CUTICULAR HYDROCARBONS AND ECOLOGICAL SPECIATION IN *DROSOPHILA LUMMEI*

By

Saniya Rose Henderson

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ABSTRACT

Ecological speciation occurs when populations diverge due to adaptation to different environmental conditions, leading to reproductive isolation. Cuticular hydrocarbons (CHCs) serve dual roles in desiccation resistance and mate choice, making them key traits to investigate in ecological divergence. This thesis examines CHC variation between two populations of *Drosophila lummei* from Japan and Russia to investigate their role in ecological speciation. Our results showed significant differences in CHC composition between the populations, with Japan exhibiting a greater diversity of CHCs. Desiccation resistance assays showed that the Japan population had higher desiccation resistance compared to the Russia population. However, reciprocal crosses between these populations did not show any significant differences in desiccation resistance, suggesting that the genetic factors effecting desiccation resistance is not sex-linked. Male mate choice assays showed no preference for females of either population, suggesting that CHCs may not influence male mate choice in this species. Additionally, reproductive assays showed that offsprings counts varied across temperature conditions, with Russia *D. lummei* producing fewer offspring than Japan *D. lummei* and hybrid crosses. These findings suggest that the CHC variation in *D. lummei* may be shaped by ecological factors but do not affect mate choice based on our limited experiments. Future research will include incorporating female choice experiments and transcriptomic analyses to determine whether CHC variation in this species affects female choice and the genetic basis underlying this variation. This study provides valuable insights into how environmental pressures may drive divergence in mating traits, contributing to our broader understanding of speciation mechanisms in *Drosophila*.

This is dedicated to all my loved ones and all of you taking the time to read this thesis.
And the color pink.

ACKNOWLEDGEMENTS

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Chapter 1:

General introduction

1.1 Ecological Speciation

Ecological speciation occurs when populations of the same species diverge into separate species due to adaptation to different environmental conditions or ecological niches (Rundle & Nosil, 2005). As populations experience different selective pressures, they evolve distinct traits, reducing gene flow between them (Rundle & Nosil, 2005). Over time, these differences may become significant enough to prevent interbreeding, resulting in the formation of new species (Mallet, 2008). An example of this is the *Heliconius* sister species *Heliconius melpomene* and *H. cydno*, which have evolved distinct wing color patterns as a form of Mullerian mimicry to avoid predation (Jiggins et al., 2004). *H. melpomene* typically mimics species with red and yellow wing patterns, while *H. cydno* mimics species with black and white patterns. These differences help each species blend into their respective mimicry groups, reducing their risk of predation (Jiggins et al., 2004). This divergence in color pattern mimicry is driven by natural selection and reinforces reproductive isolation, as individuals preferentially mate with others that share their wing coloration, further promoting speciation (Jiggins et al., 2004). These environmental differences create selective pressures that drive reproductive isolation, supporting the case for ecological speciation. Divergence in mate preference play a role in reproductive isolation and limits gene flow between species. In *Drosophila* species, reproductive isolation is crucial for maintaining species boundaries, which could result from ecological speciation (Christie & Strauss, 2019). This can manifest through behavioral differences, such as variations in mating calls or courtship rituals (Turissini et al., 2018). Understanding the mechanisms behind speciation is essential for describing how *Drosophila* species adapt to changing environments

and how new species emerge through ecological speciation (Hoikkala & Poikela, 2022). Several factors play a role in speciation and a species' ability to thrive in dynamic environments. Key elements such as mating cues, chemical communication, and the genetics of a species significantly influence its reproductive success and potential for adaptation. These factors collectively shape how species evolve and maintain their ecological niches and influence key traits for survival.

1.2 Cuticular hydrocarbons (CHCs) as dual traits in studying ecological speciation

Traits that influence both ecological divergence and mate preference are often called dual traits (Chung et al., 2014). These traits may play a key role in ecological speciation (Chung et al., 2014). Evolution in one process such as increased survival in a specific environment may lead to change in mating preference. An example of this is cuticular hydrocarbons (CHCs). CHCs are long-chain hydrocarbons (typically alkanes, alkenes, and dienes) found on the insect cuticle (Coyne et al., 2005). Insects use CHCs as a waxy layer on their cuticle to prevent desiccation as well as pheromones in chemical communication. Adaptation to environments of different humidity can lead to different populations of a single species evolving population specific CHCs, leading to potential changes in mate recognition, and reproductive isolation (Chung & Carroll, 2015). This relationship between environmental adaptation and CHC evolution is seen in many *Drosophila* species. Previous examples in the laboratory showed that in two species of *Drosophila*, the evolution in methyl-branched cuticular hydrocarbons (mbCHCs), a subset of CHCs, underlies the ecological speciation of two Australian *Drosophila* species, *D. serrata*, and *D. birchii* (Chung et al., 2014). However, this example examines CHC divergence between two species that have diverged for a long time, and do not mate and produce offspring. To provide

greater insights into ecological speciation, we aim to study populations of a single species that recently diverged and test whether CHCs do indeed contribute to ecological speciation.

1.3 *Drosophila lummei* populations as a model for the study for ecological speciation

The *Drosophila virilis* group has been used in comparative studies of reproductive isolation and hybrid sterility, illuminating how speciation occurs through genetic divergence. (Sagga & Civetta, 2011). The virilis subgroup consists of five species: *D. virilis, D. lummei, D. novamexicana, D. americana americana*, and *D. americana texana* (Sagga & Civetta, 2011). Preliminary data from our showed that two *D. lummei* populations had differences in their CHC profiles laboratory (Dr. Zinan Wang, unpublished). Populations of *D. lummei* in Japan inhabit diverse environments, ranging from temperate forests to subtropical regions, adapting to the humid and seasonal climate of the Japanese archipelago (Garbuz et al., 2003). A comparative study of *D. lummei* populations across Eurasia revealed morphological and genetic differences, further supporting the idea of regional adaptation (Haas & Tolley, 1998). In contrast, Russian populations of *D. lummei* are primarily found in temperate and boreal zones, facing harsher climatic conditions compared to Japan (Gornostaev et al., 2024). These populations exhibit resilience to cold temperatures and shorter breeding seasons, as observed in studies tracking seasonal dynamics in *Drosophila* (Gornostaev et al., 2024). Examining these differences offers insights into how environmental factors drive genetic and ecological divergence, as well as providing a framework for understanding broader evolutionary processes within the genus *Drosophila*. Studying these populations enhances our knowledge of species adaptation and sheds light on the effects of climate and geography on genetic diversity and speciation (Garbuz et al., 2003).

1.4 Aims

The first aim of this study is to quantify the CHC profile of each population (Japan and Russia) and their intraspecific crosses (Chapter 2). We hypothesize that there will be CHC differences between each parent population and its intraspecific cross due to previous unpublished data from Dr. Zinan Wang. The second aim is to determine whether CHC differences between these two populations affects desiccation resistance (Chapter 3). We hypothesize that differences in CHC composition will lead to variations in desiccation resistance between the two populations. The third aim is to assess whether CHC differences influence mating preference (Chapter 4). In aim 3.2 we investigate male mate preference in a two-choice mate assay. We hypothesize that reproductive success will be higher within the same population (Japan females × Japan males and Russia females × Russia males) than between populations (Japan females × Russia males and Russia females × Japan males). In aim 3.2 we investigate the ability for intraspecific populations to produce viable offspring. The fourth aim is to investigate the genetic basis of the CHC differences (Chapter 5). Since CHCs are produced in the oenocytes, we dissected the abdomens of different populations and sexes (12 samples total) for RNA-seq/transcriptome analysis. We hypothesize that genes potentially involved in CHC production differences may be differentially expressed between the Japan and Russia populations. This research will provide greater insights in the role of CHC in ecological speciation in *Drosophila*.

Chapter 2:

The CHCs of two *D. lummei* populations and their intraspecific crosses

2.1 Introduction

Preliminary studies in our laboratory (Dr. Zinan Wang, unpublished) suggested that CHC profiles vary between the Japan and Russian *D. lummei* populations. However, these differences were not quantified. Furthermore, the CHC profiles that are produced by the potential offspring (hybrids) of these two populations are also unknown. This chapter aims to quantify the CHC profiles of each population and their intraspecific crosses, hypothesizing that differences will be observed between parent populations and their hybrids. A reciprocal cross will also determine if the genetic mechanisms underlying the production of population-specific CHCs are sex-linked.

2.2 Materials and Methods

Drosophila flies

Drosophila lummei flies were originally obtained from the Cornell University *Drosophila* Species Stock Center by Dr. Abby Lamb (University of Michigan) and Dr. Zinan Wang (Lamb et al. 2020). The Japan population (15010-1011-0845) was originally collected from Hokkaido, Japan while the Russia population (15010-1011-01) is originally collected from Moscow, Russia, USSR (15010-1011-01) (Information from the Cornell University *Drosophila* Species Stock Center). Pinned voucher specimens of these stock are deposited in the A.J. Cook Arthropod Research Collection (Michigan State University).

Stock maintenance

The two populations were maintained at 23°C (room temperature in the laboratory) on Nutri-Fly Bloomington formulation (https://bdsc.indiana.edu/information/recipes/bloomfood.html), a standardized *Drosophila* diet to ensure consistent nutritional conditions across replicates.

CHC extraction and data analysis

For CHC extraction, 5-day-old females or males were used. Following established protocols, five flies (each replicate) were soaked in 200 µl of hexane for 10 minutes to extract surface lipids, with hexacosane (C26; 25 ng/µl) used as an internal standard for quantification (Lamb et al., 2020; Wang et al., 2022). Five biological replicates were prepared for each population to ensure statistical robustness. CHC extractions were analyzed using gas chromatography-mass spectrometry (GC/MS), following methodology adapted from previous studies in our laboratory (Lamb et al., 2020; Wang et al., 2022).

Intraspecific crosses

To generate offspring from intraspecific crosses (hybrids), unmated *D. lummei* from each population were collected and crossed to the opposite sex of the other population at seven days to control potential effects of age and developmental variation.

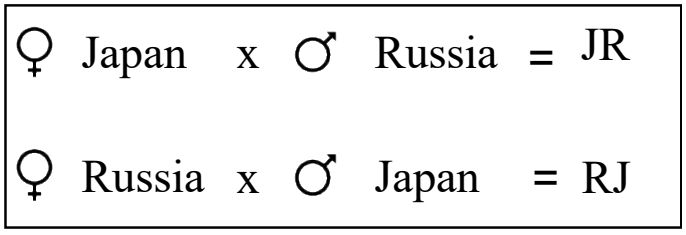


Figure 2.1 Intraspecific cross of *D. lummei* populations. Japan females are mated with Russia males, cross is denoted as JR. Russia females are mated with Japan males, cross is denoted as RJ.

Offspring were subsequently collected, sexed, and aged to maturity (7 days) under the same environmental conditions before CHC extraction and analysis. The intraspecific cross with Japan female and Russian male is denoted as JR, while the intraspecific cross with the Russian female and the Japan male is denoted as RJ.

2.3 Results and Discussion

We first analyzed the CHCs of both males and females from the Japan and Russia *D. lummei* populations (**Figure 2.2**). Overall, Japan and Russia *D. lummei* flies exhibit qualitative differences in their CHC profiles between populations, but do not exhibit qualitative differences between the sexes within one population.

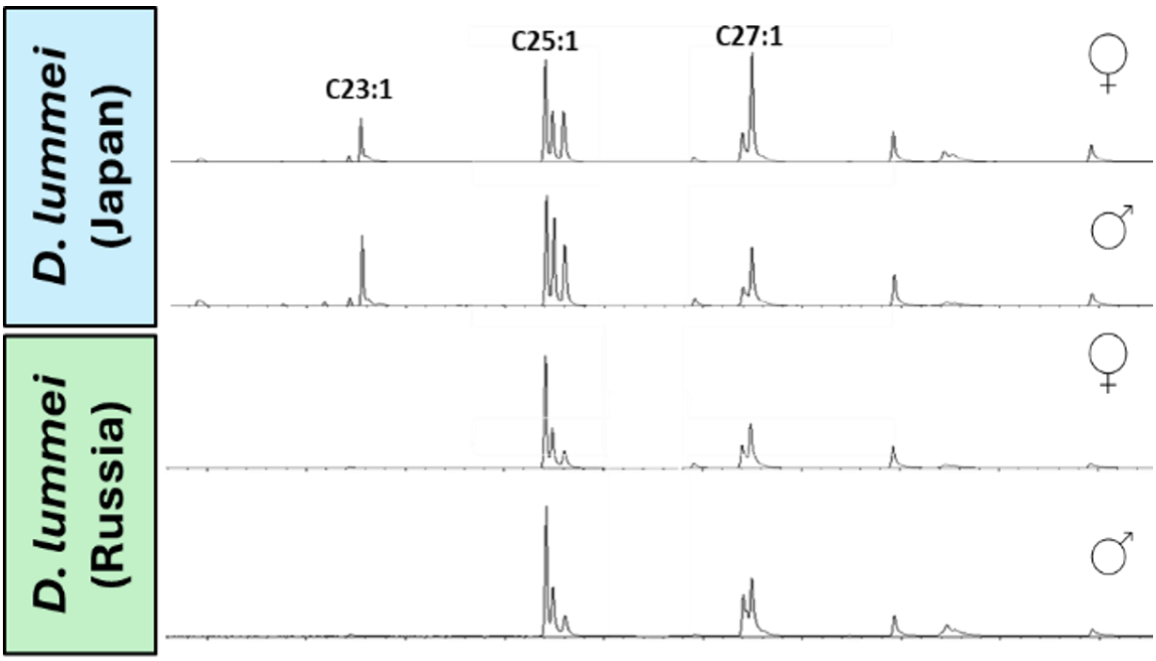
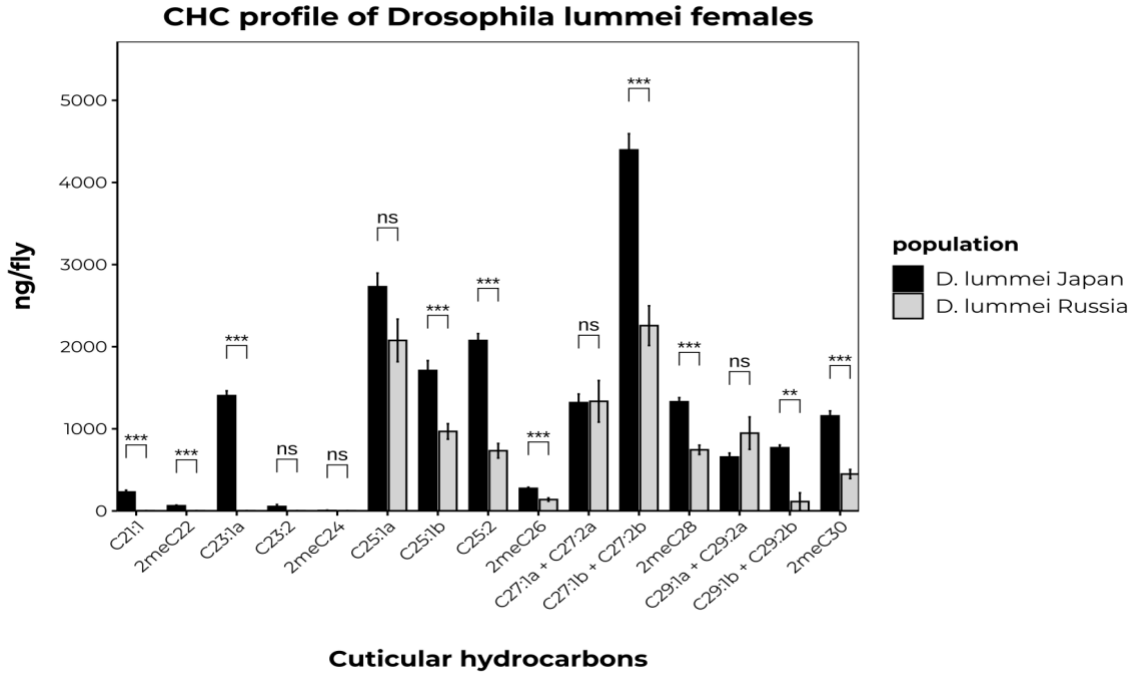


Figure 2.2 Gas Chromatography/Mass Spectrometry (GC/MS) Chromatogram. CHCs from *D. lummei* Japan and *D. lummei* Russia (both sexes). C23:1 was not detected in *D. lummei* Russia.

In females, there is a significant difference ($p<0.001$) in the total quantity of CHC compounds produced between the Japan (18174.5 ± 823.49 ng/fly) and Russia (9762.0 ± 1089.1 ng/fly). *D.*

lummei populations (**Figure 2.3A**). Compounds shorter than C25:1 were not detected in Russia females. The Japan females also have significantly more mbCHCs such as 2meC22 ($p<0.01$), 2meC26 ($p<0.01$), 2meC28 ($p<0.01$), and 2meC30 ($p<0.01$), compared to the Russia females. The CHC profile of male populations showed a similar pattern. There is a significant difference ($p<0.001$) in the total quantity of CHC compounds produced between the Japan (16906.0 ± 1018.5 ng/fly) and Russia (6522.6 ± 657.6 ng/fly). (**Figure 2.3B**). Compounds shorter than C25:1 were not detected in Russia males. The Japan male also have significantly more mbCHCs such as 2meC24 ($p<0.01$), 2meC26 ($p<0.001$), 2meC28 ($p<0.001$), and 2meC30 ($p<0.001$), compared to the Russian males.

(A)



(B)

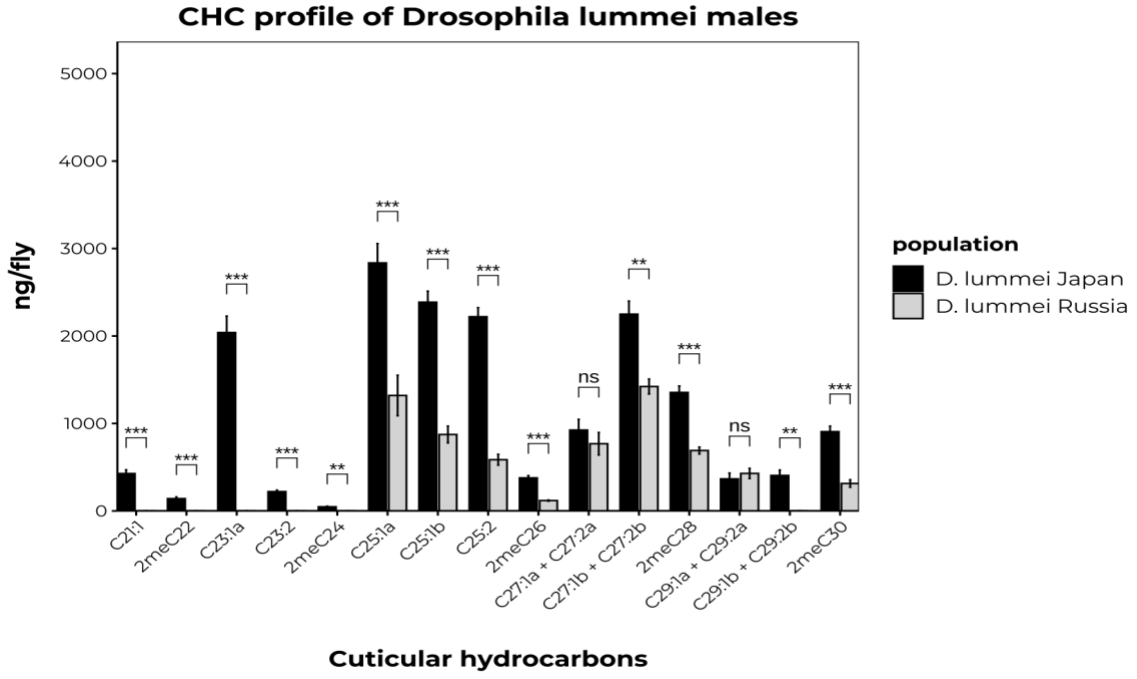
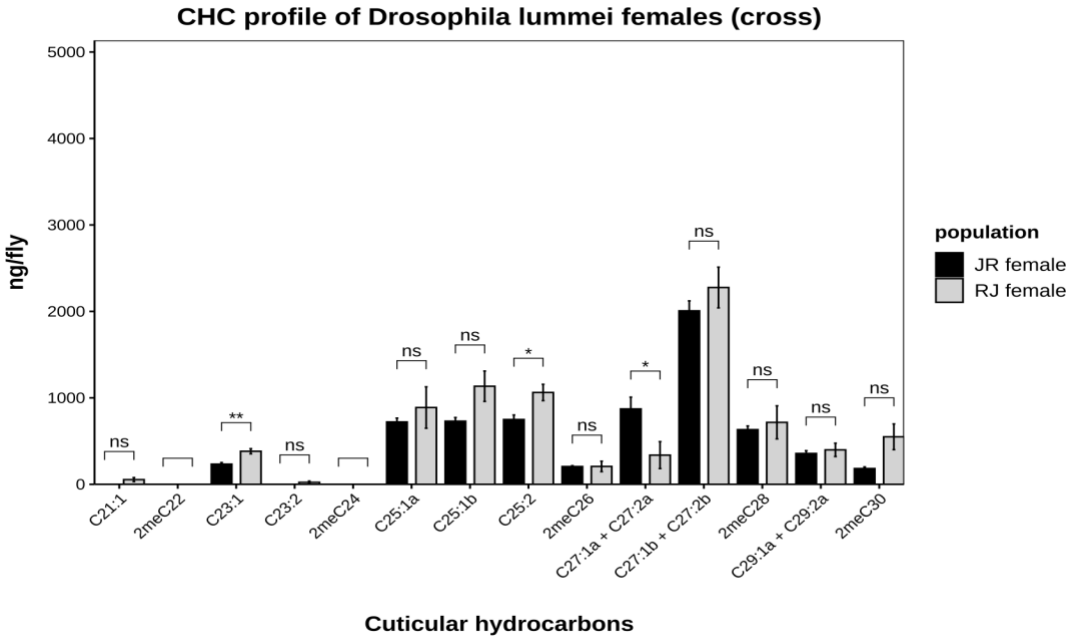


Figure 2.3 CHCs of *Drosophila lummei* populations from Japan and Russia. (A) CHC profiles of *D. lummei* females from Japan and Russia. (B) CHC profiles of *D. lummei* males from Japan and Russia. Data were analyzed using RStudio. Statistical comparisons were performed using two-tailed t-tests with significance levels indicated as ****p < 0.001, **p < 0.01, *p < 0.05,* and *p > 0.05 N.S. (not significant)*. Adjusted p-values were calculated using the Bonferroni method.

In intraspecific cross female offspring, there is no significant difference ($p>0.05$) in the total quantity of CHC compounds produced between the Japan (6690.5 ± 457.5 ng/fly) and Russia (8029.0 ± 452.37 ng/fly). There is a loss of mbCHCs in compounds 2meC22 and 2meC24 in the intraspecific JR and RJ offspring (**Figure 2.4A**). There is no difference in mbCHCs compounds 2meC26, 2meC28, and 2meC30 ($p>0.05$). C23:1 was detected in both female cross populations. In male cross populations, there is no significant difference ($p>0.05$) in the total quantity of CHC compounds produced between the Japan (5225.1 ± 186.5 ng/fly) and Russia (4540.7 ± 865.9 ng/fly). There is a loss of mbCHCs in compounds 2meC22 and 2meC24 in the intraspecific JR and RJ offspring (**Figure 2.4B**). There is no difference in mbCHCs compounds 2meC26, 2meC28, and 2meC30 ($p>0.05$). C23:1 was detected in JR but not RJ.

(A)



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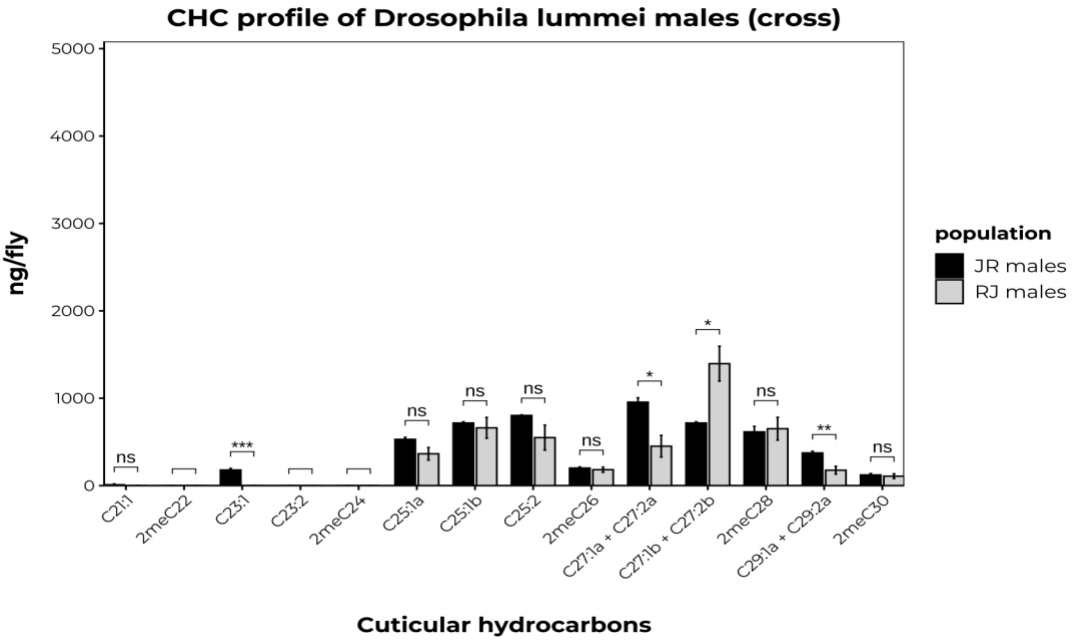

Figure 2.4 CHCs of *Drosophila lummei* intraspecific cross offspring populations. (A) CHC profiles of intraspecific *D. lummei females* from Japan and Russia. (B) CHC profiles of intraspecific *D. lummei* males from Japan and Russia. Data were analyzed using RStudio. Statistical comparisons were performed using two-tailed t-tests with significance levels indicated as ****p < 0.001, **p < 0.01, *p < 0.05,* and *p > 0.05 N.S. (not significant)*. Adjusted p-values were calculated using the Bonferroni method.

The CHC diversity observed between the Japan population and the Russia may be shaped by distinct environmental factors, particularly humidity and temperature. For example, Sapporo (Hokkaido), Japan, throughout the year, the average humidity in Sapporo is 70% (Weather-and-Climate.com, 2025). In contrast, Moscow, Russia, has an average annual relative humidity of around 76.7% (Climate.top, 2025). The lower humidity levels and higher temperature fluctuations in Hokkaido may drive the evolution of more diversity in CHCs, whereas Moscow's relatively wetter, colder climate may favor less diversity in CHCs. Similar CHC diversity has been observed in other *Drosophila* species, where populations adapted to different desert environments exhibit distinct CHC compositions, reflecting local ecological pressures (Wang et al., 2022). These findings emphasize the role of CHC variation in ecological adaptation beyond the well-studied *D. melanogaster*.

In the next chapter, we discuss CHCs influence on survival by conducting a desiccation resistance experiment. Understanding these differences provides valuable insights into how climatic pressures shape adaptation across populations.

Chapter 3:

Desiccation Resistance of *D. lummei* populations

3.1 Introduction

In the previous chapter, we showed CHC differences between *D. lummei* populations as well as with the intraspecific crosses of these populations. Understanding the role of CHCs in desiccation resistance lays the groundwork for exploring how species adapt to different climates. This chapter will investigate desiccation resistance in each parental population and the intraspecific cross. We hypothesize that differences in CHC composition may correlate to a difference in desiccation resistance in these populations.

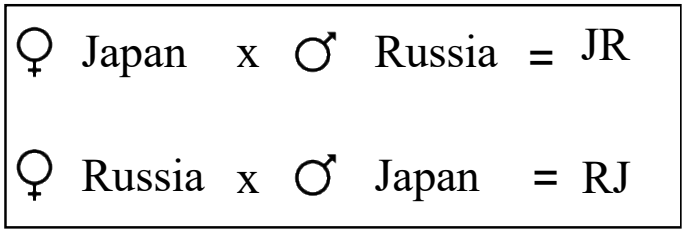

Figure 3.1 Intraspecific cross of *D. lummei* populations. Japan females are mated with Russia males, cross is denoted as JR. Russia females are mated with Japan males, cross is denoted as RJ.

3.2 Materials and Methods

Drosophila flies for desiccation experiments

Newly emerged 0-day-old male and female *Drosophila* were collected between 10:30 am and 12:00 pm and maintained at 25°C. Seven-day old flies are used for the desiccation experiment.

Desiccation resistance assay

Silica gel beads (10 grams per vial) were placed in glass vials, covered with saran wrap, for 24 hours. 10 flies of either sex was transferred to sanitized vials and covered with two 1-inch pieces of cheesecloth secured with tape (Wang et al., 2022). The vials were connected using tape and

incubated at 25°C. We score for mortality once an hour for over 30 hours until all flies died This protocol was applied to both non-reciprocal cross populations and reciprocal cross-mated individuals.

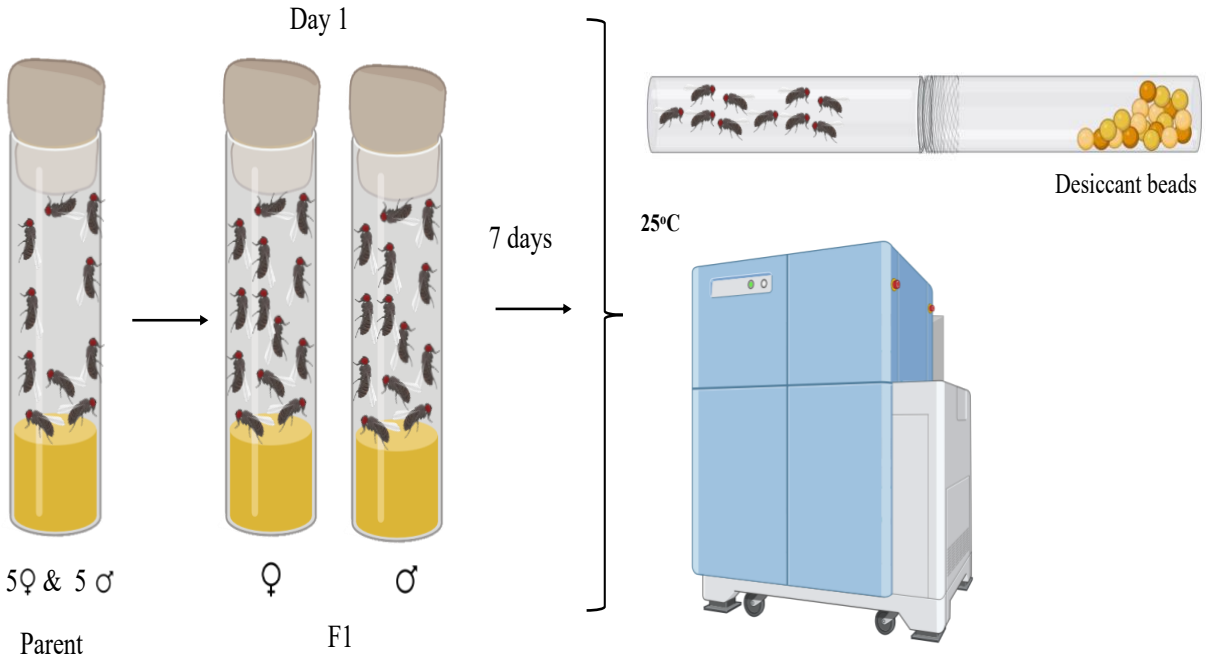

Figure 3.2 Desiccation resistance assay. Offspring were separated by sex and aged for seven days. Flies and silica gel beads were placed in 25°C incubator and mortality was checked once an hour. Figure adapted from (Wang et al., 2022). Image was created using BioRender.

3.3 Results and Discussion

We first performed a desiccation assay comparing desiccation resistance between the parental populations. Our experimental findings are consistent with previous studies in our laboratory that showed *D. lummei* had an average desiccation resistance of 20 hours (Wang et al., 2022). Japan females (20.5 ± 0. 5 h) have significantly higher desiccation resistance ($p<0.01$) compared to Russia females (18.6 ± 0.6 h) (**Figure 3.3**). Similarly, the Japan males have significant higher desiccation resistance compared to Russian males ($p<0.05$) with an average time of (20.0 ± 0. 5 h) compared with (18.4 ± 0. 5 h). The parent populations of *D. lummei* showed significant

differences ($p<0.01$) in survival under desiccation stress (**Figure 3.3**). The Russia population females had shorter survival time when compared to Japan females, indicating higher desiccation resistance in Japan females (**Figure 3.3**). Similarly, Russia males had shorter, survival time when compared to Japan males, indicating higher desiccation resistance in Japan males (**Figure 3.3**).

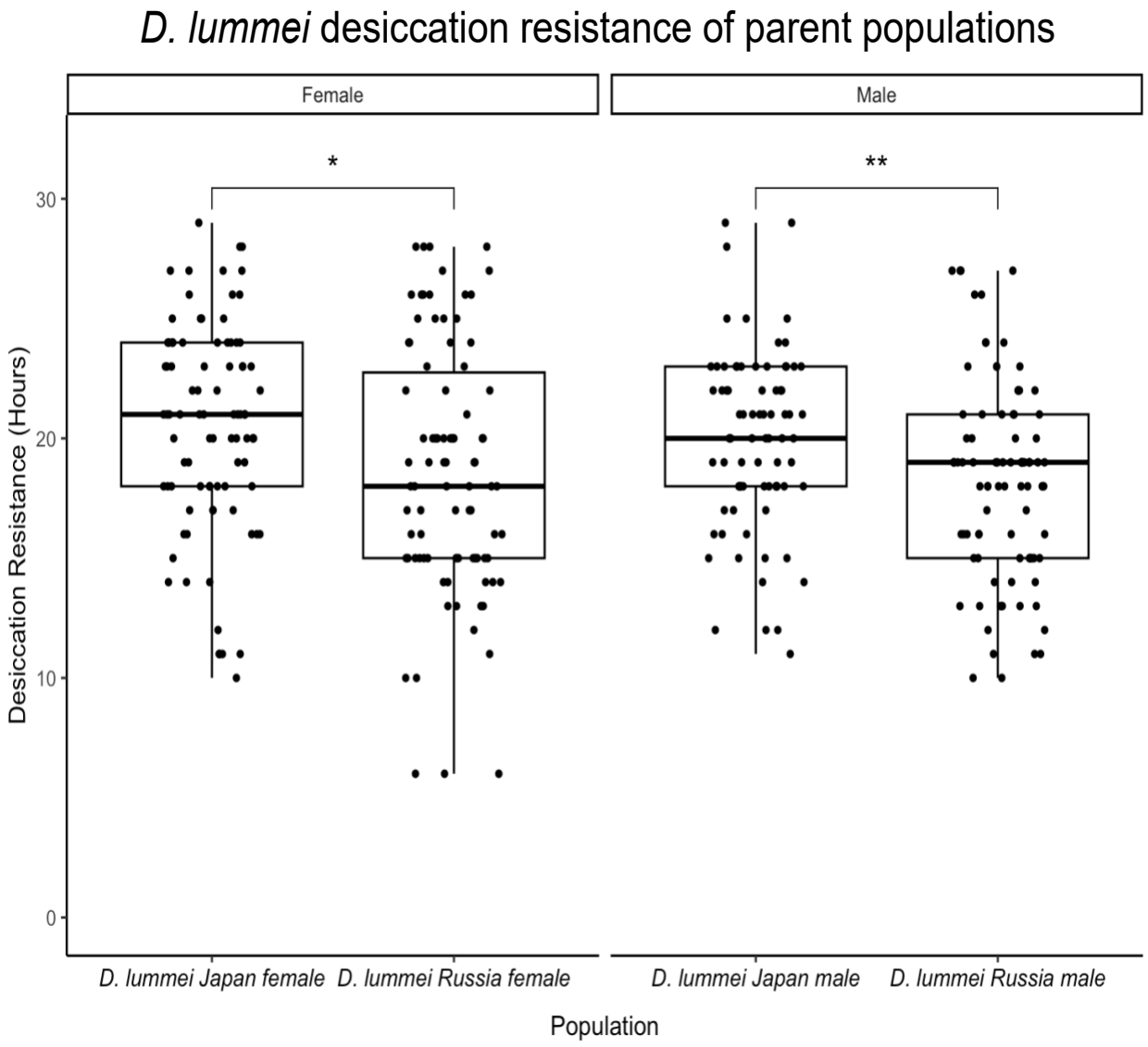

Figure 3.3. Desiccation resistance of *D. lummei* parent populations. Trials were conducted over 20 hours to measure the survival rate of each population. Data were analyzed using RStudio (version 2024.09). Statistical comparisons were performed using pairwise t-test within each sex group. The significance levels indicated as $**p < 0.01$, $*p < 0.05$, and $p > 0.05$ *N.S.*. Adjusted p-values were calculated using the Bonferroni method.

For the desiccation resistance of the offspring of *D. lummei*, within each sex, there was no significant difference ($p>0.05$) between the Japan and Russia populations. In females, there is no significant differences ($p>0.05$) in desiccation resistance between JR (20.2 ± 0. 5 h) compared to RJ (20.0 ± 0. 5 h) (**Figure 3.4**). Similarly, in males, there is no significant difference in desiccation resistance between JR (21.0 ± 0. 5 h) compared to RJ (21.7 ± 0. 5 h) (**Figure 3.4**).

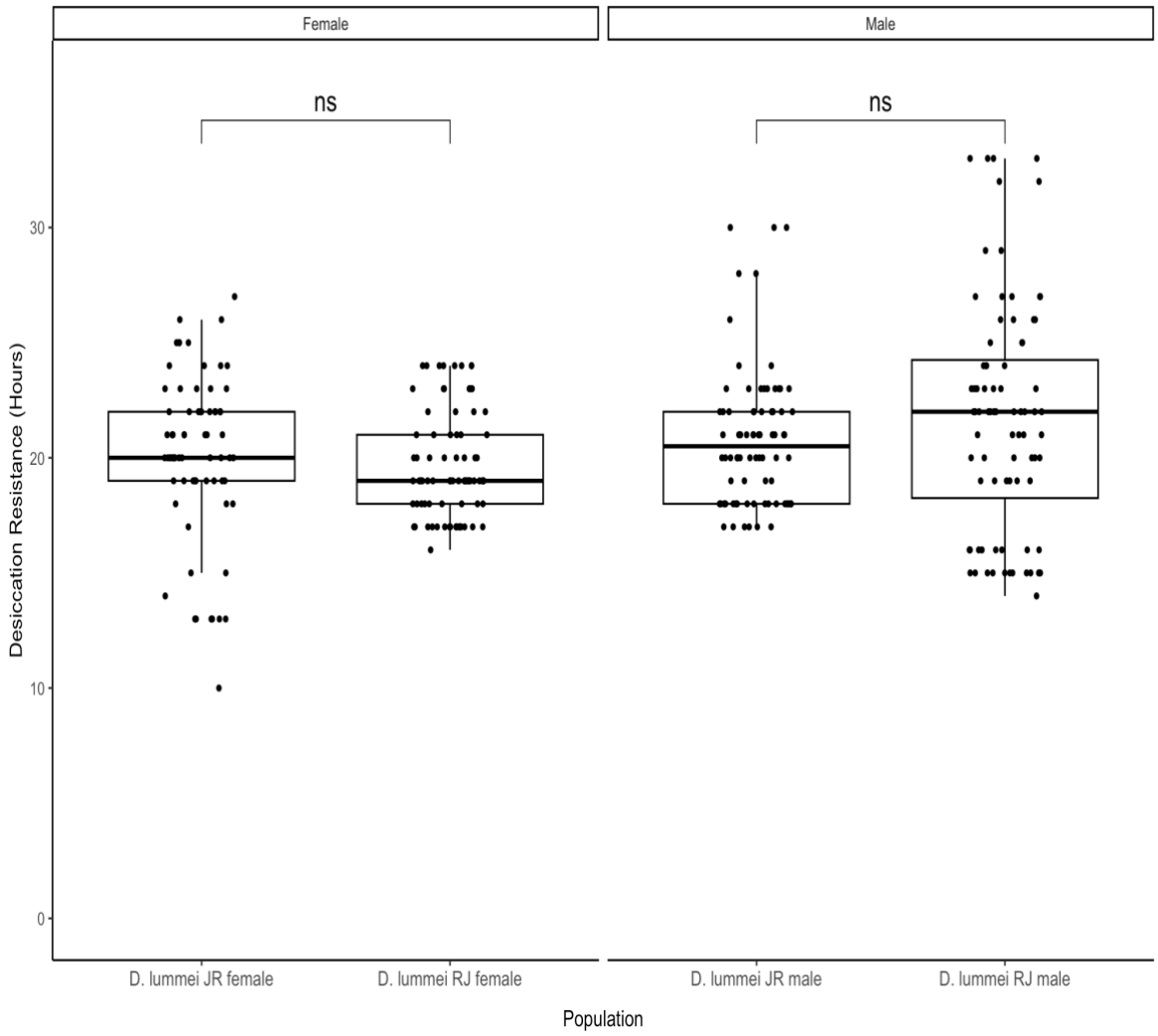

Figure 3.4. Desiccation resistance of the offspring of *D. lummei* reciprocal crosses. Trials were conducted over 20 hours to measure the survival rate of each population. Data were analyzed using RStudio (version 2024.09). Statistical comparisons were performed using pairwise t-test within each sex group. The significance levels indicated as $^{**}p < 0.01$, $^{*}p < 0.05$, and $p > 0.05$ *N.S.* Adjusted p-values were calculated using the Bonferroni method.

Since there are no significant differences in desiccation resistance between JR and RJ flies, it may suggest that the genetic mechanisms underlying this difference in desiccation resistance in the parents are non-sex linked. Additionally, the experimental conditions (25°C) may not have imposed enough stress to reveal differences between offspring of the intraspecific

populations. The combination of lower humidity and greater temperature in Hokkaido may promote a higher desiccation resistance, while Moscow's colder and relatively wetter conditions might contribute to a lower desiccation resistance. The differences in desiccation resistance observed between the Japan population and the Russia populations suggests that environmental adaptation, such as desiccation resistance, can vary across populations of a single species.

Chapter 4:

Mate choice and reproductive success in *D. lummei* populations

4.1 Introduction

In *D. lummei*, significant differences in CHC profiles have been observed between populations from Japan and Russia. Japan populations exhibit a broader range of CHC compounds both qualitatively and quantitatively compared to Russia populations (**Figures 2.1, 2.2,** and **2.3**). Additionally, significant differences in desiccation resistance have been observed between the parent populations (**Figure 3.3**). However, in the offspring of the intraspecific crosses, this difference was not statistically significant (**Figure 3.4**). Mate choice assays can used to assess mating preferences and the potential for assortative mating within a species (Ruskie & Zakas, 2023). This chapter aims to determine whether CHC polymorphism influences mate preference between two populations. Additionally, we will examine whether temperature affects total viable offspring and compare offspring viability between parent populations and intraspecific crosses. We hypothesize that assortative mating will be lower between populations (intraspecific crosses) than within parent populations.

4.2 Materials and Methods

Fly collection and experimental controls

To prevent any bias associated with color marking, each population was assigned a different color, with colors alternating across trials (as recommended by Dr. Henry Chung) to control for unintended color effects on mate choice. A small dot of acrylic paint was carefully placed on the thorax of each female to avoid interference with wing movement or other behaviors 8 days after emergence from pupal cases. After marking, females were transferred to fresh vials of food and kept undisturbed 48 hours before mating experiment. Males were also placed into fresh vials on

day 8 to maintain consistent conditions, reducing the potential influence of bacteria or environmental factors on mate selection (Coyne et al., 2005).

Mate choice experiment

To minimize CO_2 exposure to flies, a mouth aspirator was constructed using a clear fish tube. A small net was secured inside the tube, with a 1000µL pipette tip placed on top to prevent flies from moving too far inside. A 200µL pipette tip was attached to the other end as a mouthpiece, allowing precise placement of individual flies into vials (**Figure 4.1**).

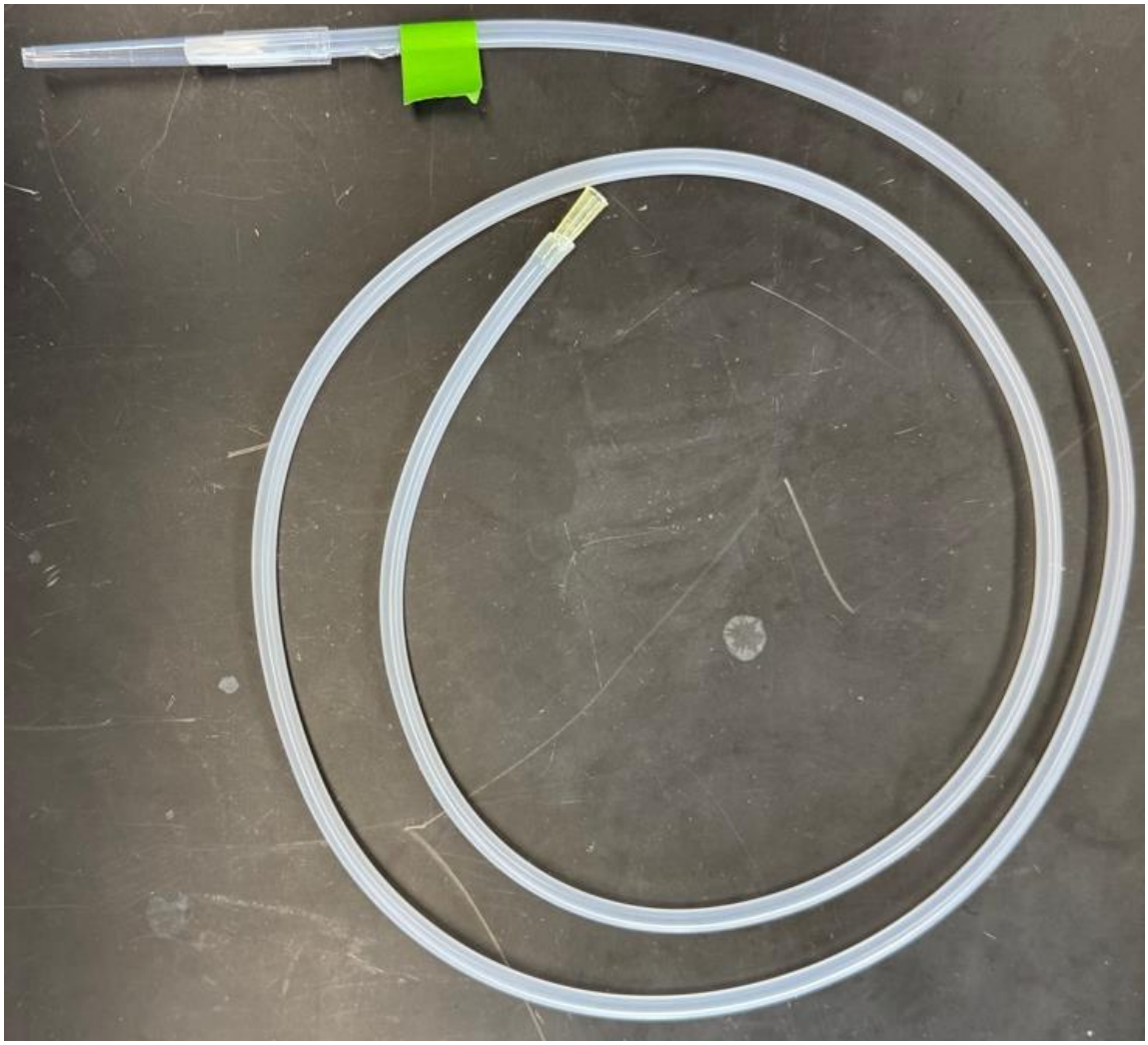

Figure 4.1 Mouth aspirator. A 1000µL pipette tip placed on one end of clear tube to move flies from one vial to another. A 200µL pipette tip was attached to the other end.

Trials were conducted in a controlled setting with minimized external disturbances to reduce potential experimental artifacts (Coyne et al., 2005). A male choice mating experiment was performed to determine if there is a preference in male choice when two females of different populations are introduced to males. One male was placed with one female from each population (Japan and Russia) in a controlled environment (**Figure 4.2**).

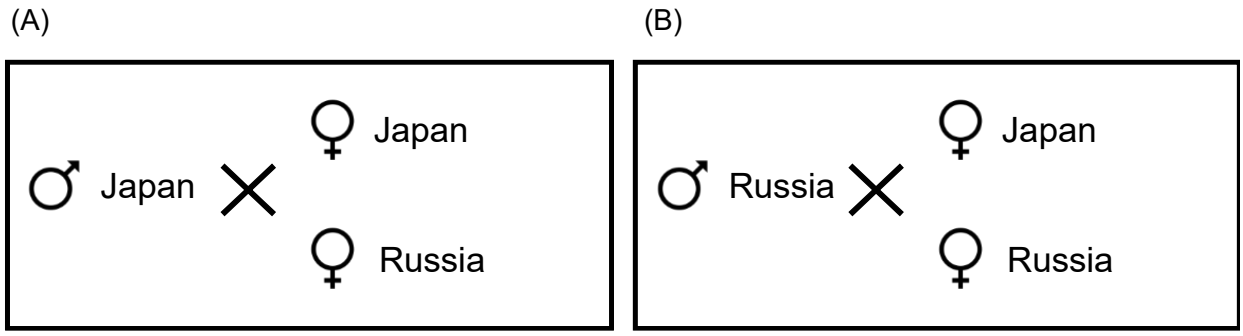

Figure 4.2. Male mate choice assay to determine *D. lummei* male preference. A single *D. lummei* Japan female and a single *D. lummei* Russia female are placed in a single vial with a single *D. lummei* Japan or *D. lummei* Russia male.

Mating trials ran from mid-morning to early afternoon (4 hours) when *Drosophila* exhibited peak mating activity in previous experiments, with a maximum observation period of four hours per trial to standardize latency measurements. Observations were made in person. Matings were recorded across multiple trials to ensure statistical robustness. For mating duration to be recorded, the pair must mate for at least 2 minutes. If they separate before the 2-minute mark, the event will not be counted in the trial. This experiment aimed to assess whether males exhibit a preference when presented with females from two different populations. Additionally, we measured mating latency and duration to further explore potential behavioral differences between populations, incorporating methodological refinements to ensure that experimental design does not artificially inflate or suppress sexual isolation effects (Coyne et al., 2005).

Reproductive success assay

Three treatment temperatures were used to determine reproductive success across parent populations and intraspecific cross populations (18ºC, 25ºC, and 27ºC). Three adult females were placed with three adult males per vial and allowed to mate for seven days. Each treatment included five replicates per cross. Adults were removed from vials and disposed of. Vials were left in their respective treatment incubator for 2 weeks or until offspring emerged. Each day viable offspring was sexed and counted, then was disposed of. This continued for 2 weeks or until offspring no longer emerged from vials.

4.3 Results and Discussion

Mate choice

We perform male mate choice experiments to determine if males from both populations exhibit mating preferences for females of the same population. We perform 80 male mate choice experiments for both Japan and Russia males. In 80 mate choice experiments each, the Japan males successful mated 54 times (**Table 4.1**), and the Russia makes mated 10 times (**Table 4.2**). This suggests that Japan males were generally more active or receptive to mating. A chi-square test was used to determine if Japan males preferred Japan females over Russia females. Japan males showed there is no significant difference in male choice between Japan females and Russia females (χ^2=2.7, *p=0.10*) (**Table 4.1**).

Table 4.1 *D. lummei* Japan male choice.

	Japan male choice	
	Japan female	Russia female
Observed	31	23
Expected	27	27
Chi-square test	2.7	
p-value	0.10	

A chi-square test was used to determine if Russia males preferred Japan females over Russia females. Russia males showed there is no significant difference in male choice between Japan males and Russia males (χ^2=0.4, *p=0.53*) (**Table 4.2**).

Table 4.2 *D. lummei* Russia male choice.

	Russia male choice	
	Japan female	Russia female
Observed	4	6
Expected	5	5
Chi-square	0.4	
p-value	0.53	

As the Russia male mate choice experiments showed a low mating rate, we focused our next analyses on the Japan male choice experiments to further determine whether mating latency and mating duration can be used to assess mate preference.

D. lummei Japan males do not show a significant difference (*p>0.05*) in mating duration when mating with either Japan females or Russia females (**Table 4.3**). Mating duration in lasted between 2-5 minutes depending on the pairing. This indicated that once mating occurred, the Japan males did not show any preference in mating duration between the Japan or Russia females.

Table 4.3. Comparison of mating duration (minutes) between *D. lummei* Japan males and Japan or Russia females.

	Japan	Russia
Mating Duration (mean)	3.8	4.4
Mating Duration (SEM)	0.2	0.3
t-test (P-value)	0.1	

Mating latency refers to the amount of time that passes from when a potential mate is introduced until mating begins. It is a key indicator of mating behavior (Eastwood & Burnet, 1977). In our experiments, the Japan males do not show a significant difference ($p>0.05$) in mating latency when mating with the Japan females or the Russia females. (Table 4.4).

Table 4. 4. Mating latency of *D. lummei* Japan and Russia.

	Japan	Russia
Mating latency (mean)	108.0	86.8
Mating latency (SEM)	12.4	14.8
t-test (P-value)	0.3	

Since Russia males exhibited low mating activity, it was not possible to determine whether Russia male populations preferred their own females over those from another population. We were also not able to determine mating duration or mating latency in Russia males. This limitation suggests that differences in overall mating behavior (rather than mate preference) may be influencing the results. In this trial we only observed male mate choice in the *D. lummei* populations. In future trials we will expand our observations to female choice. Additional experiments with a larger sample size or different experimental setups might be needed to clarify whether mate preference exists.

Reproductive success

To further understand our findings, we scored offspring in parent populations and compared it to the intraspecific crosses. We compared three different treatments groups (Treatment A 18^{o}C, Treatment C 25^{o}C, and Treatment C 27^{o}C) to determine if parent populations were produced more offspring when compared to cross population in multiple environments (**Figure 4.3**). Treatment A (18^{o}C) showed that Russia offspring produced the least amount of viable offspring. Offspring produced from Japan, JR, and RJ all produced similar amounts of offspring in this treatment. Treatment B (25^{o}C) showed similar patterns with Russia producing the least viable offspring in this treatment. We see the same pattern in treatment C (27^{o}C) (**Figure 4.3**).

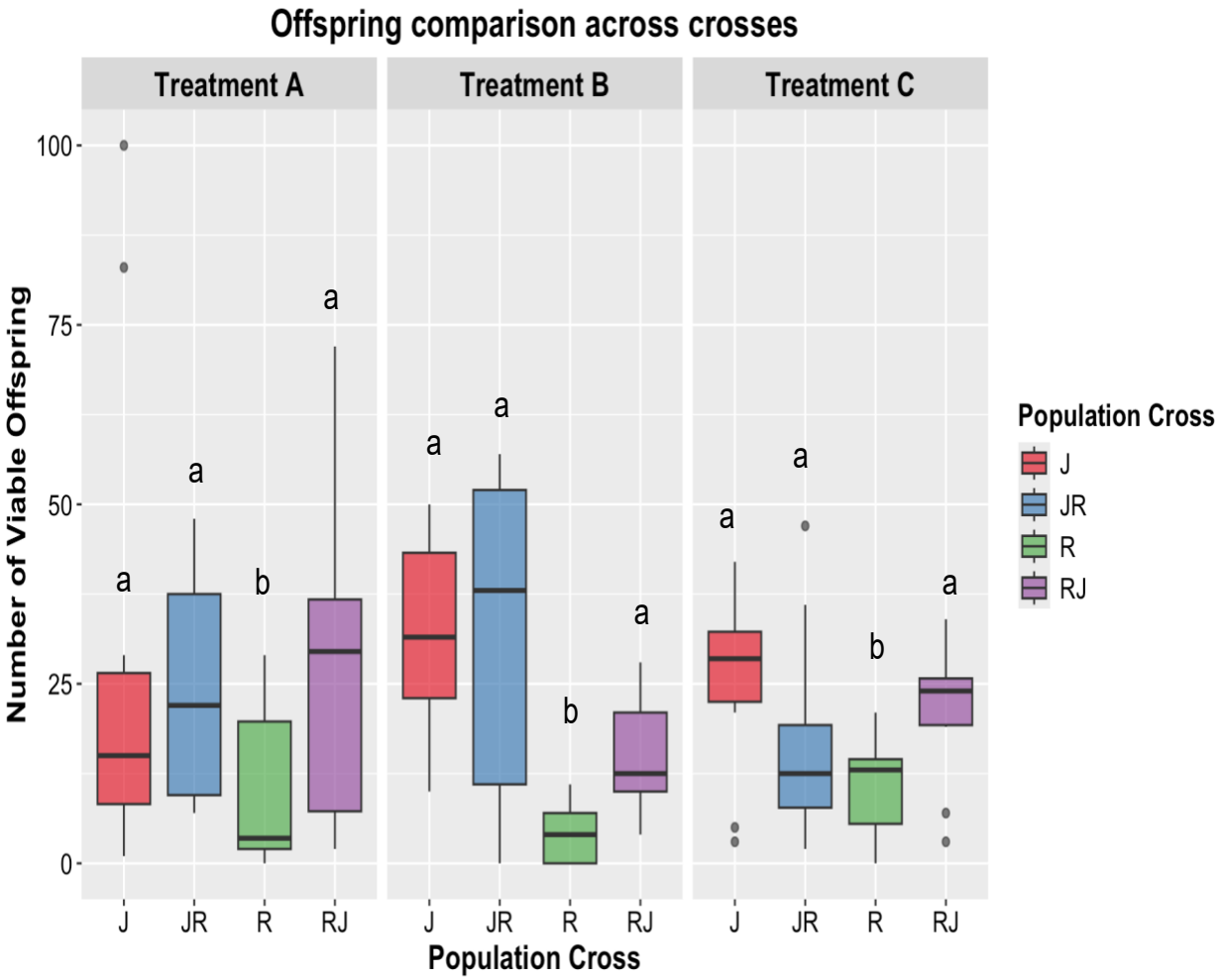

Figure 4.3 *D. lummei* total offspring. In each treatment, Treatment A 18ºC, Treatment B 25ºC, and Treatment C 27ºC, the number of viable offspring from parent population crosses was compared to that from intraspecific crosses. There is significant difference in offspring between treatment groups Japan, JR, and RJ compared to Russia. Data were analyzed using RStudio. Statistical comparisons were performed using an Anova and Tukey HSD test, with significance levels indicated as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and *N.S. (not significant)*.

These findings suggest that all these different treatment groups (18°C, 25°C, and 27°C) has no impact on offspring, with Russia consistently producing the least amount of offspring across all temperatures. These results could imply that the Russia population has some inherent biological limitations that affect its reproductive success. This suggests genetic or environmental factors may influence mating and total offspring. This pattern could be important for understanding the adaptability of different populations to changing environmental conditions.

Chapter 5:

RNA-Sequencing in *D. lummei* male and females

5.1 Introduction

Studying the transcriptome of *D. lummei* from Japan and Russia is crucial for identifying the genetic basis of CHC variation within this species. It provides insights into gene expression patterns, evolutionary adaptations, and potential genetic differences between populations. In this chapter, we aim to compare transcriptomic differences between *D. lummei* from Japan and Russia. We extracted RNA from the dissected abdominal cuticles of *D. lummei* (Japan and Russia populations) to send for RNA-seq to investigate gene expression differences between the two different populations. Given the critical role of CHCs in desiccation resistance (Chapter 3), mate choice and reproductive success (Chapter 4), and ecological adaptation. Comparing gene expression in these two populations will enhance our understanding of the genetic basis of these traits in *D. lummei*.

5.2 Materials and Methods

Sample Preparation

D. lummei populations were separated by sex upon emergence and aged for 7 days. On day 7, populations were transferred to 1.5mL low-retention microcentrifuge tubes and placed on ice. For 1 replicate, 10 abdomens were dissected, with each sex having 3 replicates.

Buffer Preparation

A 1x PBS Buffer (1mL of 10x PBS Buffer pH 7.4 and 9mL of molecular-grade water) was prepared and placed on ice.

Dissection Procedure

One fly was placed on a glass slide with chilled PBS under the microscope. Using forceps, the head and thorax were removed and placed on a separate slide. To remove the cuticle from the abdomen, a pair of vanna's spring scissors (3mm) was used to cut around the entire abdomen. The forceps were then used to remove the reproductive organs and any fat body remaining on the cuticle.

RNA Extraction and Sequencing

RNA was extracted using the Qiagen RNeasy Mini Kit following established protocols. RNA sequencing was carried out by The RTSF Genomics Core at Michigan State University. 10-15 dissected abdomen cuticles were used per replicate.

5.3 Results and Discussion

To determine the quality and quantity for RNA-seq 12 samples (3 Japan males, 3 Japan females, 3 Russia males, and 3 Russia females) were prepared dissected to send for sequencing. In the meantime, we prepared preliminary samples to determine how many dissected abdominal cuticles were needed per samples (ng/fly). We also needed to determine if the quality of each sample was good enough to use for RNA-seq. The quality of each sample was determined by the RTSF Genomics Core at Michigan State University. We found that for 20ng of RNA needed for the RNA-seq per sample using the Qubit^{TM} assay fluorometer. A total of 20 ng/sample was used, with RNA extracted from 10 dissected abdominal cuticles per sample. 10 dissected abdominal cuticles were enough for Japan populations but were not enough for Russia populations. To offset the bad quality in Russia samples, we upped the dissected abdominal cuticles from 10 to 15 (**Figure 5.1**).

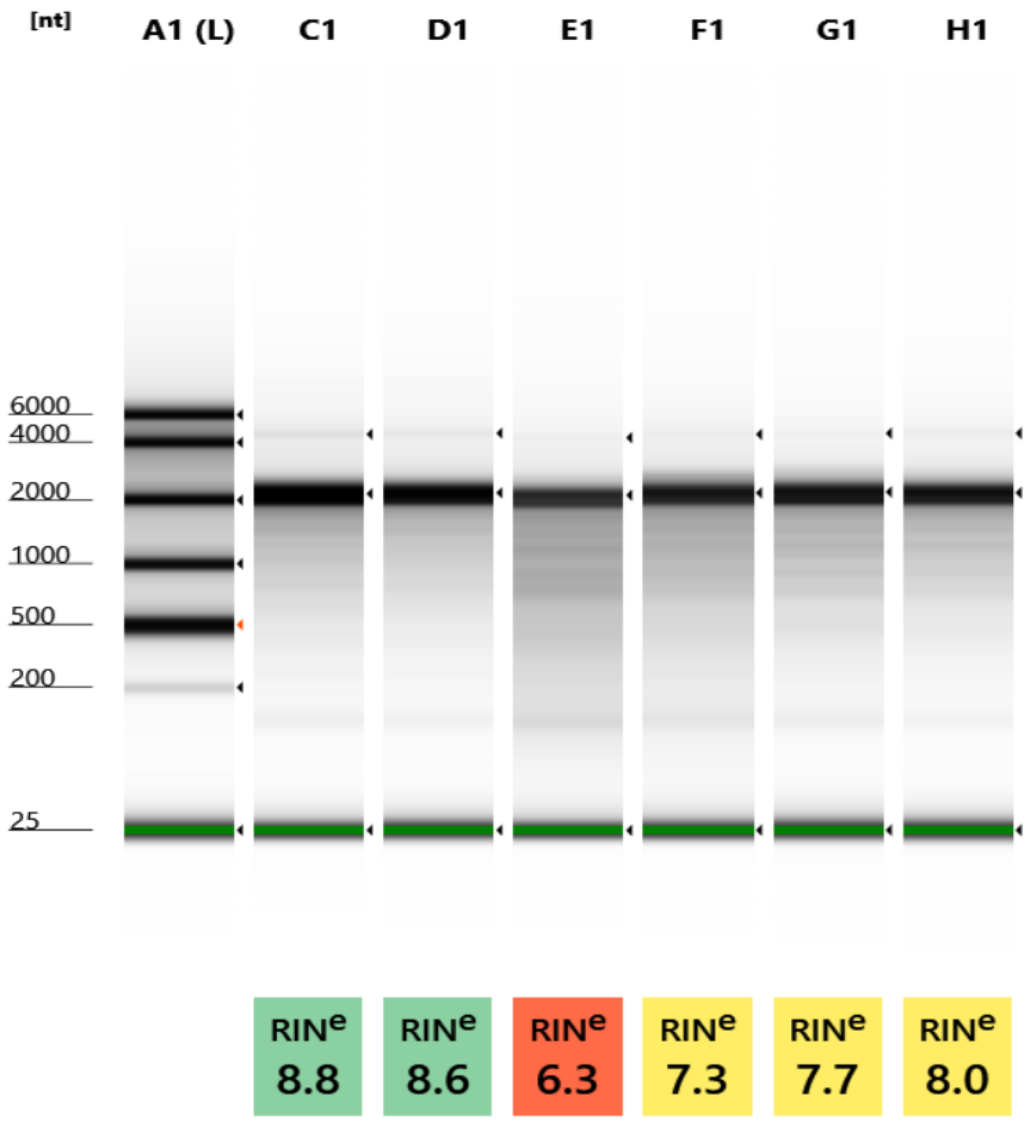

Figure 5.1. RNA Quality Check using Tape station. This figure displays the RNA extraction quality check for *D. lummei* populations. Each cell represents a distinct population, with 10 dissected abdominal cuticles used per sample. **A1**: ladder, **C1**: Japan (Male), **D1**: Japan (Female), **E1**: Russia (Male), **F1**: Russia (Female), **G1**: Russia (Male) H1: Russia (Female). Cells highlighted in yellow or red indicate samples that were excluded from RNA sequencing (RNA-seq).

RNA extraction product will be sent for sequencing at the RTSF Genomics Core at Michigan State University. Due to the short time frame results are still pending. Once available, these

results will provide insights into the genetic mechanisms underlying CHC biosynthesis, desiccation resistance, and mate choice in *D. lummei*. The data will be analyzed to determine differential gene expression between populations and sexes, contributing to a deeper understanding of ecological speciation. Population-level differences in gene expression can shed light on how ecological factors shape reproductive isolation and speciation (Castillo & Moyle, 2022). Integrating transcriptomic data with behavioral and ecological studies will provide a comprehensive understanding of the molecular drivers of reproductive divergence and evolutionary change. Future updates will incorporate these findings to further contextualize their implications within the broader scope of this study.

Chapter 6 :

Discussion

This thesis investigated the role of cuticular hydrocarbons (CHCs) in ecological speciation using two populations of *Drosophila lummei* from Japan and Russia. We found significant differences in CHC profiles between the two populations, with Japan *D. lummei* exhibiting a broader range of CHC compounds both qualitatively and quantitatively compared to Russia *D. lummei*. Desiccation resistance assays revealed that Japan populations demonstrated greater resistance to desiccation compared to Russia populations. Mate choice experiments showed no significant assortative mating between populations, and reproductive success assays indicated that the reproductive success of Russia *D. lummei* is lower across temperature treatments compared to Japan *D. lummei* and hybrid crosses.

The observed CHC differences between the Japan and Russia populations aligns with findings in other *Drosophila* species, where CHCs serve as both ecological and mating cues (Chung et al., 2014; Wang et al., 2022). CHC variation has been shown in other *Drosophila* species, where different CHC compositions correspond to different levels of desiccation resistance, further supporting the role of CHCs in ecological adaptation (Wang et al., 2022). The reduced CHC composition in Russia *D. lummei* could be a result of the colder and drier climate, which may impose selective pressures favoring a less diverse CHC profile optimized for survival in harsher conditions. The results from the desiccation resistance assays suggest that CHC variation influences desiccation resistance, supporting the hypothesis that CHC composition is an adaptive trait shaped by environmental conditions. This aligns with previous work demonstrating the role of CHCs in desiccation resistance in *Drosophila* (Chung & Carroll, 2015).

In other *Drosophila* species, CHCs serve as mating signals, leading to reproductive isolation (Chung et al., 2014). However, in the male mate choice assay, we observed no significant mate preference for Japan females or Russia females, suggesting that CHCs may not play a role in male mate choice in this species. However, the observed low mating activity in Russia *D. lummei* males may have influenced the results, potentially masking subtle mate preferences. Our findings on mate choice are inconclusive due to the low sample size of mating in males. We did not perform the female choice experiment in this thesis due to the lack of time, so we are unable to make a conclusion about CHC and female choice in this species. Our results provide insights into how CHC diversity and environmental adaptation influence *Drosophila* populations. The significant CHC differences between Japan and Russia *D. lummei* populations highlight the role of environmental pressures in shaping chemical communication and survival traits. Additionally, the lower reproduction observed in Russia *D. lummei* offspring across temperature treatments suggests potential fitness trade-offs that could impact population persistence under changing climate conditions.

One limitation of this study is the focus on male mate choice. Given that CHCs are known to influence female mating decisions in many *Drosophila* species, future experiments should incorporate female choice assays to determine whether CHCs play a more significant role in female-driven mate selection. Additionally, expanding the sample size and conducting trials under varied environmental conditions may provide more robust insights into CHC-mediated reproductive isolation. Further genetic analyses, including RNA sequencing of CHC-producing tissues, could help identify specific genes underlying CHC variation and their potential roles in ecological adaptation. Experiments in this thesis suggest that CHCs may contribute to ecological divergence between *D. lummei* populations from Japan and Russia. While CHC differences may

play a role in desiccation resistance, their role in mate choice is still inconclusive in this species. These findings provide valuable insights into how environmental factors shape chemical communication and adaptation, contributing to our broader understanding of ecological speciation.

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