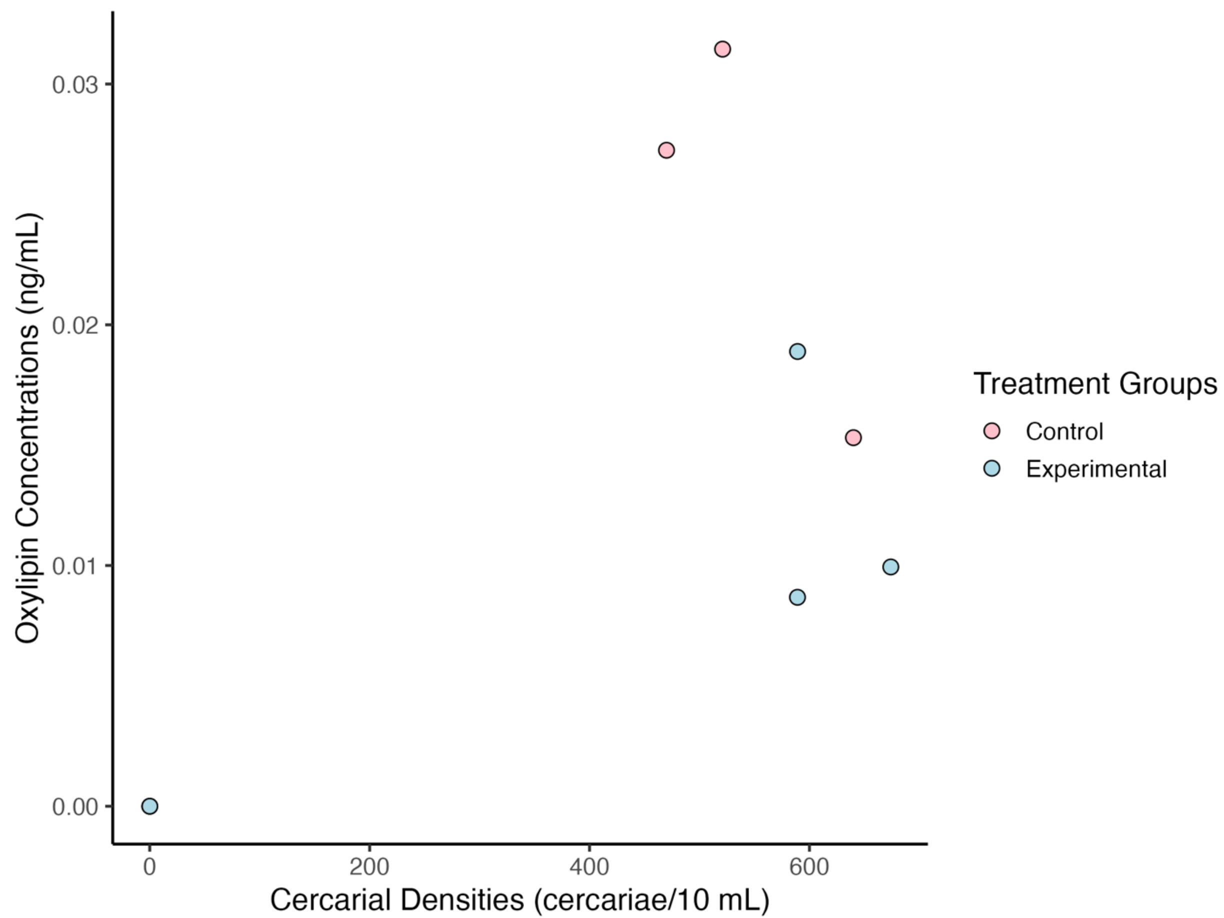

Figure 3.47. Concentration of 15-oxoETE (ng/mL) across cercarial densities (cercariae/10 mL).

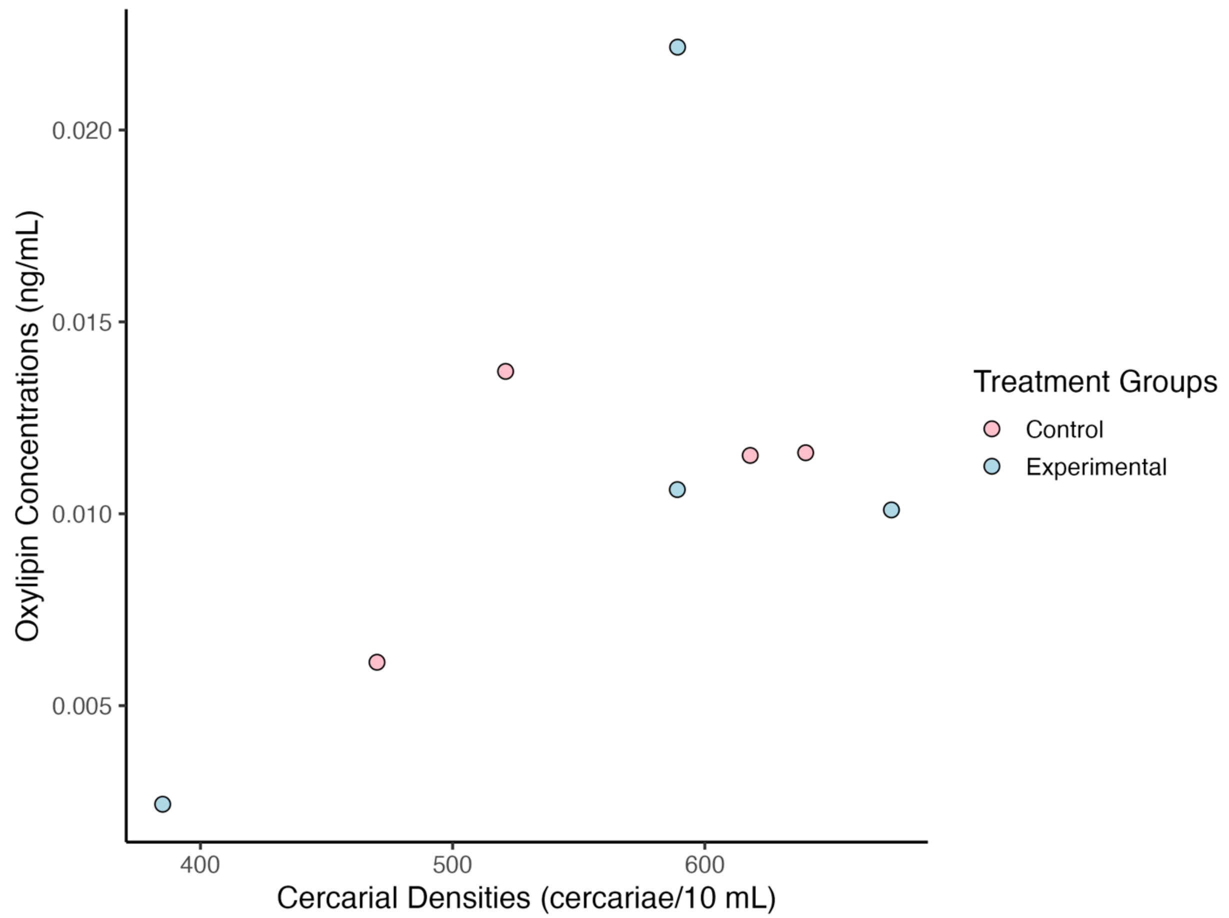

Figure 3.48. Concentration of 5-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

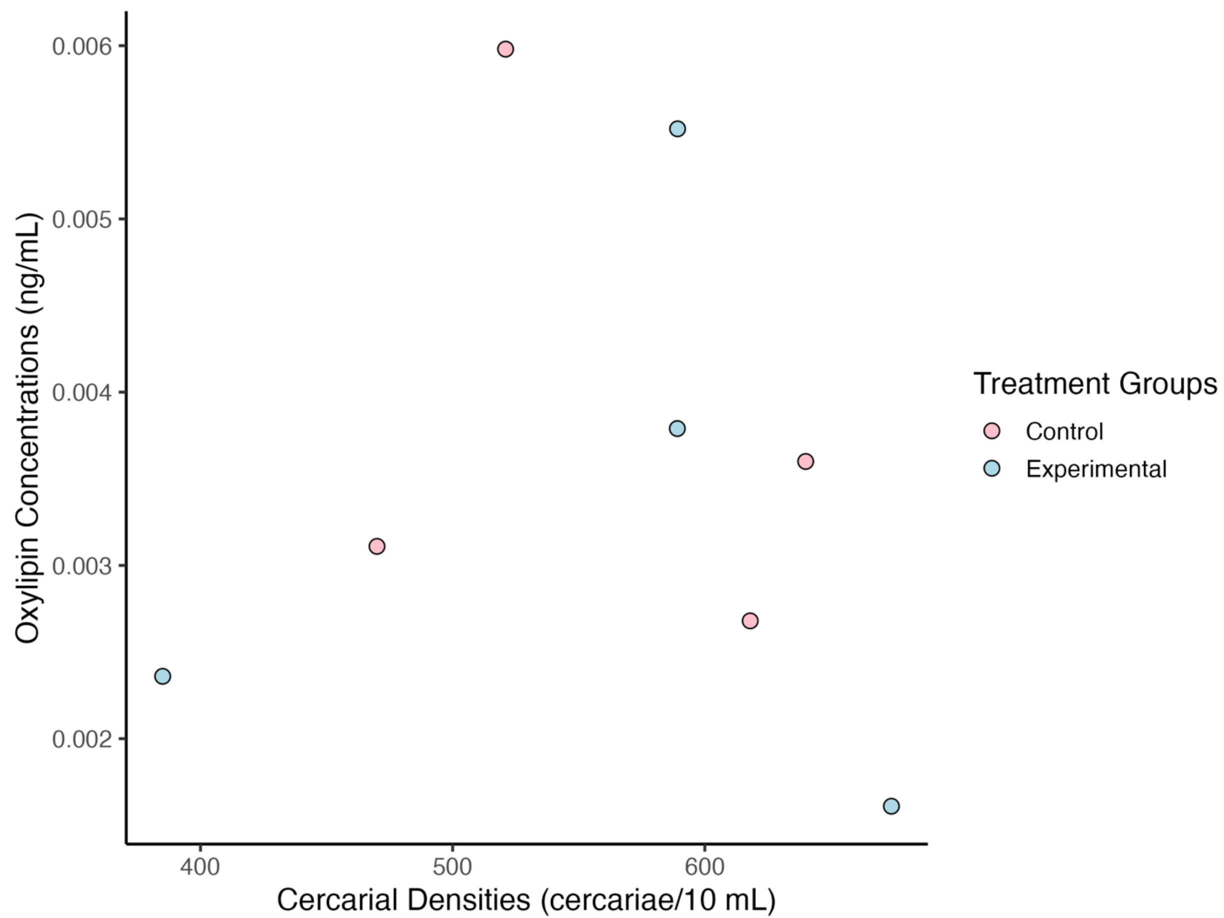

Figure 3.49. Concentration of 8-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

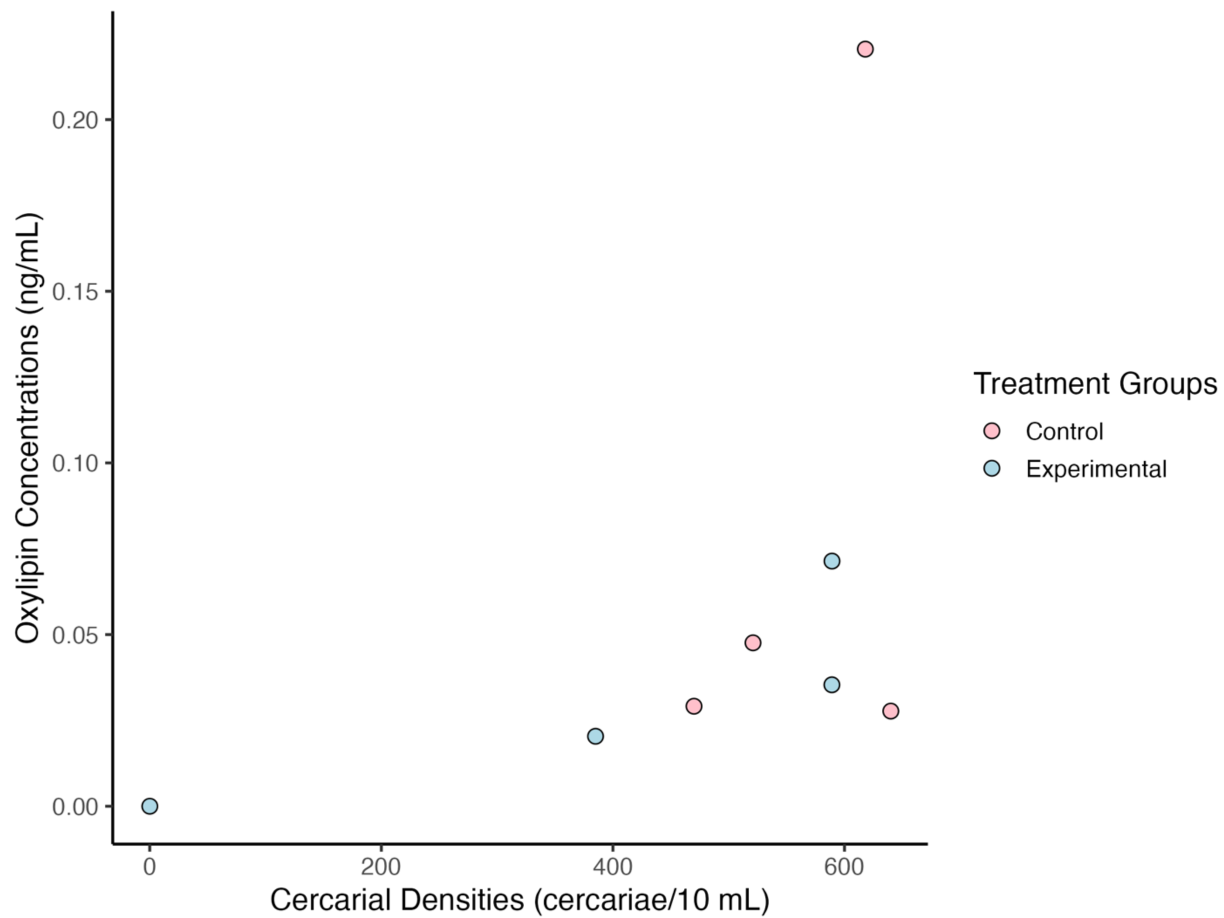

Figure 3.50. Concentration of 9-oxoOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

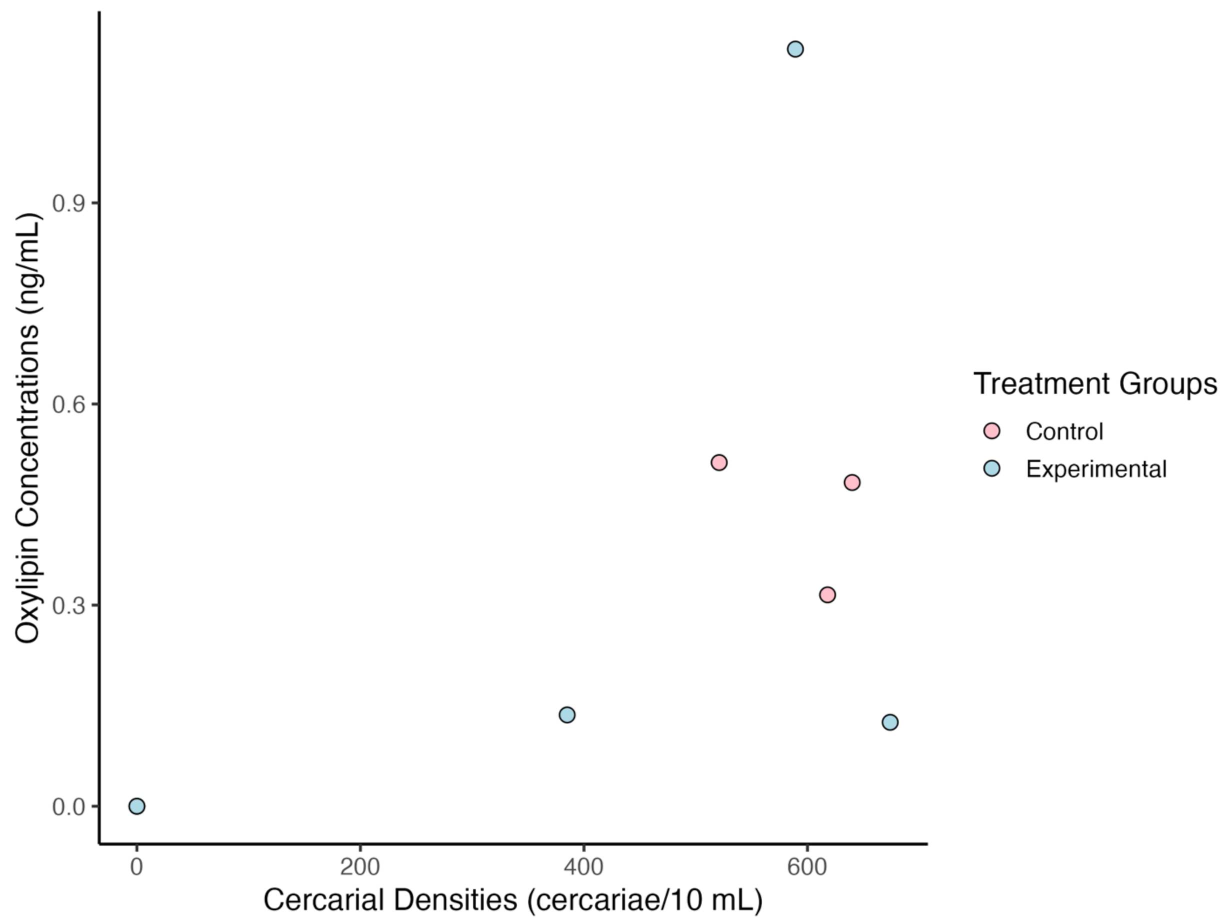

Figure 3.51. Concentration of 9-HODE (ng/mL) across cercarial densities (cercariae/10 mL).

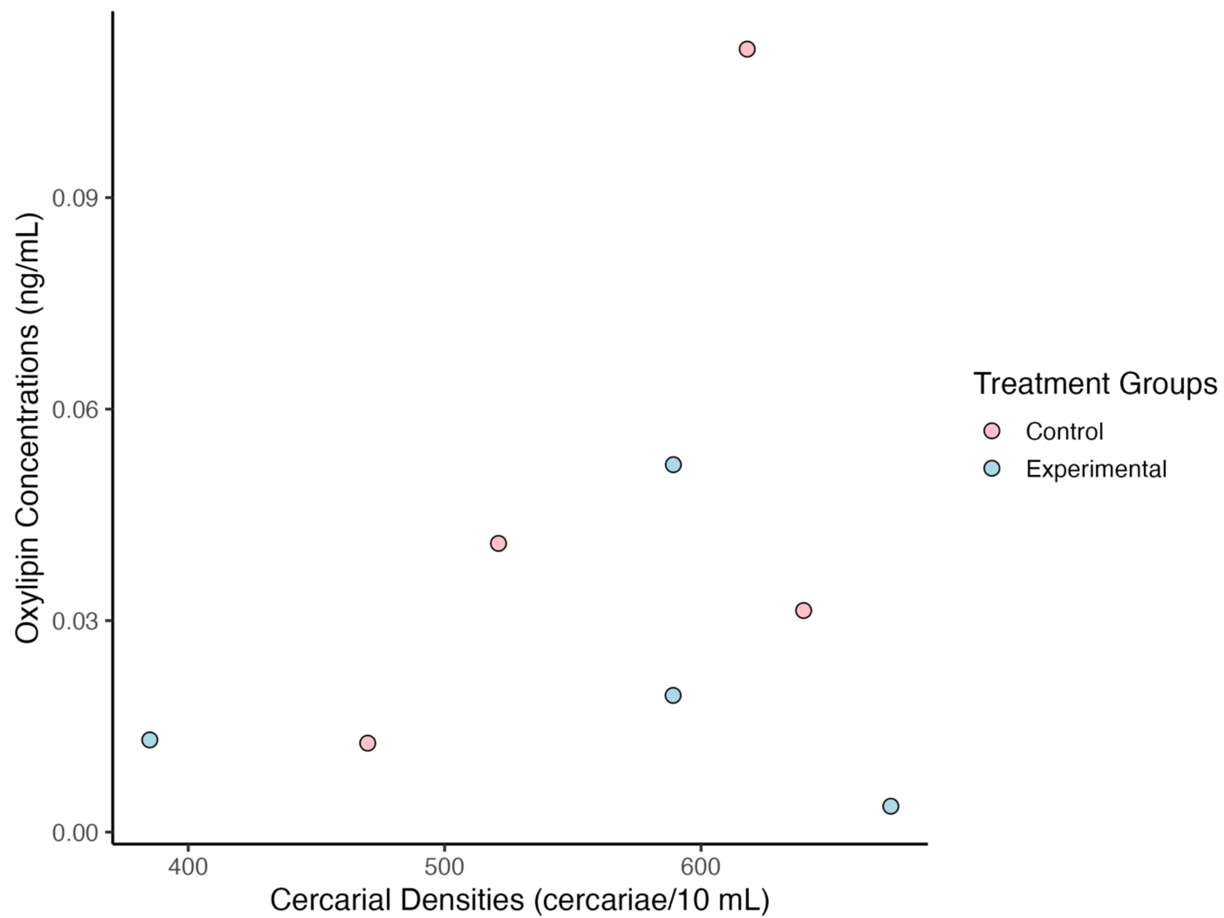

Figure 3.52. Concentration of 9-HOTrE (ng/mL) across cercarial densities (cercariae/10 mL).

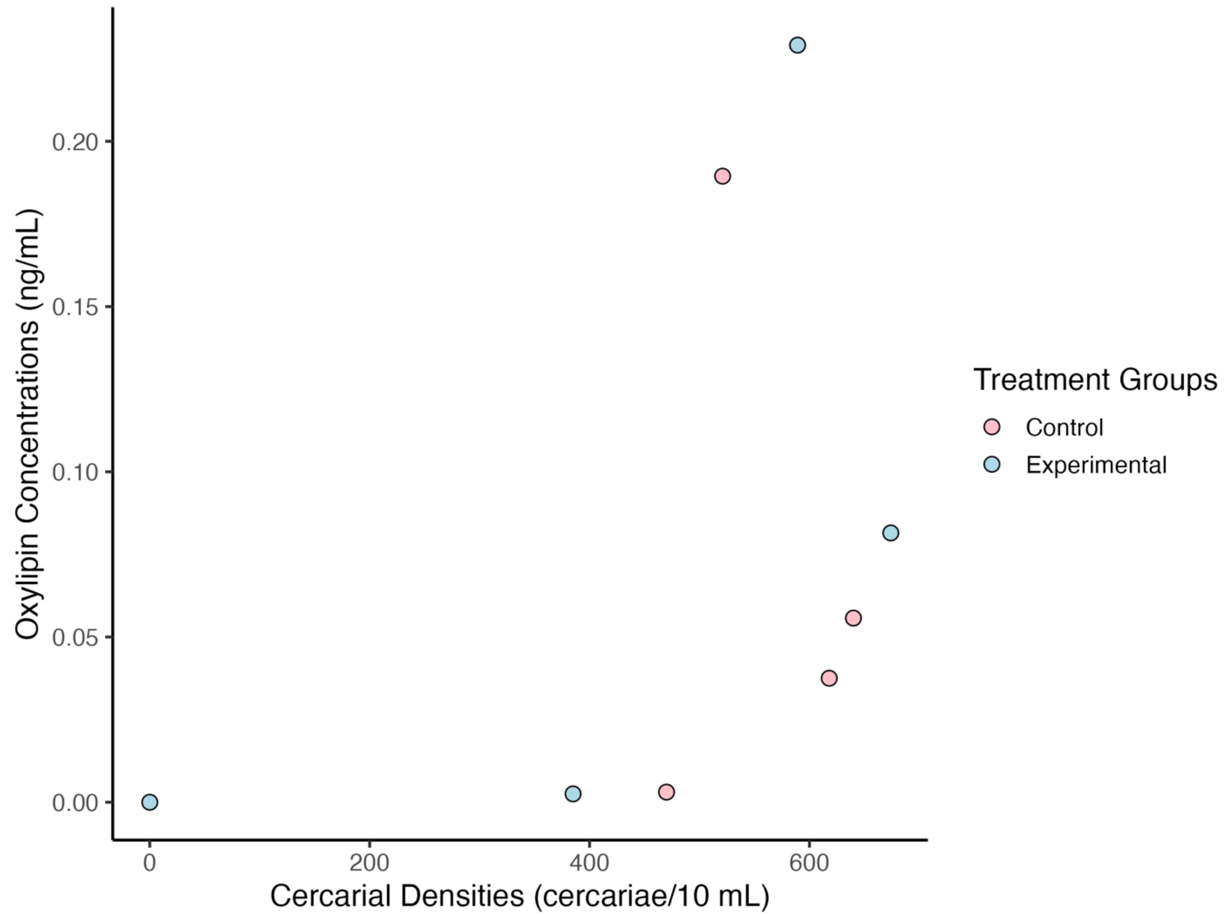

Figure 3.53. Concentration of RvD_5 (ng/mL) across cercarial densities (cercariae/10 mL).

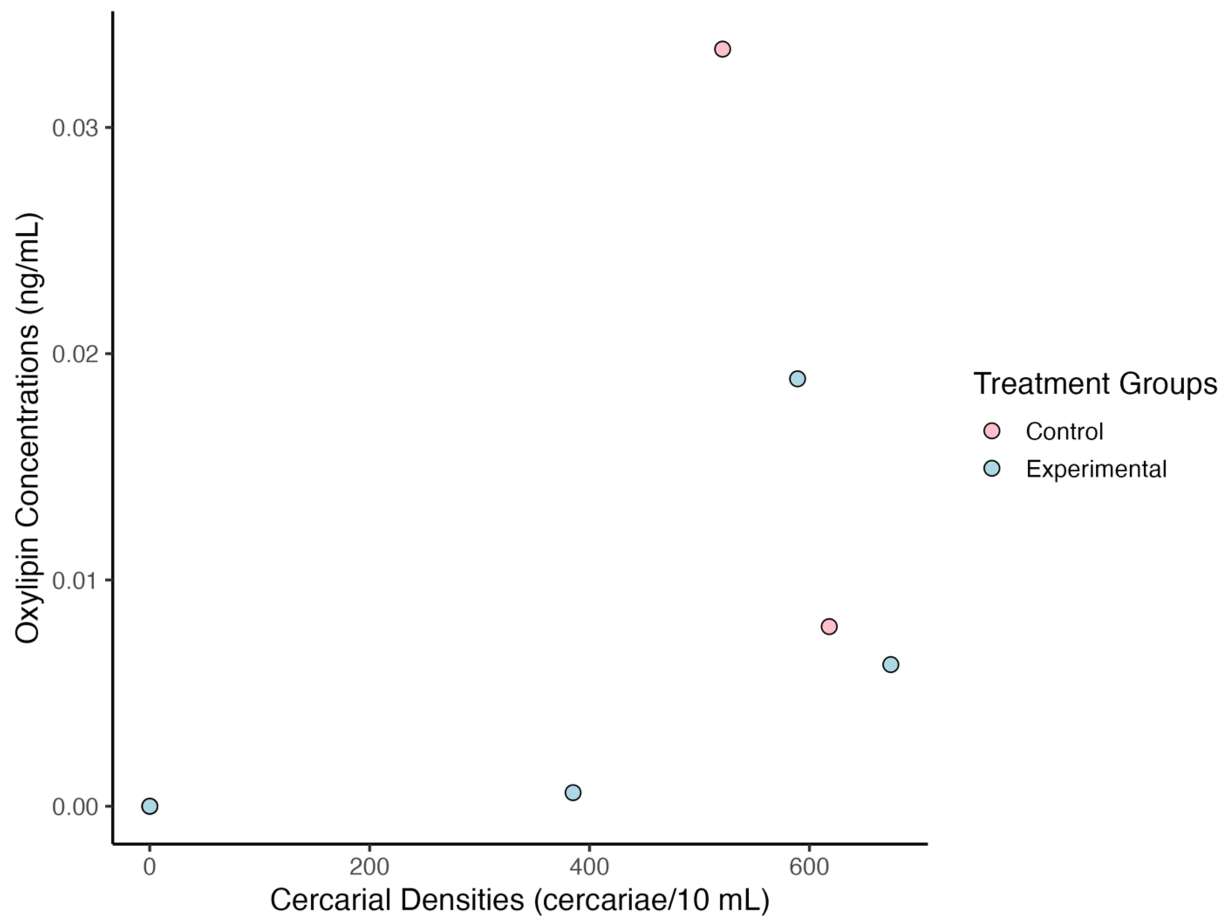

Figure 3.54. Concentration of 10S,17S-DiHDoHE (PDX) (ng/mL) across cercarial densities (cercariae/10 mL).

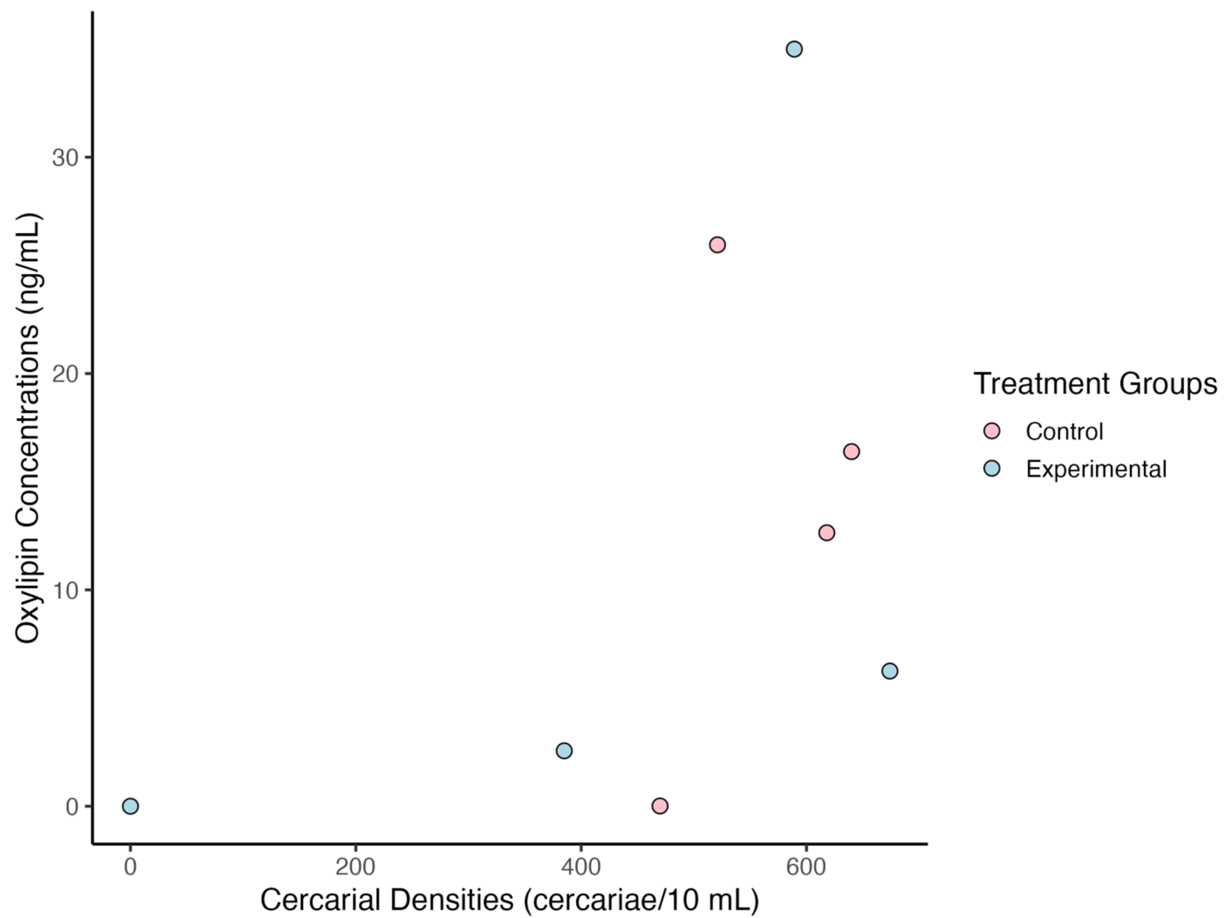

Figure 3.55. Concentration of 9,12,13-triHOME (ng/mL) across cercarial densities (cercariae/10 mL).

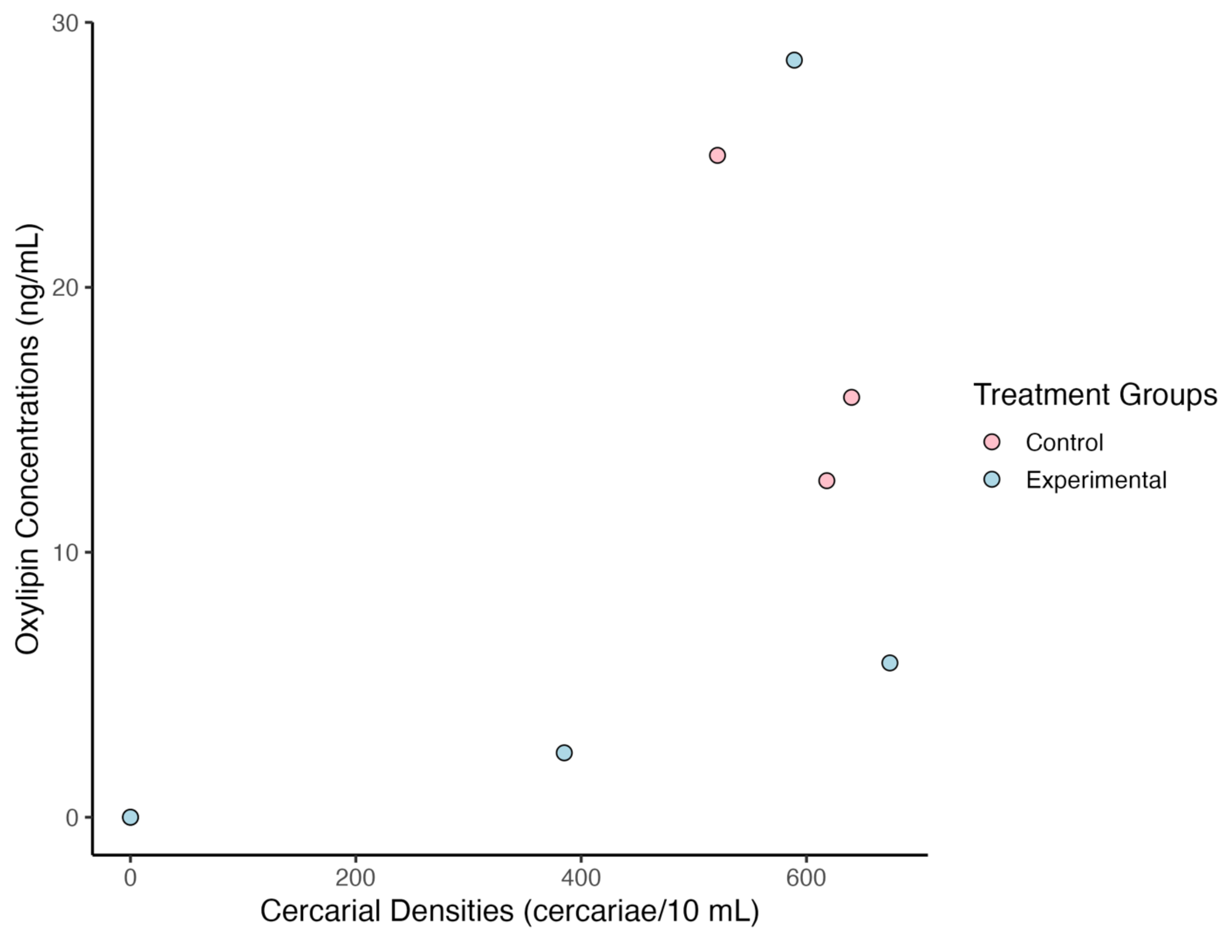

Figure 3.56. Concentration of 9,10,13-triHOME (ng/mL) across cercarial densities (cercariae/10 mL).

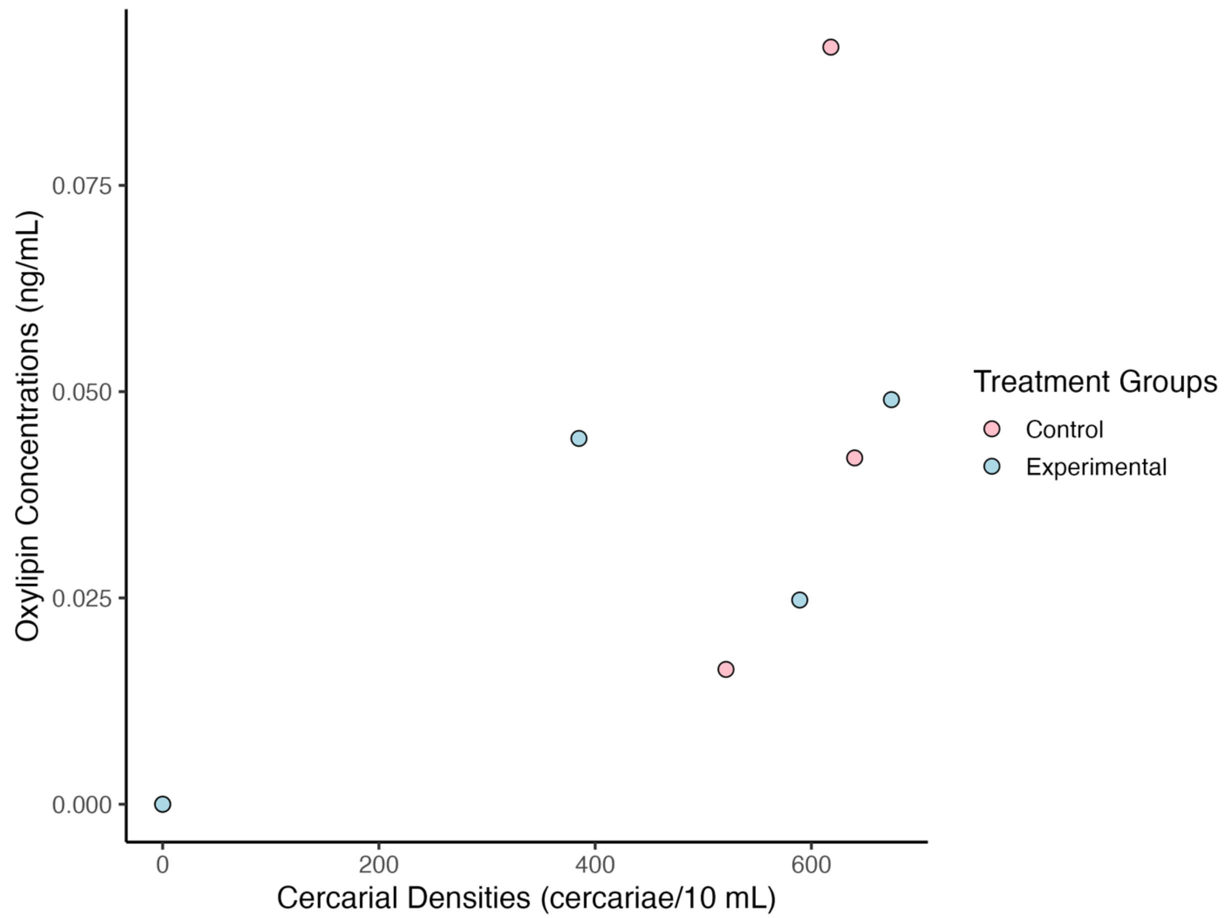

Figure 3.57. Concentration of 2,3-dinor TXB_2 (ng/mL) across cercarial densities (cercariae/10 mL).

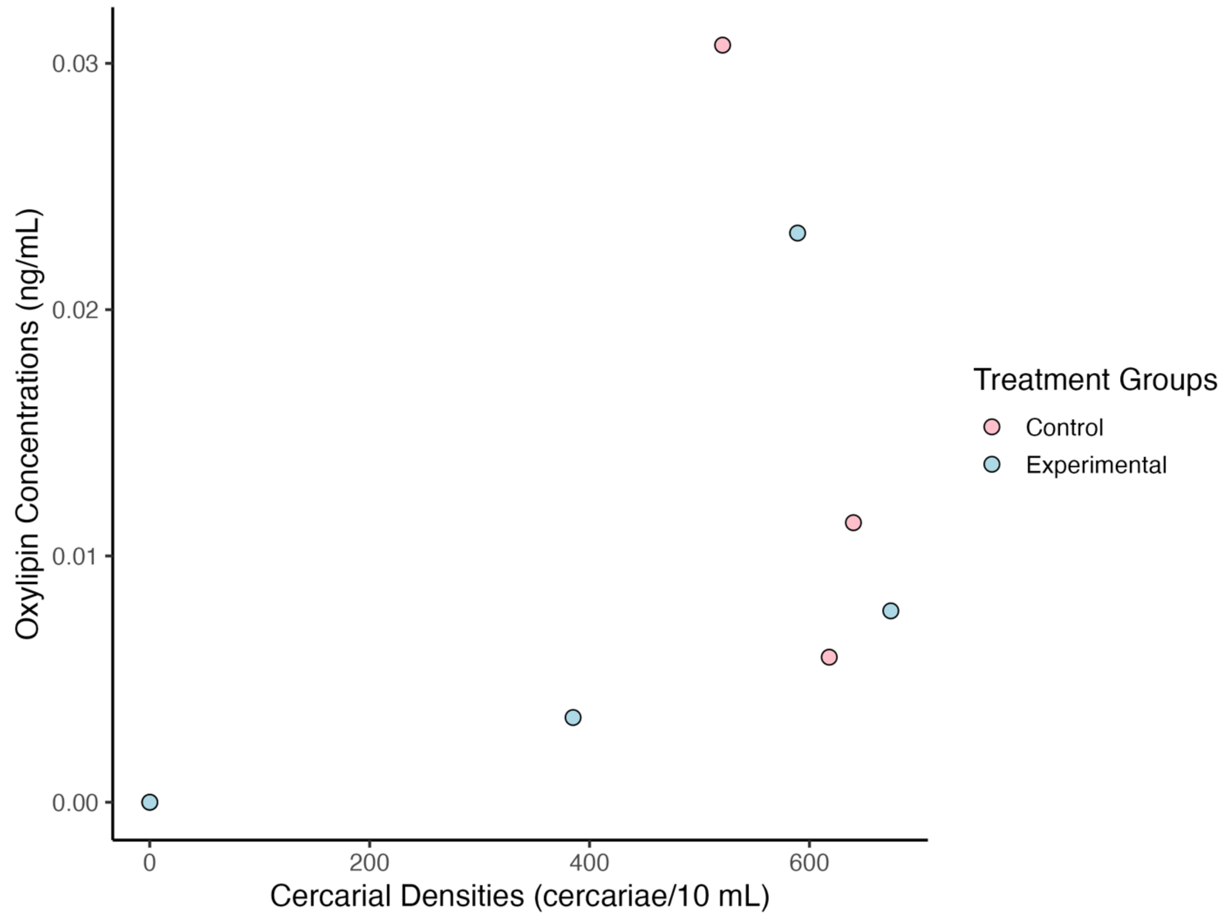

Figure 3.58. Concentration of PGD_1 (ng/mL) across cercarial densities (cercariae/10 mL).

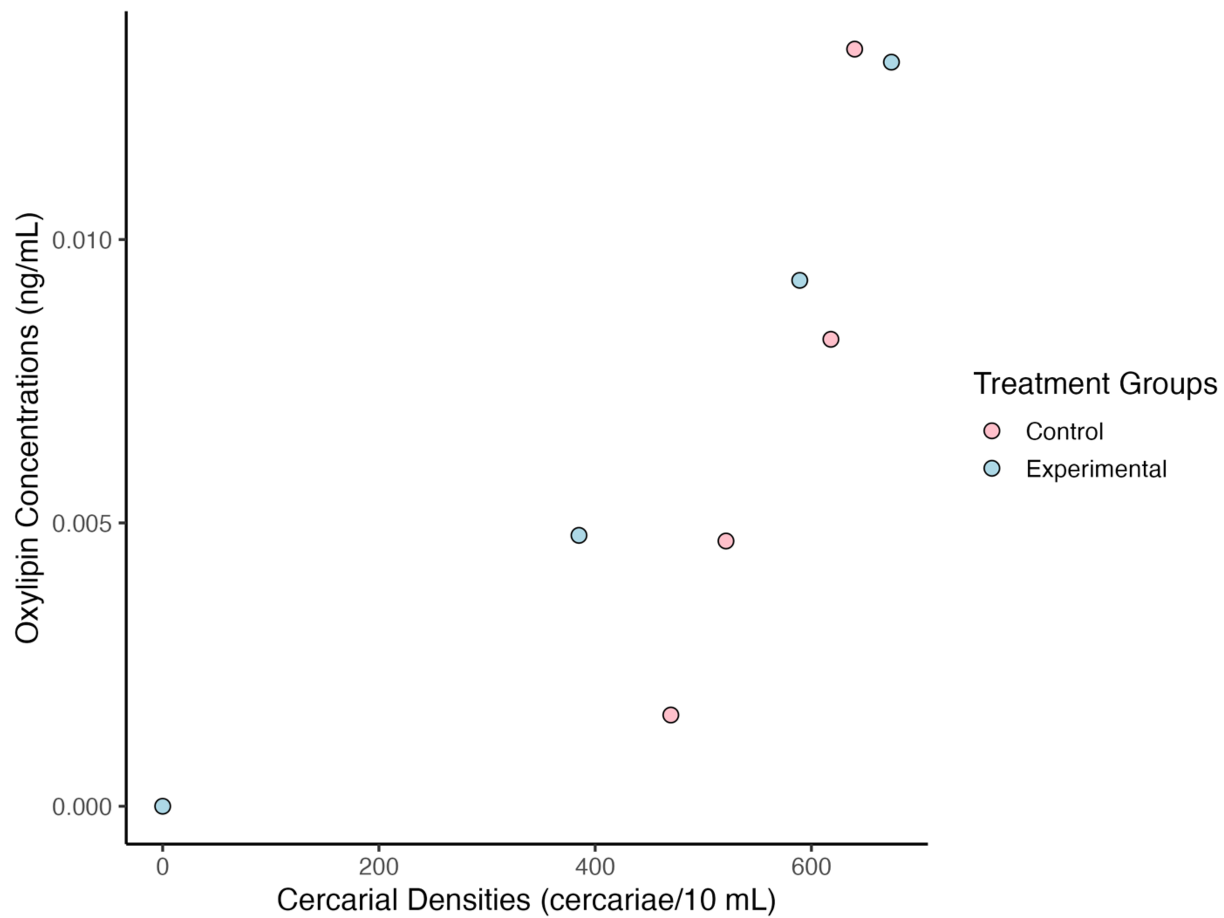

Figure 3.59. Concentration of TXB_1 (ng/mL) across cercarial densities (cercariae/10 mL).

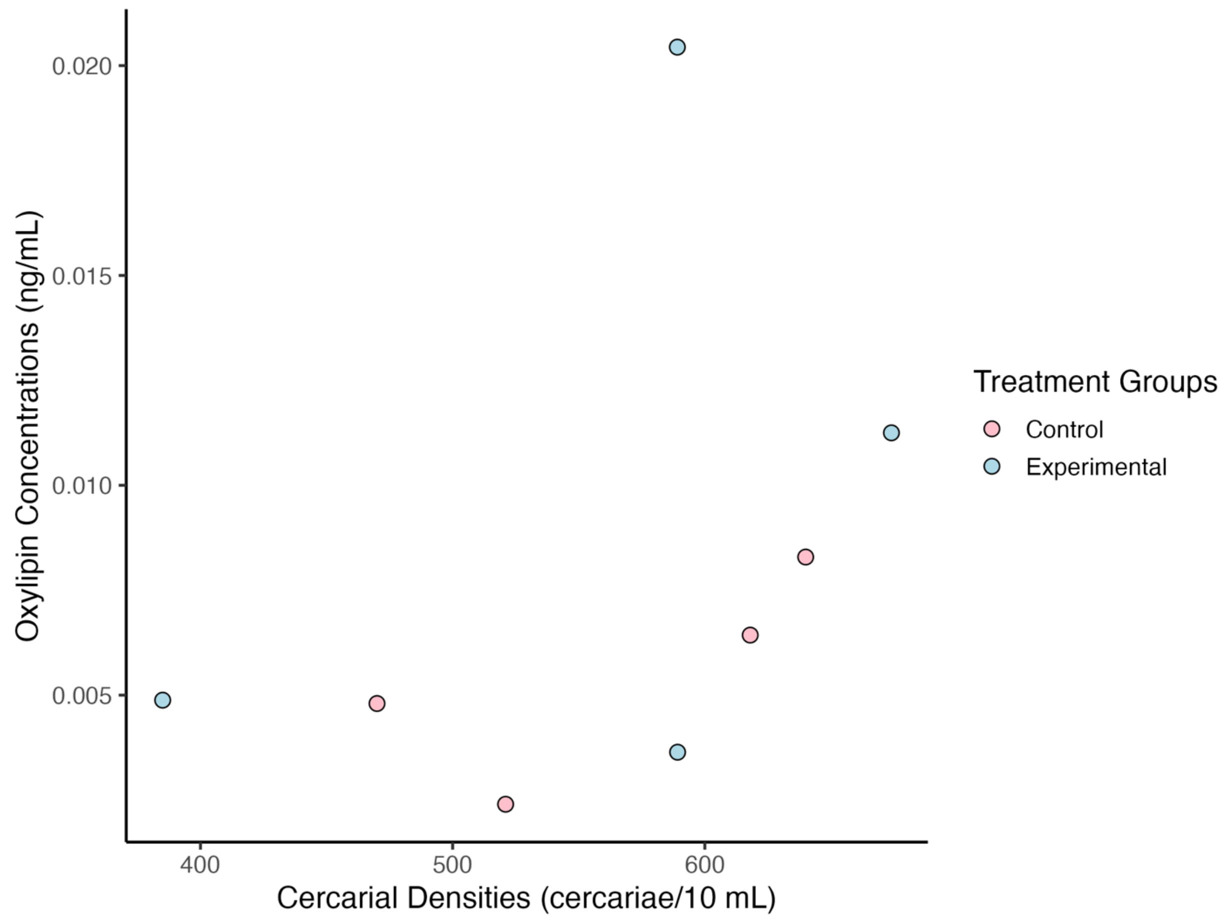

Figure 3.60. Concentration of TXB_3 (ng/mL) across cercarial densities (cercariae/10 mL).

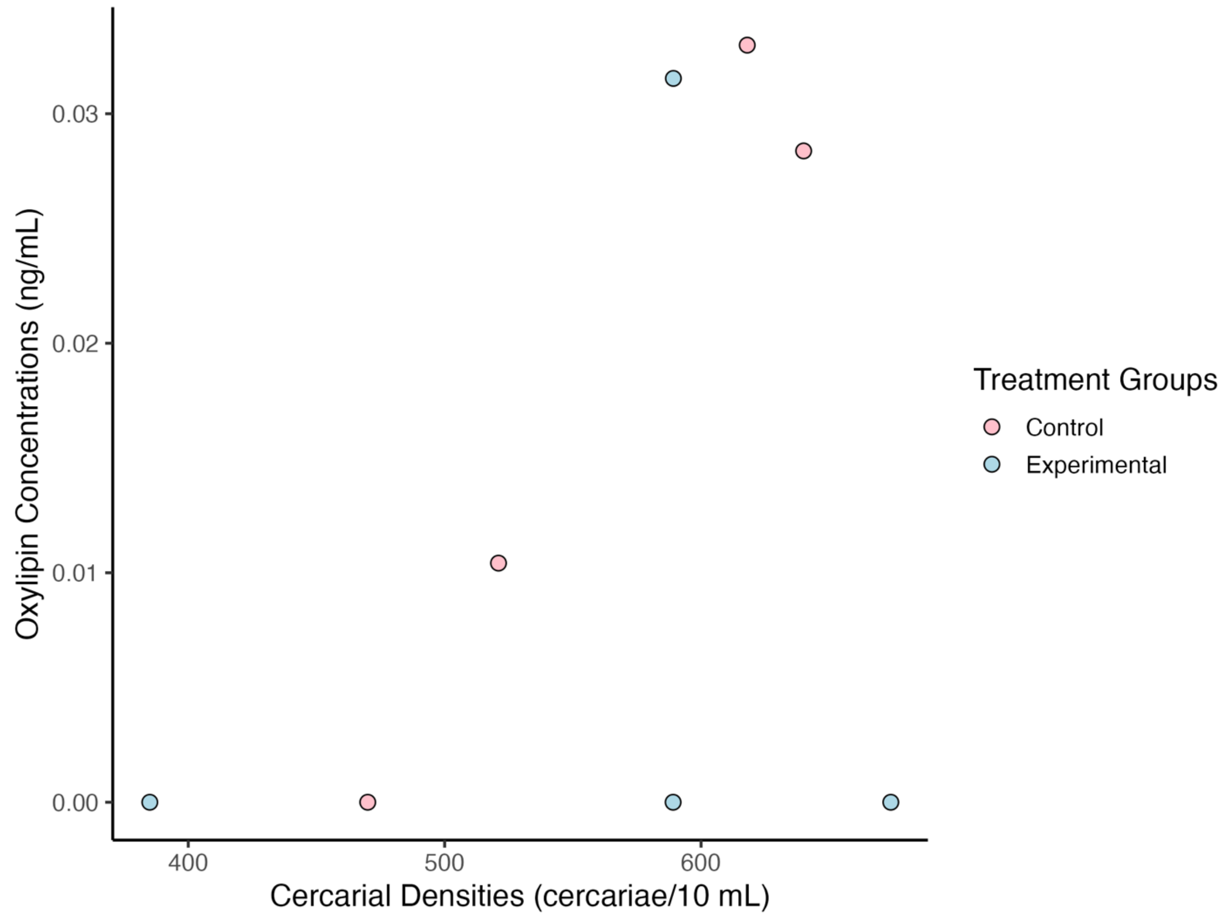

Figure 3.61. Concentration of dhk $PGF_{2\alpha}$ (ng/mL) across cercarial densities (cercariae/10 mL).

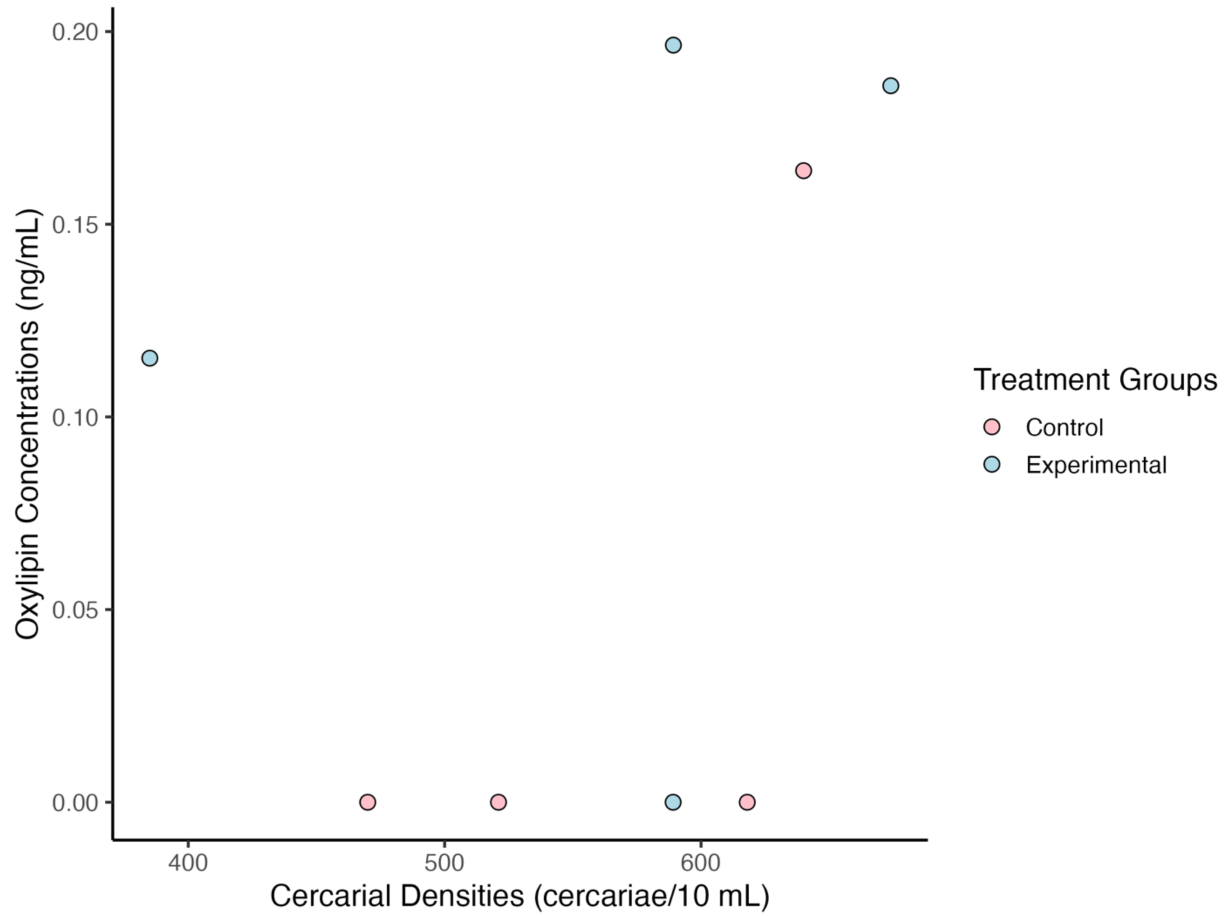

Figure 3.62. Concentration of 2,3-dinor-6k $PGF_{1\alpha}$ (ng/mL) across cercarial densities (cercariae/10 mL).

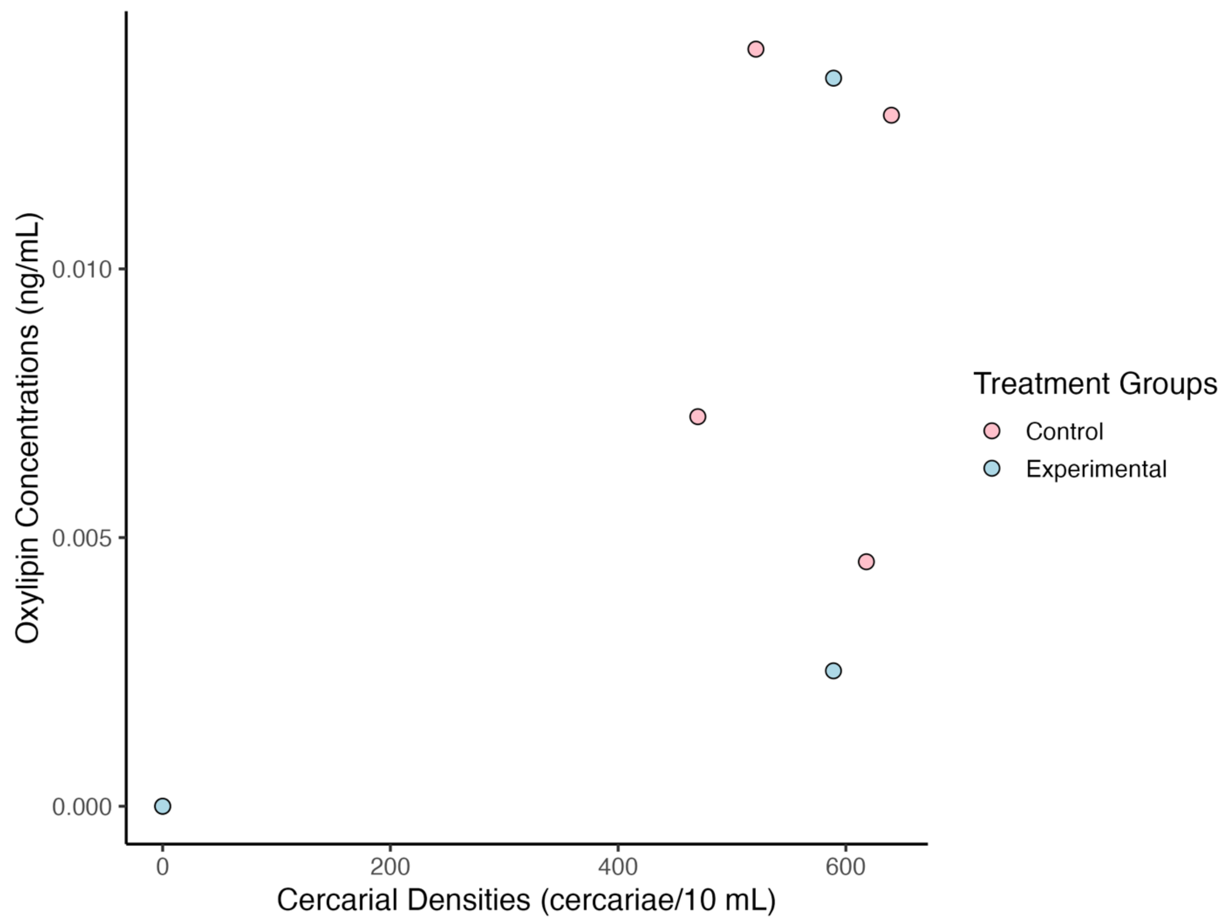

Figure 3.63. Concentration of 12,13-EpODE (ng/mL) across cercarial densities (cercariae/10 mL).

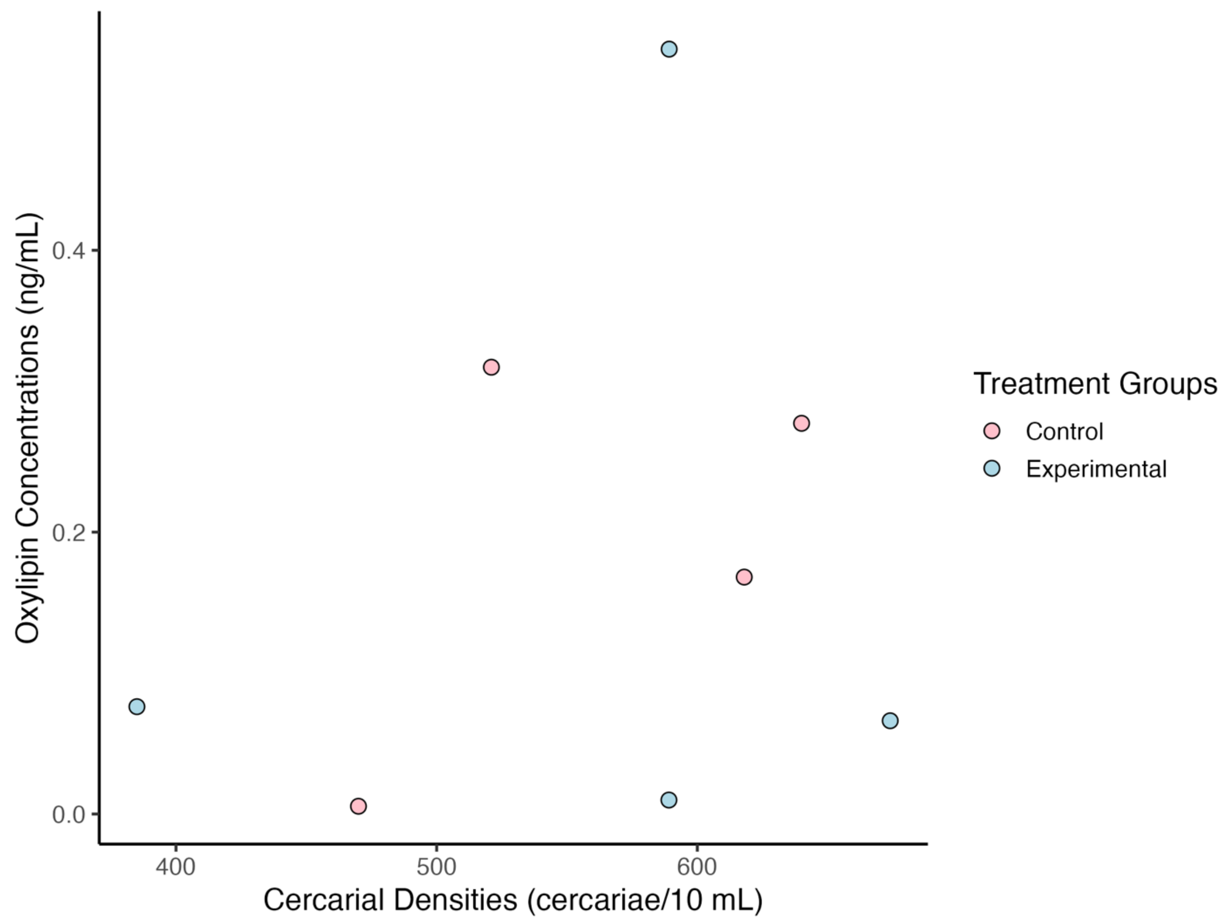

Figure 3.64. Concentration of 12,13-diHOME (ng/mL) across cercarial densities (cercariae/10 mL).

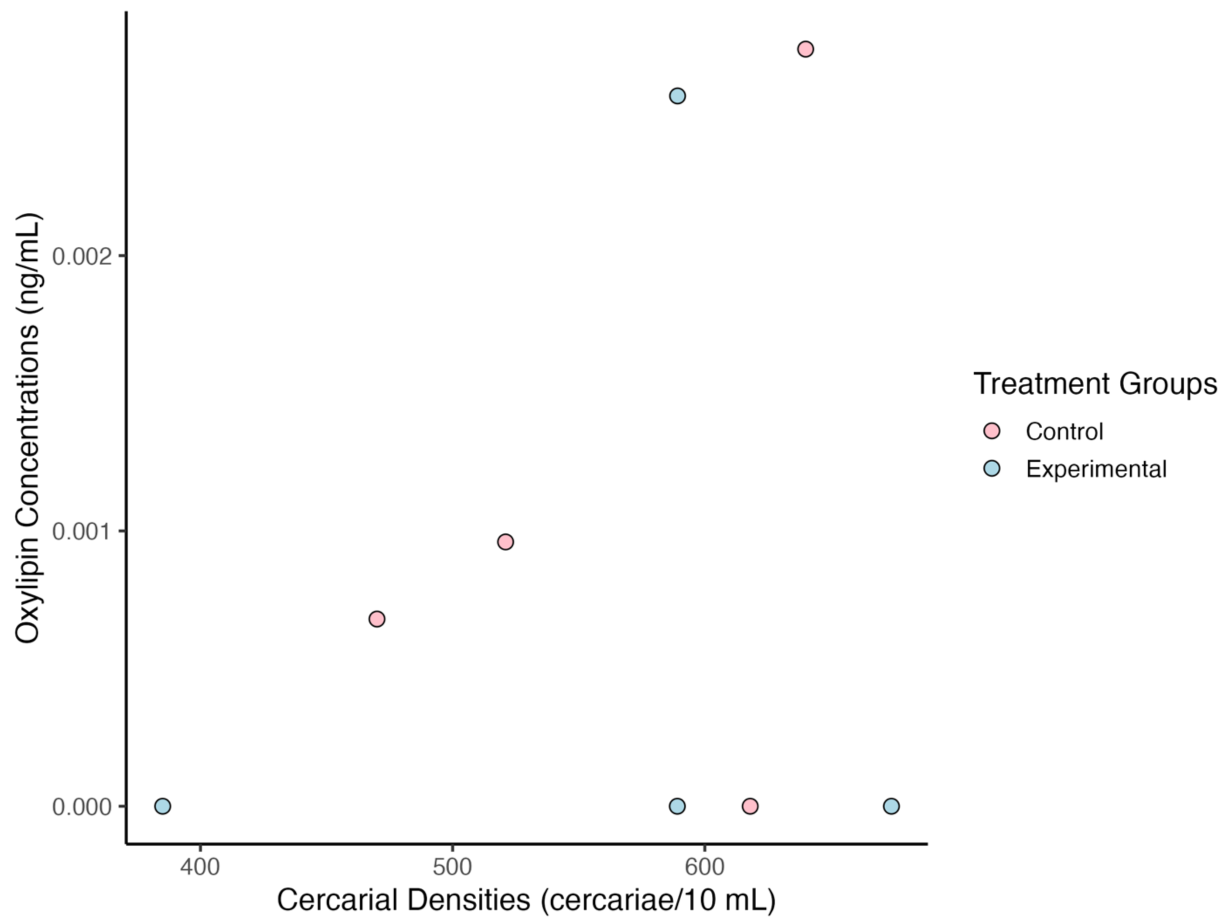

Figure 3.65. Concentration of 14,15-DiHETrE (ng/mL) across cercarial densities (cercariae/10 mL).

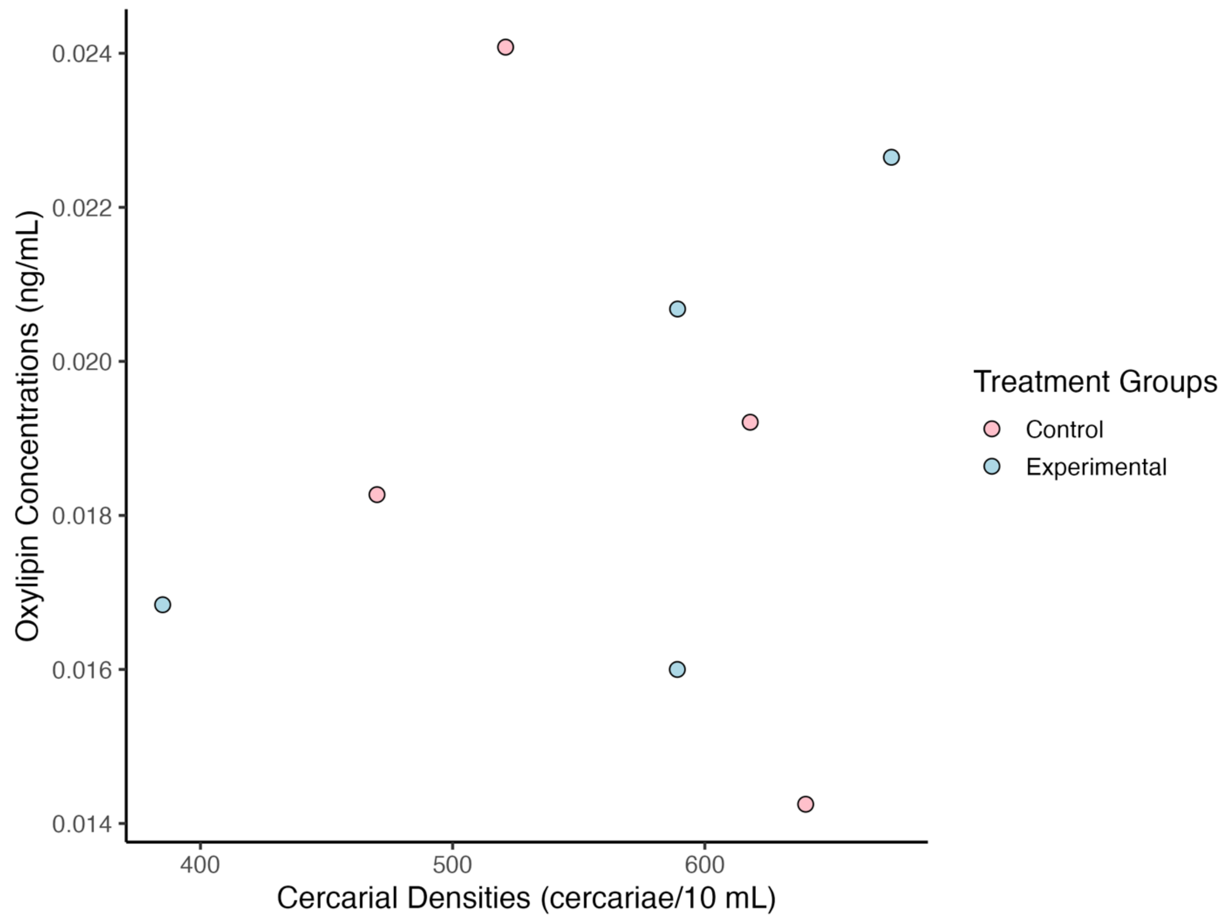

Figure 3.66. Concentration of 16-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

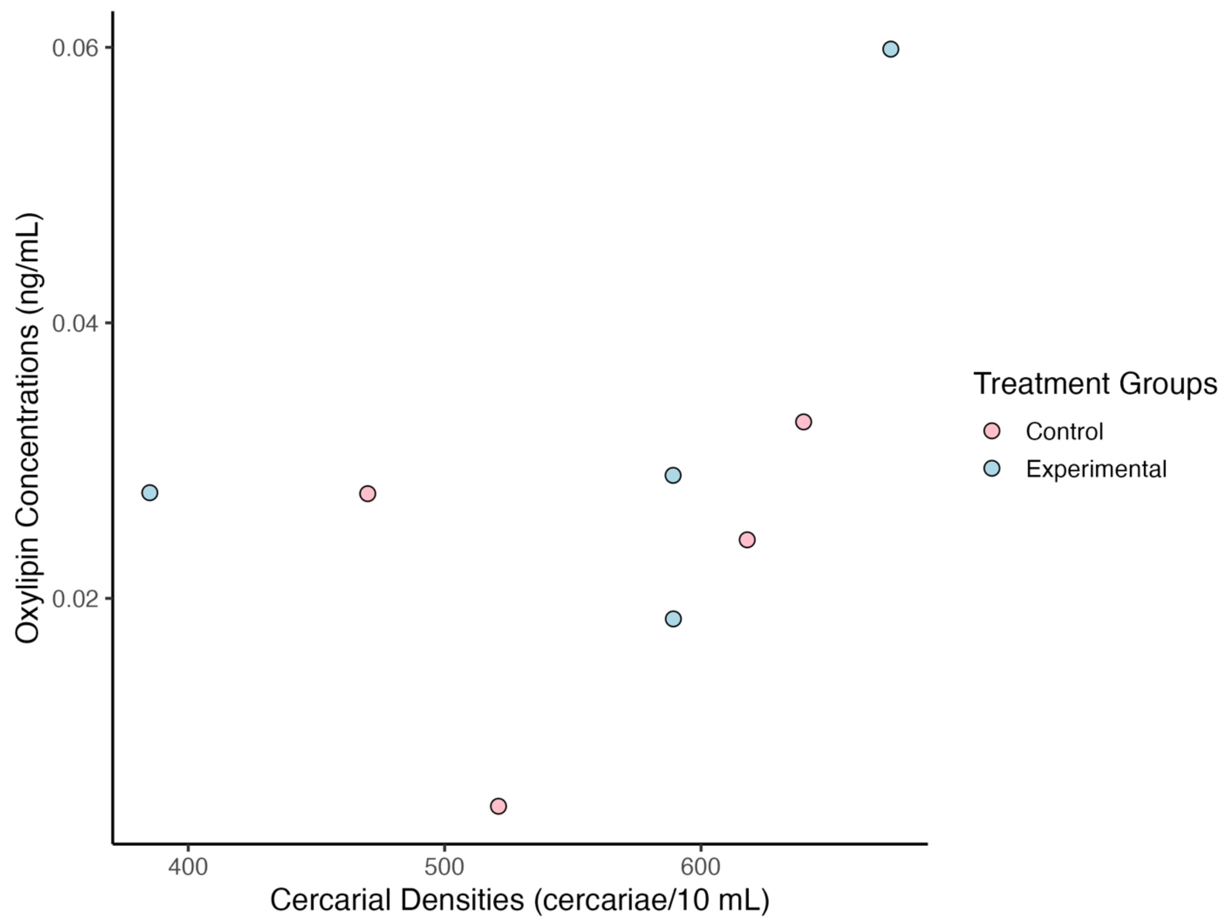

Figure 3.67. Concentration of 20-HETE (ng/mL) across cercarial densities (cercariae/10 mL).

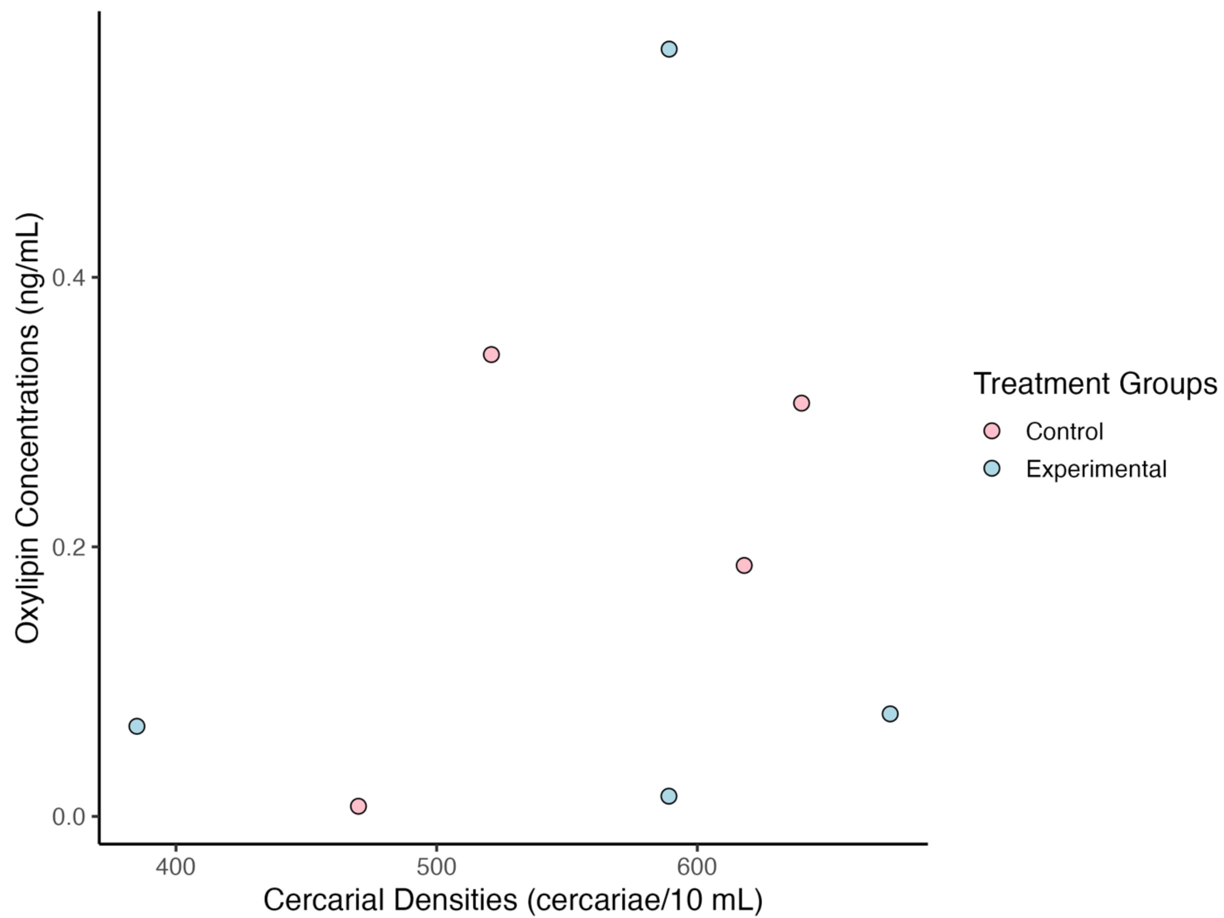

Figure 3.68. Concentration of 9,10-diHOME (ng/mL) across cercarial densities (cercariae/10 mL).

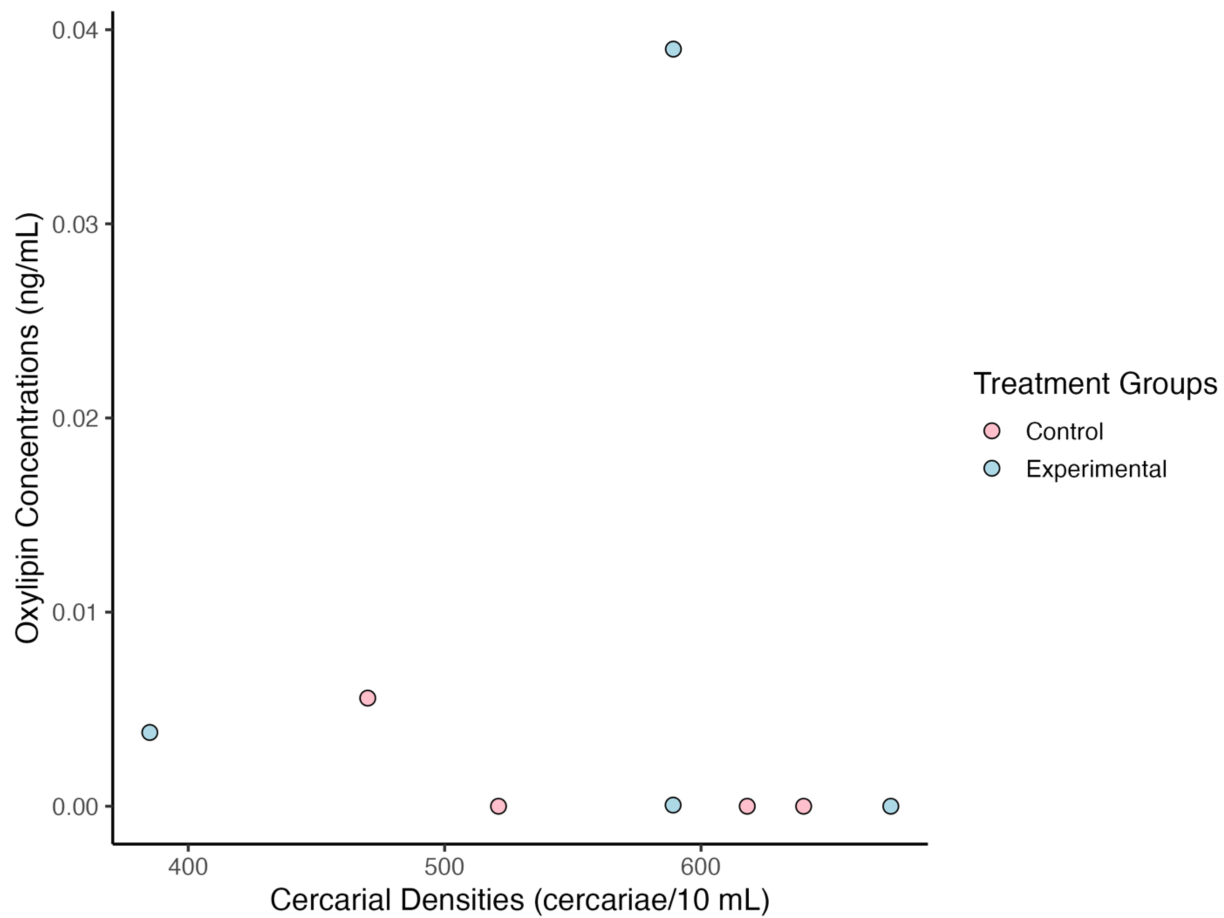

Figure 3.69. Concentration of 9,10-EpOME (ng/mL) across cercarial densities (cercariae/10 mL).

Table 3.1. Full list of oxylipins scanned for in samples using HPLC-MS/MS (n = 158 oxylipins).

Oxylipins
10-HDoHE
10-Nitrooleate
10S,17S-DiHDoHE (PDX)
11-HDoHE
11-HEPE
11-HETE
11,12 DiHETrE
11,12 EpETrE
11b PGE2
11bdhk PGF2a
11bPGF2a
11d-TXB2
12-HEPE
12-HETE
12-HHTrE
12-oxoETE
12,13 diHODE
12,13 diHOME
12,13 EpODE
12,13 EpOME
12epi LTB4
12oxo LTB4
13 oxoOTrE
13-HDoHE
13-HODE
13-HOTrE
13-HOTrE-y
13-oxoODE
14-HDoHE
14,15 diHETE
14,15 DiHETrE
14,15 EpETE
14,15 EpETrE
14,15-LTC4 (EXC4)
14,15-LTD4 (EXD4)
14,15-LTE4 (EXE4)
15-HEPE
15-HETE
15-HETrE
15-oxoEDE
15-oxoETE
15,16 diHODE
15d PGA2
15d PGD2
15d PGJ2
15k PGD2
15k PGE1
15k PGE2
15k PGF1a
15k PGF2a
15R-LXA4
15t PD1

16-HDoHE
16-HETE
16,17 DiHDoPE
16,17 EpDPE
17-HDoHE
17-HETE
17,18 diHETE
17,18 EpETE
17k DHA
17k DPA
18-HEPE
18-HETE
19-HETE
19,20 DiHDoPE
19,20 EpDPE
2,3-dinor 11b PGF2a
2,3-dinor 8-iso PGF2a
2,3-dinor TXB2
2,3-dinor-6k PGF1a
20-HDoHE
20-HETE
20cooh AA
20cooh LTB4
20oh LTB4
4-HDoHE
4k DHA
5-HEPE
5-HETE
5-HETrE
5-iso PGF2aVI
5-oxoETE
5,15 diHETE
5,6 diHETE
5,6 DiHETrE
5,6 EpETrE
6,15-dk-,dh-PGF1a
6k PGE1
6k PGF1a
6R-LXA4
6S-LXA4
6t LTB4
6t, 12epi LTB4
7-HDoHE
7R Maresin-1
8-HDoHE
8-HEPE
8-HETE
8-HETrE
8-iso 15k PGF2b
8-iso PGF2aIII
8-iso PGF3a
8,15 diHETE
8,9 DiHETrE
8,9 EpETrE
9 oxoOTrE
9-HEPE

9-HETE
9-HODE
9-HOTrE
9-Nitrooleate
9-oxoODE
9,10 diHOME
9,10 EpOME
9,10,13 triHOME
9,12,13 triHOME
bicyclo PGE2
d17 6k PGF1a
dh PGF2a
dhk PGD2
dhk PGE2
dhk PGF2a
dihomo 15d PGD2
dihomo PGD2
dihomo PGE2
dihomo PGF2a
dihomo PGJ2
HXA3
LTB4
LTC4
LTD4
LTE4
LXA5
LXB4
PD1
PGA2
PGB2
PGD1
PGD2
PGD3
PGE1
PGE2
PGE3
PGF1a
PGF2a
PGF3a
PGJ2
PGK1
PGK2
RvD1
RvD2
RvD5
RvE1
tetranor 12-HETE
TXB1
TXB2
TXB3

Table 3.2. Extended version of gas chromatography results of snail tissues from control and experimental groups. Peak area percentages are measured as the proportion of a fatty acid's chromatographic area in comparison to the total detected fatty acid peak area in a sample. Abbreviations: SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, SCFA = short-chain fatty acid, MCFA = medium-chain fatty acid, LCFA = long-chain fatty acid, n-3 = omega-3, n-6 = omega-6, DNS = de novo synthesis (synthesized by organism), DS = dietary sources (obtained from diet), and DNS/DS = de novo synthesis/dietary sources (not synthesized by organism, but can be synthesized from precursors obtained from diet).

			Area (%)			
			Head-Foot Area (%)		**Gonads Area (%)**	
Fatty Acid	**Fatty Acid Derivation**	**Fatty Acid Synthesis**	**Control (n = 1)**	**Experimental (n = 2; average)**	**Control (n = 1)**	**Experimental (n = 2; average)**
C4:0 (Butyric Acid)	SFA – SCFA	DNS	0	0	0	0
C6:0 (Caproic Acid)	SFA – MCFA	DNS	0	0	0	0
C8:0 (Caprylic Acid)	SFA – MCFA	DNS	0	0	0	0
C10:0 (Capric Acid)	SFA – MCFA	DNS	0	0	0	0
C12:0 (Lauric Acid)	SFA – MCFA	DNS	0	0	0	0
C14:0 (Myristic Acid)	SFA – LCFA	DNS	1.158	0.711	0.496	0.785
C14:1 (Myristoleic Acid)	MUFA – MCFA	DNS	0	0	0	0
C15:0 (Pentadecanoic Acid)	SFA – LCFA	DS	1.038	0.42	0.487	0.2005
C16:0 (Palmitic Acid)	SFA – LCFA	DNS	12.866	7.8125	11.469	6.7995
C16:1t (Trans-Palmitoleic Acid)	MUFA – LCFA	DS	0.643	0.145	0.234	0.179
C16:1 (Palmitoleic Acid)	MUFA – LCFA	DNS	0.777	0.3275	0.724	0.439
C17:0 (Heptadecanoic Acid)	SFA – LCFA	DS	0.812	0.467	0.544	0.4825
C17:1 (Heptadecenoic Acid)	MUFA – LCFA	DS	0.398	0.0915	0.166	0.1125

C18:0 (Stearic Acid)	SFA – LCFA	DNS	8.887	5.8635	5.961	6.274
C18:1 (Oleic Acid)	MUFA – LCFA	DNS	5.352	2.166	2.591	2.394
C18:1n7c (Vaccenic Acid)	MUFA – LCFA	DS	1.8	0.6815	1.544	0.893
C18:2 (Linoleic Acid)	n-6 PUFA – LCFA	DS	7.693	36.5095	10.492	35.1245
C18:3n6 (Gamma-Linolenic Acid)	n-6 PUFA – LCFA	DNS/DS	0.208	0.157	0.172	0.28
C18:3n3 (Alpha-Linolenic Acid)	n-3 PUFA – LCFA	DS	12.488	11.178	32.514	9.24
C20:0 (Arachidic Acid)	SFA – LCFA	DNS	0.102	0.116	0.167	0.103
C20:1 (Gondoic Acid)	MUFA – LCFA	DNS	0.675	0.243	0.294	0.269
C20:2 (Eicosadienoic Acid)	n-6 PUFA – LCFA	DNS/DS	9.585	13.146	9.269	14.4075
C20:3n6 (Dihomo-Gamma-Linolenic Acid)	n-6 PUFA – LCFA	DNS/DS	0.702	0.6335	0.666	0.726
C20:4 (Arachidonic Acid)	n-6 PUFA – LCFA	DNS/DS	21.13	12.983	8.843	13.383
C20:3n3 (Eicosatrienoic Acid)	n-3 PUFA – LCFA	DNS/DS	3.358	1.0765	3.84	1.191
C20:5 (Eicosapentaenoic Acid)	n-3 PUFA – LCFA	DNS/DS	3.72	1.465	3.598	2.128
C22:0 (Behenic Acid)	SFA – LCFA	DNS	0.081	0.0775	0.147	0.061
C22:1 (Erucic Acid)	MUFA – LCFA	DNS	0.055	0.026	0.051	0.0265
C22:2 (Docosadienoic Acid)	n-6 PUFA – LCFA	DNS/DS	0.062	0.104	0.07	0.119
C22:4 (Adrenic Acid)	n-6 PUFA – LCFA	DNS/DS	4.759	2.228	2.154	2.3965
C22:5n6 (Docosapentaenoic Acid)	n-6 PUFA – LCFA	DNS/DS	0.04	0.075	0.087	0.0825
C22:5n3 (Docosapentaenoic Acid)	n-3 PUFA – LCFA	DNS/DS	1.44	1.1155	3.102	1.7365

C22:6n3 (Docosahexaenoic Acid)	n-3 PUFA – LCFA	DNS/DS	0.064	0.063	0.106	0.0925
C24:0 (Lignoceric Acid)	SFA – LCFA	DNS	0.106	0.0875	0.133	0.0515
C24:1 (Nervonic Acid)	MUFA – LCFA	DNS	0.002	0.0305	0.079	0.023

*n refers to the number of samples

Table 3.3. Cercarial production across shedding weeks for control and experimental groups. For each week, the number of infected snails, pooled cercarial densities (mean ± SE), total cercariae summed across pools, and average number of cercariae per snail are reported.

Treatment Group	Shedding Week	Number of Infected Snails	Cercarial Density Pools (mean ± SE)	Sum of Cercariae Across Pools	Average Number of Cercariae per Snail
Control	9	4	Pool 1 = 521 ± 64	521	130
Experimental	9	4	Pool 1 = 946 ± 96; Pool 2 = 589 ± 70	1535	384
Control	10	4	Pool 1 = 618 ± 139	618	155
Experimental	10	4	Pool 1 = 975 ± 236; Pool 2 = 742 ± 214	1717	429
Control	11	3	Pool 1 = 470 ± 141	470	157
Experimental	11	3	Pool 1 = 884 ± 117; Pool 2 = 674 ± 205	1558	519
Control	13	3	Pool 1 = 640 ± 159	640	213
Experimental	13	3	Pool 1 = 839 ± 120; Pool 2 = 776 ± 144; Pool 3 = 589 ± 98	2204	735
Control	15	0	Pool 1 = 0 ± 0	0	0
Experimental	15	3	Pool 1 = 691 ± 77; Pool 2 = 385 ± 93	1076	359

Chapter 2 R Code

```
#Load and install libraries
library(dplyr)
library(ggplot2)
library(tidyr)
library(car)
library(purrr)
library(broom)
library(tidyverse)
library(vegan)
library(viridis)
library(ggforce)
library(lmtest)
library(MASS)
library(patchwork)
library(tibble)
library(ggrepel)

#Set working directory
setwd("/Users/joshitasehgal/Desktop")

#Set output directory
output_dir <- "/Users/joshitasehgal/Desktop/Chapter2Figures"
if (!dir.exists(output_dir)) dir.create(output_dir)

#Load data file
library(readr)
JS_Chapter2CercarialOxylipins <- read_csv("JS_Chapter2CercarialOxylipins.csv")
View(JS_Chapter2CercarialOxylipins)

#Notes on dataset
#1) Scannned total of 169 chemicals originally using HPLC with Tanja - see Joshita Results
March142025 file
#2) From 169 chemicals, omitted 7 precursors (ARA, ADA, EPA, DHA, ALA, LA, and gLA)
AND omitted 4 oxylipins (15,16 EpODE; 9,10 diHODE; 9,10 EpODE; HXB3 - these oxylipins
are ratios according to Tanja)
#3) Omit oxylipins quantified in only 1 sample (may need to omit oxylipins quantified in only 2
samples, but discuss with Jillian and Harold)
#4) Omit sample of cercarial density 544 (discussed replacing this sample and running another
sample instead of this sample as this sample detected fewer oxylipins than all other samples)
#Use Viridis for consistent colour palette (purples, blues, greens, and yellows)

#Define precursors and oxylipins to omit
precursors_to_omit <- c("ARA", "ADA", "EPA", "DHA", "ALA", "LA", "gLA")
oxylipins_to_omit <- c("15,16 EpODE", "9,10 diHODE", "9,10 EpODE", "HXB3")
```

```
#Define and sort treatment groups
control_densities <- sort(c(618, 640, 470, 521))
experimental_densities <- sort(c(589.1, 589.2, 674, 385))
ordered_density_levels <- c(control_densities, experimental_densities)

#Filter dataset and assign treatment group
filtered_data <- JS_Chapter2CercarialOxylipins %>%
filter(CercarialDensity %in% ordered_density_levels &
`OxylipinConcentration(ng)` > 0 &
!OxylipinName %in% c(precursors_to_omit, oxylipins_to_omit)) %>%
mutate(TreatmentGroup = ifelse(CercarialDensity %in% control_densities, "Control",
"Experimental"),
CercarialDensity = factor(CercarialDensity, levels = ordered_density_levels))

#Get oxylipin levels
oxylipin_levels <- levels(as.factor(filtered_data$OxylipinName))

#Tally oxylipins per cercarial density
tally_oxylipin_per_cercdensitysample <- filtered_data %>%
group_by(CercarialDensity) %>%
summarise(tally_oxylipin_per_cercdensitysample = n_distinct(OxylipinName)) %>%
ungroup() %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = ordered_density_levels),
CercarialDensity_numeric = as.numeric(CercarialDensity_factor))

#Tally detections per oxylipin
tally_oxylipin_detected <- filtered_data %>%
group_by(OxylipinName) %>%
summarise(tally_oxylipin_detected = n_distinct(CercarialDensity)) %>%
ungroup() %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels),
OxylipinName_numeric = as.numeric(OxylipinName_factor))

#View tallies
print(tally_oxylipin_per_cercdensitysample, n = Inf)
print(tally_oxylipin_detected, n = Inf)

#Create plot
Chapter2AllOxylipins <- ggplot(filtered_data, aes(x = CercarialDensity, y =
factor(OxylipinName, levels = oxylipin_levels), fill = TreatmentGroup)) +
geom_tile(color = "grey", width = 0.9, height = 0.9) +
scale_fill_manual(values = c("Control" = "lightpink", "Experimental" = "lightblue")) +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
```

```
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
legend.position = "right",
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +
geom_text(data = tally_oxylipin_per_cercdensitysample,
aes(x = CercarialDensity_factor, y = length(oxylipin_levels) + 0.5, label =
tally_oxylipin_per_cercdensitysample),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
geom_text(data = tally_oxylipin_detected,
aes(x = length(ordered_density_levels) + 0.5, y = OxylipinName_factor, label =
tally_oxylipin_detected),
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
expand_limits(y = length(oxylipin_levels) + 1, x = length(ordered_density_levels) + 1) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins",
fill = "Treatment Group")

#Display plot
print(Chapter2AllOxylipins)

#Define pathways
lox <- c("5-HETE", "8-HETE", "11-HETE", "12-HETE", "15-HETE",
"16-HETE", "20-HETE",
"9-HODE", "13-HODE", "13-oxoODE",
"9-HOTrE", "13-HOTrE", "13-HOTrE-y",
"10-HDoHE", "14-HDoHE", "16-HDoHE", "4-HDoHE",
"12-HEPE", "18-HEPE",
"15-HETrE", "15-oxoETE", "9 oxoOTrE",
"8-HETrE")

cox <- c("PGE2", "PGD1", "PGF3a", "2,3-dinor 8-iso PGF2a", "2,3-dinor TXB2",
"2,3-dinor-6k PGF1a", "TXB1", "TXB3", "dhk PGD2", "dhk PGE2",
"dhk PGF2a")

cyp <- c("12,13 EpODE", "9,10 EpODE", "15,16 EpODE",
"12,13 diHOME", "9,10 diHOME",
"9,10,13 triHOME", "9,12,13 triHOME",
"14,15 DiHETrE", "17,18 EpETE",
"19,20 EpDPE",
"9,10 EpOME",
"RvD2", "RvD5",
"10S,17S-DiHDoHE (PDX)",
"15,16 diHODE")
```

```
#Join pathway info into filtered_data
pathway_map <- tibble::tibble(OxylipinName = c(lox, cox, cyp),
Pathway = c(rep("LOX", length(lox)),
rep("COX", length(cox)),
rep("CYP", length(cyp))))

filtered_data <- filtered_data %>%
left_join(pathway_map, by = "OxylipinName")

make_pathway_plot <- function(data, pathway_name, tag_label) {
data_sub <- data %>% filter(Pathway == pathway_name)
oxylipins <- unique(data_sub$OxylipinName)

#Tallies per density (top)
tally_x <- data_sub %>%
group_by(CercarialDensity) %>%
summarise(tally = n_distinct(OxylipinName), .groups = "drop") %>%
mutate(CercarialDensity_factor = factor(CercarialDensity,
levels = ordered_density_levels))

#Tallies per oxylipin (right)
tally_y <- data_sub %>%
group_by(OxylipinName) %>%
summarise(tally = n_distinct(CercarialDensity), .groups = "drop") %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipins))
n_x <- length(ordered_density_levels)
n_y <- length(oxylipins)
ggplot(data_sub,
aes(x = CercarialDensity,
y = factor(OxylipinName, levels = oxylipins),
fill = TreatmentGroup)) +
geom_tile(color = "grey90", width = 0.9, height = 0.9) +
scale_fill_manual(values = c(Control = "lightpink",
Experimental = "lightblue")) +
geom_text(data = tally_x,
aes(x = CercarialDensity_factor,
y = n_y + 0.5,
label = tally),
vjust = 0,
size = 3.5,
inherit.aes = FALSE) +
geom_text(data = tally_y,
aes(x = n_x + 0.5,
y = OxylipinName_factor,
label = tally),
```

```
hjust = 0,
size = 3.5,
inherit.aes = FALSE) +
expand_limits(y = n_y + 1, x = n_x + 1) +
coord_cartesian(clip = "off") +
theme_minimal(base_size = 13) +
theme(axis.text.x  = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 13),
axis.title.x = element_text(size = 13),
axis.text.y  = element_text(size = 11),
axis.ticks   = element_blank(),
panel.grid   = element_blank(),
plot.margin  = margin(t = 28, r = 55, b = 10, l = 10),
plot.title   = element_text(face = "bold", size = 15),
legend.position = "right") +
labs(x     = "Cercarial Densities (cercariae/10 mL)",
y     = "Oxylipins",
fill  = "Treatment Group",
title = tag_label)}

#Create plots
plot_A <- make_pathway_plot(filtered_data, "LOX", "A")
plot_B <- make_pathway_plot(filtered_data, "COX", "B")
plot_C <- make_pathway_plot(filtered_data, "CYP", "C")

#Combine plots horizontally
Chapter2AllOxylipinsPanel <- plot_A | plot_B | plot_C + plot_layout(nrow = 1)

#Display plot
print(Chapter2AllOxylipinsPanel)

#Define and sort treatment groups
control_densities <- sort(c(618, 640, 470, 521))
experimental_densities <- sort(c(589.1, 589.2, 674, 385))
ordered_density_levels <- c(control_densities, experimental_densities)

#Filter oxylipins quantified in at least 6 samples
oxylipins_to_keep <- tally_oxylipin_detected %>%
filter(tally_oxylipin_detected >= 6) %>%
pull(OxylipinName)

#Filter original data
filtered_data_Chapter2SelectOxylipins <- filtered_data %>%
filter(OxylipinName %in% oxylipins_to_keep) %>%
mutate(TreatmentGroup = ifelse(CercarialDensity %in% control_densities, "Control",
"Experimental"),
```

```
CercarialDensity = factor(CercarialDensity, levels = ordered_density_levels))

#Update factor levels
oxylipin_levels_6 <- levels(as.factor(filtered_data_Chapter2SelectOxylipins$OxylipinName))
density_levels_6 <- ordered_density_levels

#Tally oxylipins per cercarial density
tally_oxylipin_per_cercdensitysample_6 <- filtered_data_Chapter2SelectOxylipins %>%
group_by(CercarialDensity) %>%
summarise(tally_oxylipin_per_cercdensitysample_6 = n_distinct(OxylipinName)) %>%
ungroup() %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = density_levels_6))

#Tally detections per oxylipin
tally_oxylipin_detected_6 <- filtered_data_Chapter2SelectOxylipins %>%
group_by(OxylipinName) %>%
summarise(tally_oxylipin_detected_6 = n_distinct(CercarialDensity)) %>%
ungroup() %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels_6))

#Create plot
Chapter2SelectOxylipins <- ggplot(
filtered_data_Chapter2SelectOxylipins,
aes(x = factor(CercarialDensity, levels = density_levels_6),
y = factor(OxylipinName, levels = oxylipin_levels_6),
fill = TreatmentGroup)) +
geom_tile(color = "grey", width = 0.9, height = 0.9) +
scale_fill_manual(values = c("Control" = "lightpink", "Experimental" = "lightblue")) +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
legend.position = "right",
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +
geom_text(data = tally_oxylipin_per_cercdensitysample_6,
aes(x = CercarialDensity_factor,
y = length(oxylipins_to_keep) + 0.5,
label = tally_oxylipin_per_cercdensitysample_6),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
geom_text(data = tally_oxylipin_detected_6,
aes(x = length(density_levels_6) + 0.5,
```

```
y = OxylipinName_factor,
label = tally_oxylipin_detected_6),
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
expand_limits(y = length(oxylipins_to_keep) + 1,
x = length(density_levels_6) + 1) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins",
fill = "Treatment Group")

#Display plot
print(Chapter2SelectOxylipins)

#Define 22 oxylipins to keep
oxylipins_to_keep <- c("11-HETE", "12,13 EpODE", "12,13 diHOME", "12-HEPE",
"13-HOTrE", "15-HETE", "15-oxoETE", "16-HETE",
"2,3-dinor TXB2", "20-HETE", "5-HETE", "8-HETE",
"9 oxoOTrE", "9,10 diHOME", "9,10,13 triHOME", "9,12,13 triHOME",
"9-HODE", "9-HOTrE", "PGD1", "RvD5", "TXB1", "TXB3")

#Define pathway groups
lox <- c("5-HETE", "8-HETE", "11-HETE", "12-HEPE", "13-HOTrE", "15-HETE",
"15-oxoETE", "16-HETE", "20-HETE", "9 oxoOTrE", "9-HODE", "9-HOTrE")

cox <- c("PGD1", "2,3-dinor TXB2", "TXB1", "TXB3")

cyp <- c("12,13 EpODE", "12,13 diHOME", "9,10 diHOME", "9,10,13 triHOME",
"9,12,13 triHOME", "RvD5")

#Create mapping tibble
pathway_map <- tibble(
OxylipinName = c(lox, cox, cyp),
Pathway = c(rep("LOX", length(lox)),
rep("COX", length(cox)),
rep("CYP", length(cyp))))

#Filter and join with pathway info
filtered_data <- filtered_data %>%
filter(OxylipinName %in% oxylipins_to_keep) %>%
left_join(pathway_map, by = "OxylipinName") %>%
mutate(Pathway = coalesce(Pathway.x, Pathway.y)) %>%
dplyr::select(., !starts_with("Pathway."))

#Define plot-making function
make_pathway_plot <- function(data, pathway_name, tag_label) {
data_sub <- data %>% filter(Pathway == pathway_name)
oxylipins <- unique(data_sub$OxylipinName)
```

```
#Tallies per density (top)
tally_x <- data_sub %>%
group_by(CercarialDensity) %>%
summarise(tally = n_distinct(OxylipinName), .groups = "drop") %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = ordered_density_levels))

#Tallies per oxylipin (right)
tally_y <- data_sub %>%
group_by(OxylipinName) %>%
summarise(tally = n_distinct(CercarialDensity), .groups = "drop") %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipins))

n_x <- length(ordered_density_levels)
n_y <- length(oxylipins)

ggplot(data_sub,
aes(x = CercarialDensity,
y = factor(OxylipinName, levels = oxylipins),
fill = TreatmentGroup)) +
geom_tile(color = "grey90", width = 0.9, height = 0.9) +
scale_fill_manual(values = c(Control = "lightpink", Experimental = "lightblue")) +
geom_text(data = tally_x,
aes(x = CercarialDensity_factor,
y = n_y + 0.5,
label = tally),
vjust = 0,
size = 3.5,
inherit.aes = FALSE) +
geom_text(data = tally_y,
aes(x = n_x + 0.5,
y = OxylipinName_factor,
label = tally),
hjust = 0,
size = 3.5,
inherit.aes = FALSE) +
expand_limits(y = n_y + 1, x = n_x + 1) +
coord_cartesian(clip = "off") +
theme_minimal(base_size = 13) +
theme(axis.text.x  = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 13),
axis.title.x = element_text(size = 13),
axis.text.y  = element_text(size = 11),
axis.ticks   = element_blank(),
panel.grid   = element_blank(),
plot.margin  = margin(t = 28, r = 55, b = 10, l = 10),
```

```
plot.title   = element_text(face = "bold", size = 15),
legend.position = "right") +
labs(x     = "Cercarial Densities (cercariae/10 mL)",
y     = "Oxylipins",
fill  = "Treatment Group",
title = tag_label)}

#Create plots
plot_A <- make_pathway_plot(filtered_data, "LOX", "A")
plot_B <- make_pathway_plot(filtered_data, "COX", "B")
plot_C <- make_pathway_plot(filtered_data, "CYP", "C")

#Combine plots horizontally
Chapter2SelectOxylipinsPanel <- plot_A | plot_B | plot_C + plot_layout(nrow = 1)

#Display plot
print(Chapter2SelectOxylipinsPanel)

#Define control and experimental densities
control_densities <- sort(c(618, 640, 470, 521))
experimental_densities <- sort(c(589.1, 589.2, 674, 385))

#Filter data for control and experimental groups
filtered_control <- filtered_data %>% filter(CercarialDensity %in% control_densities)
filtered_experimental <- filtered_data %>% filter(CercarialDensity %in%
experimental_densities)

#Oxylipins quantified at least 3 times in control group
oxylipins_control_3plus <- filtered_control %>%
group_by(OxylipinName) %>%
summarise(tally_control = n_distinct(CercarialDensity)) %>%
filter(tally_control >= 3) %>%
pull(OxylipinName)

filtered_control_3plus <- filtered_control %>%
filter(OxylipinName %in% oxylipins_control_3plus)

#Levels for plotting
oxylipin_levels_control <- levels(factor(filtered_control_3plus$OxylipinName))
density_levels_control <- levels(factor(filtered_control_3plus$CercarialDensity))

#Tally control oxylipins per density sample
tally_per_density_control <- filtered_control_3plus %>%
group_by(CercarialDensity) %>%
summarise(tally_per_density = n_distinct(OxylipinName)) %>%
ungroup() %>%
```

```
mutate(CercarialDensity_factor = factor(CercarialDensity, levels = density_levels_control))

#Tally control oxylipins quantified across densities
tally_detected_control <- filtered_control_3plus %>%
group_by(OxylipinName) %>%
summarise(tally_detected = n_distinct(CercarialDensity)) %>%
ungroup() %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels_control))

#Create plot
Chapter2ControlOxylipins <- ggplot(filtered_control_3plus, aes(
x = factor(CercarialDensity, levels = density_levels_control),
y = factor(OxylipinName, levels = oxylipin_levels_control))) +
geom_tile(fill = "lightpink", color = "grey", width = 0.9, height = 0.9) +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +
geom_text(data = tally_per_density_control,
aes(x = CercarialDensity_factor, y = length(oxylipins_control_3plus) + 0.5, label =
tally_per_density),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
geom_text(data = tally_detected_control,
aes(y = OxylipinName_factor, label = tally_detected),
x = length(density_levels_control) + 0.5,
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
expand_limits(y = length(oxylipins_control_3plus) + 1, x = length(density_levels_control) + 1)
+
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins")

#Display plot
print(Chapter2ControlOxylipins)

#Oxylipins quantified at least 3 times in experimental group
oxylipins_experimental_3plus <- filtered_experimental %>%
group_by(OxylipinName) %>%
summarise(tally_experimental = n_distinct(CercarialDensity), .groups = "drop") %>%
filter(tally_experimental >= 3) %>%
pull(OxylipinName)
```

```
filtered_experimental_3plus <- filtered_experimental %>%
filter(OxylipinName %in% oxylipins_experimental_3plus)

#Levels for plotting
oxylipin_levels_experimental <- levels(factor(filtered_experimental_3plus$OxylipinName))
density_levels_experimental <- levels(factor(filtered_experimental_3plus$CercarialDensity))

#Tally experimental oxylipins per density sample
tally_per_density_experimental <- filtered_experimental_3plus %>%
group_by(CercarialDensity) %>%
summarise(tally_per_density = n_distinct(OxylipinName)) %>%
ungroup() %>%
mutate(CercarialDensity_factor = factor(CercarialDensity, levels =
density_levels_experimental))

#Tally experimental oxylipins quantified across densities
tally_detected_experimental <- filtered_experimental_3plus %>%
group_by(OxylipinName) %>%
summarise(tally_detected = n_distinct(CercarialDensity)) %>%
ungroup() %>%
mutate(OxylipinName_factor = factor(OxylipinName, levels = oxylipin_levels_experimental))

#Create plot
Chapter2ExperimentalOxylipins <- ggplot(filtered_experimental_3plus, aes(
x = factor(CercarialDensity, levels = density_levels_experimental),
y = factor(OxylipinName, levels = oxylipin_levels_experimental))) +
geom_tile(fill = "lightblue", color = "grey", width = 0.9, height = 0.9) +
theme_minimal(base_size = 15) +
theme(axis.text.x = element_text(angle = 45, hjust = 1),
axis.title.y = element_text(size = 15),
axis.title.x = element_text(size = 15),
axis.ticks = element_blank(),
panel.grid = element_blank(),
panel.border = element_blank(),
plot.background = element_rect(fill = "white", color = "white"),
panel.background = element_rect(fill = "white", color = "white"),
plot.margin = margin(t = 30, r = 40, b = 10, l = 10)) +
geom_text(data = tally_per_density_experimental,
aes(x = CercarialDensity_factor, y = length(oxylipins_experimental_3plus) + 0.5, label =
tally_per_density),
vjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
geom_text(data = tally_detected_experimental,
aes(y = OxylipinName_factor, label = tally_detected),
x = length(density_levels_experimental) + 0.5,
hjust = 0, size = 4, color = "black", inherit.aes = FALSE) +
```

```
expand_limits(y = length(oxylipins_experimental_3plus) + 1, x =
length(density_levels_experimental) + 1) +
labs(x = "Cercarial Densities (cercariae/10 mL)",
y = "Oxylipins")

#Display plot
print(Chapter2ExperimentalOxylipins)

#Define vectors
precursors_to_omit <- c("ARA", "ADA", "EPA", "DHA", "ALA", "LA", "gLA")
oxylipins_to_omit <- c("15,16 EpODE", "9,10 diHODE", "9,10 EpODE", "HXB3")

lox <- c("5-HETE", "8-HETE", "11-HETE", "12-HETE", "15-HETE",
"16-HETE", "20-HETE",
"9-HODE", "13-HODE", "13-oxoODE",
"9-HOTrE", "13-HOTrE", "13-HOTrE-y",
"10-HDoHE", "14-HDoHE", "16-HDoHE", "4-HDoHE",
"12-HEPE", "18-HEPE",
"15-HETrE", "15-oxoETE", "9 oxoOTrE",
"8-HETrE")

cox <- c("PGE2", "PGD1", "PGF3a",
"2,3-dinor 8-iso PGF2a", "2,3-dinor TXB2", "2,3-dinor-6k PGF1a",
"TXB1", "TXB3",
"dhk PGD2", "dhk PGE2", "dhk PGF2a")

cyp <- c("12,13 EpODE",
"12,13 diHOME", "9,10 diHOME",
"9,10,13 triHOME", "9,12,13 triHOME",
"14,15 DiHETrE", "17,18 EpETE",
"19,20 EpDPE",
"9,10 EpOME",
"RvD2", "RvD5",
"10S,17S-DiHDoHE (PDX)",
"15,16 diHODE")

all_oxylipins <- c(lox, cox, cyp)

control_densities <- sort(c(618, 640, 470, 521))
experimental_densities <- sort(c(589.1, 589.2, 674, 385))
ordered_density_levels <- c(control_densities, experimental_densities)

#Filter treatment groups
filtered_data <- JS_Chapter2CercarialOxylipins %>%
filter(CercarialDensity %in% ordered_density_levels,
`OxylipinConcentration(ng)` > 0,
```

```
!OxylipinName %in% c(precursors_to_omit, oxylipins_to_omit),
OxylipinName %in% all_oxylipins) %>%
mutate(TreatmentGroup = ifelse(CercarialDensity %in% control_densities, "Control",
"Experimental"),
CercarialDensity = factor(CercarialDensity, levels = ordered_density_levels))

#Count detections for oxylipins and treatment groups
detection_summary <- filtered_data %>%
group_by(OxylipinName, TreatmentGroup) %>%
summarise(n_detected = n_distinct(CercarialDensity), .groups = "drop") %>%
complete(OxylipinName = all_oxylipins,
TreatmentGroup = c("Control", "Experimental"),
fill = list(n_detected = 0)) %>%
pivot_wider(names_from = TreatmentGroup, values_from = n_detected) %>%
mutate(chi_square_p = purrr::map2_dbl(Control, Experimental, function(ctrl, exp) {
mat <- matrix(c(ctrl, exp), nrow = 2)
if (sum(mat) > 0) {
suppressWarnings(chisq.test(mat)$p.value)
} else {
NA_real_
}}))

#Print tibble with detection counts per group for all oxylipins of interest
print(detection_summary, n = Inf)

#Extract oxylipins quantified 3 or more times only in control
oxylipins_control_only <- detection_summary %>%
filter(Control >= 3, Experimental < 3) %>%
pull(OxylipinName)

#Extract oxylipins quantified 3 or more times only in experimental
oxylipins_experimental_only <- detection_summary %>%
filter(Experimental >= 3, Control < 3) %>%
pull(OxylipinName)

#Extract oxylipins quantified 3 or more times in both control and experimental
oxylipins_both <- detection_summary %>%
filter(Control >= 3, Experimental >= 3) %>%
pull(OxylipinName)

#View results
list(Control_Only = oxylipins_control_only,
Experimental_Only = oxylipins_experimental_only,
Both = oxylipins_both)

#Combine all oxylipins quantified 3 or more times in control and/or experimental
```

```
oxylipins_to_test <- unique(c(
detection_summary %>% filter(Control >= 3) %>% pull(OxylipinName),
detection_summary %>% filter(Experimental >= 3) %>% pull(OxylipinName)))

#View combined list
print(oxylipins_to_test)

#Prepare results tibble to store values
mw_test_results <- tibble(
OxylipinName = character(),
mean_control = numeric(),
mean_experimental = numeric(),
p_value = numeric(),
significant = logical())
for (ox in oxylipins_to_test) {
data_sub <- filtered_data %>% filter(OxylipinName == ox)

#Extract concentrations for each group
control_values <- data_sub %>% filter(TreatmentGroup == "Control") %>%
pull(`OxylipinConcentration(ng)`)
experimental_values <- data_sub %>% filter(TreatmentGroup == "Experimental") %>%
pull(`OxylipinConcentration(ng)`)
mean_ctrl <- if(length(control_values) > 0) mean(control_values) else NA_real_
mean_exp <- if(length(experimental_values) > 0) mean(experimental_values) else NA_real_

#Run Mann-Whitney U or Wilcoxon rank sum test
if (length(control_values) > 0 & length(experimental_values) > 0) {
test_res <- wilcox.test(control_values, experimental_values, exact = FALSE)
p_val <- test_res$p.value
} else {
p_val <- NA}

#Display results
mw_test_results <- mw_test_results %>% add_row(
OxylipinName = ox,
mean_control = mean_ctrl,
mean_experimental = mean_exp,
p_value = p_val,
significant = ifelse(!is.na(p_val) & p_val < 0.05, TRUE, FALSE))}

#Merge Mann-Whitney and Chi-square results
final_results <- mw_test_results %>%
left_join(detection_summary %>% dplyr::select(OxylipinName, chi_square_p), by =
"OxylipinName")

#Print tibble
```

```
print(mw_test_results, n = Inf)

#Export tibble
print(final_results, n = Inf)
write.csv(final_results, "Mann-Whitney U Test Results.csv", row.names = FALSE)

#Load data file
library(readr)
JS_Chapter2CercarialOxylipinsNMDS <-
read_csv("JS_Chapter2CercarialOxylipinsNMDS.csv")
View(JS_Chapter2CercarialOxylipinsNMDS)

#Extract oxylipins matrix
oxylipin_matrix <- JS_Chapter2CercarialOxylipinsNMDS[, 3:171]

#Run NMDS using Bray-Curtis distance
nmds_result <- metaMDS(oxylipin_matrix, distance = "bray", k = 2, trymax = 100,
autotransform = FALSE)

#Check stress value
nmds_result$stress

#Extract site scores
scores_df <- as.data.frame(scores(nmds_result)$sites)
scores_df$CercarialDensity <- JS_Chapter2CercarialOxylipinsNMDS$CercarialDensity
scores_df$TreatmentGroup <- JS_Chapter2CercarialOxylipinsNMDS$TreatmentGroup

#Create plot
Chapter2CercariaeOxylipinsNMDS <- ggplot(scores_df, aes(NMDS1, NMDS2, label =
CercarialDensity)) +
geom_point(aes(fill = TreatmentGroup),
shape = 21,
color = "black",
size = 4,
stroke = 0.8) +
geom_text_repel(size = 4.5) +
theme_minimal() +
labs(x = "NMDS1",
y = "NMDS2",
fill = "Treatment Group") +
scale_fill_manual(values = c("Control" = "pink", "Experimental" = "lightblue")) +
theme(panel.grid = element_blank(),
panel.border = element_rect(color = "black", fill = NA, linewidth = 1),
axis.ticks = element_line(color = "black"),
axis.ticks.length = unit(0.2, "cm"),
axis.ticks.x.top = element_blank(),
```

```
axis.ticks.y.right = element_blank(),
axis.title = element_text(size = 15, color = "black"),
axis.text = element_text(size = 14, color = "black"),
legend.title = element_text(size = 14),
legend.text = element_text(size = 13),
plot.background = element_rect(fill = "white", color = NA))

#Load data
data <- read.csv("JS_Chapter2CercarialOxylipinsNMDS.csv")

#Extract oxylipin data
oxylipins <- data[, 3:171]

#Ensure TreatmentGroup is a factor
data$TreatmentGroup <- as.factor(data$TreatmentGroup)

#Calculate Bray-Curtis dissimilarity
dist_matrix <- vegdist(oxylipins, method = "bray")

#Run PERMANOVA to test if TreatmentGroup affects oxylipin profile
permanova_result <- adonis2(dist_matrix ~ TreatmentGroup, data = data)

#Print results
print(permanova_result)

#Load data file
library(readr)
JS_Chapter1and2CercariaeReproduction <-
read_csv("JS_Chapter1and2CercariaeReproduction.csv")
View(JS_Chapter1and2CercariaeReproduction)

#Filter for chapter 2
chapter2_data <- JS_Chapter1and2CercariaeReproduction %>%
filter(ChapterUse == 2) %>%
mutate(TreatmentGroup = dplyr::recode(as.character(TreatmentGroup),
"ControlGroup" = "Control",
"ExperimentalGroup" = "Experimental"))

#Create plot
Chapter2AverageNumberofCercariaeShedLine <- ggplot(chapter2_data, aes(x = SheddingWeek,
y = AverageCercariaeShedPerSnail, fill = TreatmentGroup)) +
geom_point(shape = 21,
size = 4,
stroke = 0.8,
color = "black",
alpha = 0.7) +
```

```
geom_smooth(aes(color = TreatmentGroup), method = "loess", se = FALSE) +
labs(x = "Shedding Week",
y = "Average Number of Cercariae",
fill = "Treatment Group",
color = "Treatment Group") +
scale_fill_manual(values = c("Control" = "pink", "Experimental" = "lightblue")) +
scale_color_manual(values = c("Control" = "pink", "Experimental" = "lightblue")) +
theme_minimal() +
theme(panel.grid = element_blank(),
panel.border = element_rect(color = "black", fill = NA, linewidth = 1),
axis.ticks = element_line(color = "black"),
axis.ticks.length = unit(0.2, "cm"),
axis.ticks.x.top = element_blank(),
axis.ticks.y.right = element_blank(),
legend.title = element_text(size = 14),
legend.text = element_text(size = 13),
axis.title = element_text(size = 15, color = "black"),
axis.text = element_text(size = 14, color = "black"),
plot.background = element_rect(fill = "white", color = NA))

#Create plot
Chapter2AverageNumberofCercariaeShedScatter <- ggplot(chapter2_data, aes(
x = SheddingWeek,
y = AverageCercariaeShedPerSnail,
fill = TreatmentGroup)) +
geom_point(shape = 21,
size = 4,
stroke = 0.8,
color = "black",
alpha = 0.7) +
labs(x = "Shedding Week",
y = "Average Number of Cercariae",
fill = "Treatment Group") +
scale_fill_manual(values = c("Control" = "pink", "Experimental" = "lightblue")) +
theme_minimal() +
theme(panel.grid = element_blank(),
panel.border = element_rect(color = "black", fill = NA, linewidth = 1),
axis.ticks = element_line(color = "black"),
axis.ticks.length = unit(0.2, "cm"),
axis.ticks.x.top = element_blank(),
axis.ticks.y.right = element_blank(),
legend.title = element_text(size = 14),
legend.text = element_text(size = 13),
axis.title = element_text(size = 15, color = "black"),
axis.text = element_text(size = 14, color = "black"),
plot.background = element_rect(fill = "white", color = NA))
```

```
#Summarize mean cercariae shed
summary_data <- chapter2_data %>%
group_by(SheddingWeek, TreatmentGroup) %>%
summarise(mean_cerc = mean(AverageCercariaeShedPerSnail, na.rm = TRUE),
.groups = "drop")

#Create plot
Chapter2AverageNumberofCercariaeShedBar <- ggplot(summary_data,
aes(x = factor(SheddingWeek), y = mean_cerc, fill = TreatmentGroup)) +
geom_col(position = position_dodge(0.8), width = 0.7, color = "black") +
labs(x = "Shedding Week",
y = "Average Number of Cercariae",
fill = "Treatment Group") +
scale_fill_manual(values = c("Control" = "pink", "Experimental" = "lightblue")) +
theme_minimal() +
theme(panel.grid = element_blank(),
panel.border = element_rect(color = "black", fill = NA, linewidth = 1),
axis.ticks = element_line(color = "black"),
axis.ticks.length = unit(0.2, "cm"),
axis.ticks.x.top = element_blank(),
axis.ticks.y.right = element_blank(),
legend.title = element_text(size = 14),
legend.text = element_text(size = 13),
axis.title = element_text(size = 15, color = "black"),
axis.text = element_text(size = 14, color = "black"),
plot.background = element_rect(fill = "white", color = NA))

#Convert oxylipin lists into vectors
control_oxylipins <- c("12-HEPE", "12-HETE", "12,13 EpODE", "13-HODE", "14,15
DiHETrE", "dhk PGF2a")
experimental_oxylipins <- c("10S,17S-DiHDoHE (PDX)", "2,3-dinor-6k PGF1a", "9,10
EpOME")
both_oxylipins <- c("11-HETE", "12,13 diHOME", "13-HOTrE", "15-HETE", "15-oxoETE",
"16-HETE",
"2,3-dinor TXB2", "20-HETE", "5-HETE", "8-HETE", "9 oxoOTrE", "9-HODE",
"9-HOTrE", "9,10 diHOME", "9,10,13 triHOME", "9,12,13 triHOME",
"PGD1", "RvD5", "TXB1", "TXB3")

#Define oxylipins and densities
control_oxylipins <- c("12-HEPE", "12-HETE", "12,13 EpODE", "13-HODE", "14,15
DiHETrE", "dhk PGF2a")
experimental_oxylipins <- c("10S,17S-DiHDoHE (PDX)", "2,3-dinor-6k PGF1a", "9,10
EpOME")
both_oxylipins <- c("11-HETE", "12,13 diHOME", "13-HOTrE", "15-HETE", "15-oxoETE",
"16-HETE", "2,3-dinor TXB2", "20-HETE", "5-HETE", "8-HETE",
```

```
"9 oxoOTrE", "9-HODE", "9-HOTrE", "9,10 diHOME",
"9,10,13 triHOME", "9,12,13 triHOME", "PGD1", "RvD5", "TXB1", "TXB3")

control_densities <- sort(c(618, 640, 470, 521))
experimental_densities <- sort(c(589.1, 589.2, 674, 385))

#Create plots
plot_oxylipins_by_density <- function(data, oxylipins, densities, treatment_group, label) {
for (ox in oxylipins) {
plot_data <- data %>%
filter(OxylipinName == ox,
TreatmentGroup == treatment_group,
CercarialDensity %in% densities)

p <- ggplot(plot_data, aes(x = as.factor(CercarialDensity), y = `OxylipinConcentration(ng)`)) +
geom_jitter(width = 0.2, size = 2.5, alpha = 0.8, color = "black") +
labs(title = paste0(ox, " - ", treatment_group, " Group"),
x = "Cercarial Density (cercariae/10 mL)",
y = "Oxylipin Concentration (ng)") +
theme_minimal(base_size = 13) +
theme(plot.background = element_rect(fill = "white", color = NA),
panel.background = element_rect(fill = "white", color = NA))
filename <- paste0(label, "_", gsub("[ /(),]", "_", ox), ".png")
ggsave(filename = file.path(output_dir, filename), plot = p, width = 9, height = 6, dpi = 300, bg =
"white")}}

#Save plots
ggsave(filename = file.path(output_dir, "Chapter2AllOxylipins.png"), plot =
Chapter2AllOxylipins, width = 12, height = 12, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2AllOxylipinsPanel.png"), plot =
Chapter2AllOxylipinsPanel, width = 34, height = 12, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2SelectOxylipins.png"), plot =
Chapter2SelectOxylipins, width = 8, height = 8, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2SelectOxylipinsPanel.png"), plot =
Chapter2SelectOxylipinsPanel, width = 34, height = 12, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2ControlOxylipins.png"), plot =
Chapter2ControlOxylipins, width = 8, height = 7, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2ExperimentalOxylipins.png"), plot =
Chapter2ExperimentalOxylipins, width = 8, height = 7, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2CercariaeOxylipinsNMDS.png"), plot =
Chapter2CercariaeOxylipinsNMDS, width = 10, height = 6, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2AverageNumberofCercariaeShedLine.png"),
plot = Chapter2AverageNumberofCercariaeShedLine, width = 9, height = 6, dpi = 300)
ggsave(filename = file.path(output_dir, "Chapter2AverageNumberofCercariaeShedScatter.png"),
plot = Chapter2AverageNumberofCercariaeShedScatter, width = 9, height = 6, dpi = 300)
```

```
ggsave(filename = file.path(output_dir, "Chapter2AverageNumberofCercariaeShedBar.png"),
plot = Chapter2AverageNumberofCercariaeShedBar, width = 9, height = 6, dpi = 300)
plot_oxylipins_by_density(filtered_data, control_oxylipins, control_densities, "Control",
"Chapter2ControlOnly")
plot_oxylipins_by_density(filtered_data, experimental_oxylipins, experimental_densities,
"Experimental", "Chapter2ExperimentalOnly")
plot_oxylipins_by_density(filtered_data, both_oxylipins, control_densities, "Control",
"Chapter2ControlBoth")
plot_oxylipins_by_density(filtered_data, both_oxylipins, experimental_densities,
"Experimental", "Chapter2ExperimentalBoth")
```