A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Comparative Study on the Effects of Dietary Macronutrient Composition on Life-History Traits in Two Sibling Species, *Drosophila melanogaster* and *D. simulans*

음식물의 거대영양소 조성이 자매종인 노랑초파리(*Drosophila melanogaster*)와 어리노랑초파리(*D. simulans*)의 생활사 형질에 미치는 영향에 관한 비교연구

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Program in Entomology

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UNDER THE DIRECTION OF ADVISER KWANG PUM LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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August, 2021

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FOR THE DEGREE OF MASTER OF SCIENCE
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ABSTRACT

Comparative Study on the Effects of Dietary Macronutrient Composition on Life-History Traits in Two Sibling Species, *Drosophila melanogaster* and *D. simulans*

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Recent advances in nutritional ecology suggest that the intake of macronutrients, such as protein and carbohydrate, is one of the most decisive determinants of evolutionary fitness in insects. The two sibling species of fruit fly (Diptera: Drosophilidae), *Drosophila melanogaster* Meigen and *D. simulans* Sturtevant, have long been used as the key model organisms in ecological and evolutionary research. These two species diverged from a common ancestor about 2 million years ago and are known to coexist all over the globe, including Korea. Despite their phylogenetic closeness, the two species are reported to differ substantially in many aspects of their biology. While there is a wealth of studies comparing thermal responses between the two species, studies that explicitly compared how these two species respond to dietary macronutrient composition are rare.

The major goal of this thesis is to conduct a comparative analysis on the

effects of dietary protein and carbohydrate composition on multiple life-history traits between *D. melanogaster* and *D. simulans*. Using the natural populations of these two species, I performed two separate experiments in this thesis. In Experiment 1, I tested the effect of dietary ratio of protein-to-carbohydrate (P:C ratio) on key life-history traits expressed during the larval and adult stages in *D. melanogaster* and *D. simulans*. Here, *D. melanogaster* and *D. simulans* were subjected to one of eight chemically defined diets that differed in P:C ratio (1:16, 1:8, 1:4, 1:2. 1:1, 2:1, 4:1, or 8:1) but with the fixed total protein and carbohydrate (P+C) concentration (120 g l^{-1}). Compared to *D. simulans, D. melanogaster* took longer to complete the preadult stage but exhibited higher preadult survivorship and heavier body mass at adult emergence. For both species, an increase in dietary P: C ratio resulted in improved larval survivorship, increased body mass, and faster development. The body mass of *D. melanogaster* peaked at the P:C ratio of 1:4 and decreased as the ratio either increased or decreased from this optimal P:C ratio. In contrast, the body mass of *D. simulans* was insensitive to dietary P:C ratio. Lifespan was significantly longer for females as compared to males in both species. Regardless of sex, *D. melanogaster* lived longer in low-protein, high-carbohydrate diets, but exhibited significantly reduced lifespan as dietary P:C ratio rose above 1:2. Strikingly, the lifespan of *D. simulans* was not significantly influenced by dietary P:C ratio. For both species, egg production rate increased with rising dietary P:C ratio, but the extent of such increase was more pronounced in *D. simulans* than in *D. melanogaste*r. Female fecundity was thus significantly greater for *D. simulans* versus *D. melanogaster* at dietary P:C ratios higher than 1:2.

Experiment 1 was designed to examine only the effect of dietary P:C ratio and so it was not feasible to assess the separate and interactive effects of different macronutrients. In order to overcome this limitation, I applied the Nutritional

Geometry in Experiment 2 to construct nutritional performance landscapes for various life-history traits and measures of fitness in *D. melanogaster* and *D. simulans*. In this experiment, two species were assigned to one of 28 chemically defined diets that varied in dietary P:C ratio (1:16, 1:8, 1:4, 1:2. 1:1, 2:1, or 4:1) and in P+C concentration (60, 120, 180, or 240 g l^{-1}). Similar to Experiment 1, *D. melanogaster* had higher preadult survivorship, longer development time, and heavier body mass than *D. simulans*. Overall, the shape of the nutritional performance landscapes was not significantly different between two species, indicating that two species responded to dietary protein and carbohydrate in a qualitatively similar manner. In this study, the fitness of two sibling species was directly measured across a wide spectrum of dietary protein and carbohydrate, which was possible by quantifying the net reproductive rate (R_0) and intrinsic rate of population increase (r). For both species, these parameters of fitness increased progressively as a function of increasing dietary protein concentration. However, the extent to which fitness parameters increased with increasing protein concentration was more pronounced in *D. simulans* as compared to *D. melanogaster.* Despite its lower preadult survivorship, *D. simulans* had a significantly higher fitness than *D. melanogaster*.

In this thesis, I have compared how dietary protein and carbohydrate composition influenced the life-history traits and evolutionary fitness of two closely-related *Drosophila* species. By comparing the nutritional performance landscapes between these two species, the nutritional niche of these two species was suggested to be largely overlapped with one another. There are several candidate mechanisms that may play role in maintaining the coexistence of these sibling species in nature, including the trade-off between reproduction and the probability of survival, environmental heterogeneity, and resource partitioning. Collectively, the results reported in this thesis highlight the important yet

neglected role played by nutrition in mediating evolutionary process and ecological interactions in these two sibling species.

Key words: Development, Fitness, Fruitfly, Lifespan, Nutritional Geometry, Reproduction, Nutritional niche, Coexistence

Student number; 2019-27355

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INTRODUCTION

Understanding the process of speciation is one of fundamental challenges in evolutionary biology (Mayr, 1963; Coyne, 1994; Coyne and Orr, 2004). One powerful approach to study the mechanisms underlying speciation is the comparative analysis of morphological, physiological, behavioral and ecological traits among closely related species (Harvey and Pagel, 1991). To date, the most important model system for studying speciation and organismal evolution is the group of fruit flies species belonging to the genus *Drosophila* (Diptera: Drosophilidae) (Coyne and Orr, 1989; 1997; Orr et al., 2007). There are over 1,450 described species in the entire genus *Drosophila* (Markow and O'Grady, 2005), including the most well-known *Drosopohila melanogaster. D. melanogaster* has long been used as the key study organisms in many disciplines in biological research, including genetics, physiology, ecology, and evolution (Kohler, 1994; Powell, 1997; Jennings, 2011; Buchon et al., 2014; Ma et al., 2018). This species originated from sub-Saharan Africa (Lachaise et al., 1988) and successfully colonized temperate regions in all continents and islands (Markow, 2015). *D. simulans* is a sibling species of *D. melanogaster.* These two species diverged from their common ancestor just about 2 million years ago and are morphologically almost indistinguishable from one another (Powell, 1997). They

are known to share approximately 96% of the genome and to produce their sterile unisexual hybrid progeny (Andrew et al., 1996). Just like *D. melanogaster*, *D. simulans* is distributed across the globe (David and Capy, 1988; Lachaise et al., 1988; Andolfatto, 2005; Markow, 2015) and coexists with *D. melanogaster*. Together with *D. hydei*, *D. immigrans*, and *D. busckii*, these two sibling species are known to form so-called 'cosmopolitan guild' of *Drosophila* (Atkinson and Shorrocks, 1977; Markow, 2015).

Despite their phylogenetic closeness, two species are reported to differ substantially in many aspects of their biology, including courtship behavior, foraging, life-histories, ecophysiology, protein polymorphisms, etc (Parsons, 1975, 1983; Parsons and Stanley, 1981; David et al., 1983, 2004; Gilbert et al., 2004; Capy et al., 2004), making them ideal subjects for studying speciation through ecological adaptation. It has been generally demonstrated that *D. melanogaster* has longer preadult development time, heavier body mass, longer lifespan, and higher fecundity than *D. simulans* (David et al., 2004; Gilbert et al., 2004). Moreover, *D. melanogaster* is reported to be more refractory to environmental stressors, such as starvation, desiccation, alcohol, acetic acid, and CO_2 than its sibling species (see literature cited in David et al., 2004). One of the most studied aspects of difference between the two species is the way in which each species responds to temperature. Numerous studies have documented that *D. melanogaster* is generally less susceptible to heat and cold stress and has higher

upper thermal limits for larval development than *D. simulans* (see literature cited in David et al., 2004). Furthermore, the thermal parameters that characterize the thermal niche of a species, such as developmental zero, optimum temperature, and temperature of maximum rate, are shown to be slightly higher in *D. melnoagster* than in *D. simulnas* (Gilbert et al., 2004). Despite such wealth of information on their thermal responses, studies that explicitly compared the nutritional responses of these two sibling species of *Drosophila* are rare (but see Watanabe et al., 2019, Watada et al., 2020).

Nutrition dominates nearly all biological processes in all organisms, including insects (Simpson and Raubenheimer, 2012). However, our understanding of the role played by nutrition in the ecology and evolution of an organism has been greatly hampered by its complex and multivariate nature. Nutrition constitutes multiple components, such as macronutrients (protein, carbohydrate, lipids) and micronutrients (vitamin, salt, trace elements, etc.), and these multiple components operate interactively (Simpson et al., 2015). For example, the addition of a specific macronutrient to the diet not only increases its own concentration but also changes the overall balance of multiple macronutrients, which is known to have profound consequences for fitness in many insects in its own right. It is also important to note that the effects of these macronutrients are not always linear (Simpson et al., 2015). In order to resolve these problems, Stephan Simpson and David Raubenheimer have devised an integrative, multi-

dimensional state-space modeling framework that is now known as Nutritional Geometry (NG), which enabled researchers to analyze and interpret the complex interactions among multivariate nutritional factors with unprecedented accuracy and clarity (reviewed in Raubeheimer and Simpson, 1993; Simpson and Raubenheimer, 1993, Simpson et al., 2015). In this analytical framework, the nutritional status of individuals, its optimal nutritional requirement, and the nutritional composition of the foods can be visually represented as coordinates or rails in the two-dimensional nutrient space where the gradients of two different macronutrients, such as protein and carbohydrate, are shown on each axis. The core innovation of the NG is that it has enabled us to construct three-dimensional nutritional performance landscapes visualizing how the continuous variation in dietary protein and carbohydrate content affects the phenotypic expression of a focal trait. The nutritional optimum for a focal trait can be identified by locating the summit of the performance mountain (Lee et al., 2008; Simpson and Raubeheimer, 2012). The NG has been established as the standard methodology for studying nutritional ecology in diverse organisms ranging from microbes to primates (reviewed by Simpson and Raubenheimer, 2012). Through inspecting the topography of the nutritional landscapes, one can predict 1) how a species has adapted to its past and present nutritional environment, 2) how it will respond to changing nutritional circumstances, and 3) how and to what extent nutrition alters trade-offs among life-history traits.

In recent years, NG has emerged as a powerful and innovative analytical platform for studying ecological niche from a nutritional perspective (Kearney et al., 2010; Machovsky-Capusak et al., 2016; Shik and Dussutour, 2020). The ecological niche is defined as a hyper-volume in multi-dimensional environmental space within which stable populations can be maintained (Hutchinson, 1957; Chase and Leibold, 2003; Schoener, 2009). There have been increasing cases of studies that applied NG to explore multidimensional, nutritional niches in insects (Behmer and Joern, 2008; Krabbe et al., 2019; Crumière et al., 2020; Shik et al., 2021). Using NG, for example, Behmer and Joern (2008) compared the patterns of protein and carbohydrate selection in seven coexisting grasshopper species distributed across the prairies of North America and found evidence that these grasshoppers are partitioning their nutritional niche by occupying different protein-carbohydrate coordinates in the nutrient space. In order to define the nutritional niche, it is necessary to determine the nutritional conditions that support positive net population growth. However, studies that actually characterized the nutritional niche based on the actual measurement of population growth are scarce.

The main objective of this thesis was to compare the nutritional landscapes for fitness and multiple life-history traits between the two sibling species of *Drosophila*, *D. melanogaster* and *D. simulans*. In nature, these two sibling species consume yeasts and other microbes growing in rotten or ripened

fruits as their main diet during their entire life stages (Begon, 1982; Starmer and Fogleman, 1986; Markow and O'Grady; 2005). This indicates that protein and carbohydrate are the two main macronutrients primarily consumed by these species in nature while the dietary contribution of lipids is likely to be rather minor. This thesis consists of two separate but interconnected experiments. In the first experiment (Experiment 1, henceforth), I first tested the effect of dietary ratio of protein-to-carbohydrate (P:C ratio) on key life-history traits expressed during the larval and adult stages in *D. melanogaster* and *D. simulans*. It is widely held that the balance between protein and carbohydrate is the most influential determinant of lifespan, reproduction, and other life-history traits in a wide variety of insects, including *drosophilid flies* (Lee et al., 2008; Jang and Lee, 2018). In this experiment, I measured multiple larval and adult life-history traits from two sibling species that received ad libitum supply of one of eight chemically defined diets with differing P:C ratio. The total concentration of protein plus carbohydrate (P+C) was fixed in all these eight diets. The species differences in the life-history response to dietary P:C ratio was graphically compared by plotting continuous nutritional reaction norms where lines were drawn to connect the trait phenotypes expressed along a wide range of continuous nutritional gradients for each species. Experiment 1 was designed to consider only the effect of dietary P:C ratio among nutritional factors and so it was not possible to assess the separate and interactive effects of different macronutrients. In order to overcome this limitation, a large

scale NG-based experiment was performed in the second experiment (Experiment 2). In this experiment, I measured larval life-history traits and fitness reared on one of 28 chemically defined diets that varied systematically in the P:C ratio (P:C) and in P+C concentrations and used these data to construct the nutritional performance landscapes of the two sibling species . The most important aspect of Experiment 2 is the direct quantification of Darwinian or evolutionary fitness, which enabled me to characterize and compare the fundamental nutritional niche of the two sibling species of *Drosophila*. (Lee, 2018). In this thesis, there are three specific research questions to be addressed. First, are there any significant differences in Darwinian fitness between the natural population of *D. melanogaster* and *D. simulans*? Second, how do these two sibling species differ in their fitness responses to dietary protein and carbohydrate? In other words, do these two species differ in their nutritional niche? Third, is there any experimental evidence or sign for nutritional niche partitioning between these two coexisting species?

MATERIALS AND METHODS

1. Experimental flies

All experiments in this study were conducted using the wild-caught natural populations of *D. melanogaster* and *D. simulans*. Following the advice of David et al (2005), these outbred natural populations were founded from numerous isofemale lines for each species. Flies used for founding the natural populations of these two species were collected in early October 2019 from various locations around Mt. Gwanak, Seoul, Republic of Korea (37°48´N, 126°93´E) using 150-ml fly rearing bottles containing 20 ml of 10% molasses fixed with 4% agar and a piece of banana as traps. Ten banana-molasses traps were placed along the southern edge of the mountain at the intervals of ca. 500 meters to sample flies representing a broad range of natural populations in the region. To establish isofemale lines for each species, all adult flies captured in these ten traps were pooled and sexed by the inspection of the sexcomb. I randomly selected 100 females from this pool. These female individuals were then individually transferred to 20-ml of fly vials containing 7 ml of the standard culturing medium (90.6 g dextrose, 68 g dry yeast, 42.8 g cornmeal, 6.5 g agar, 4.5 mL propionic acid. 1 g Nipagin per 1 l distilled water) and the larvae hatched from these eggs were raised until adulthood. The species of flies assigned to each vial was

identified according to the morphological differences in the genital arch of their male progeny (Markow and Grady, 2005). For each species, I established ca. 40 isofemale lines. These newly found isofemale lines were cultured for three generations on the standard culturing medium before they were pooled for founding the outbred populations for each species. To establish the outbred population of each species, freshly eclosed virgin male and female adults emerged from all isofemale lines (ca. 100 male and female flies per line) were put together and released into a plastic cage (21 cm × 41 cm × 21 cm) containing molasses-agar plates (10% molasses fixed in 4% agar in 9-cm diameter Petri dishes) seeded with live yeast paste as oviposition substrate. Flies were allowed to feed and mate for 2 days before they received a fresh oviposition substrate on which they laid eggs for 4 h. Eggs laid on the oviposition substrate was washed with 1×phosphate-buffered saline (PBS) and poured into a 50 ml conical falcon tube. When all eggs sunk to the bottom of the falcon tube, 20 μl of this precipitated egg suspension (ca. 200-250 eggs) was transferred using micropipette and seeded into each 150 ml fly bottles containing 25 ml of the standard rearing diet (Clancy and Kennington, 2001). For each species, more than 15 bottles were seeded to start the first generation of the outbred natural population. Since their establishment, the outbred populations of *D. melanogaster* and *D. simulans* had been maintained on the standard rearing diet in an incubator set at 23℃ under a 12 h: 12 h light:dark photoregime and 70% relative humidity. To avoid overcrowding, I ensured that all

flies were raised at a constant density of 200-250 larvae per bottle.

2. Experimental diets

In this study, I prepared a total of 29 chemically defined diets following the protocol described by Jang and Lee (2018). These experimental diets contained one of four concentrations of protein plus carbohydrate (P+C = 60, 120, 180, or 240 g l^{-1}), each with seven or eight ratios of protein-to-carbohydrate (P:C = 1:16, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, or 8:1). The exact protein and carbohydrate concentrations of these 29 experimental diets are summarized in Table 1. Sodium caseinate (Sigma C8654) and sucrose (Sigma S8378) were used as the source of protein and carbohydrate, respectively. Apart from these two macronutrients, all diets comprised fixed concentrations of dietary lipids (0.3 g l^{-1} cholesterol, 4 g l^{-1} lecithin), salts (0.71 g l^{-1} KH_2PO_4, 3.73 g l^{-1} K_2HPO_4, 0.62 g l^{-1} $MgSO_4$; 1 g l^{-1} $NaHCO_3$), nucleic acids (0.57 g l^{-1} uridine, 0.64 g l^{-1} inosine), vitamins (0.002 g l^{-1} thiamine, 0.01 g l^{-1} riboflavin, 0.012 g l^{-1} nicotinic acid, 0.0167 g l^{-1} calcium pantothenate, 0.0025 g l^{-1} pyridoxine, 0.0002 g l^{-1} biotin, 0.003 g l^{-1} folic acid), and preservatives (1 g l^{-1} nipagin, 0.3 % propionic acid) (Jang and Lee, 2018). Diets were prepared by homogeneously dissolving all pre-weighed ingredients except vitamins and preservatives in 2% agar solution. The suspension was autoclaved at 121℃ for 10–15 min. Vitamins and preservatives were later added

to the autoclaved suspension when it had cooled down to <50℃ and distilled water was added to adjust the final volume of the medium. After vigorous stirring, the agar-gelled medium was dispensed into a 20 ml polystyrene fly vial in 4 or 7 ml aliquots, stabilized at room temperature for 6 h, and stored at 4℃ until use.

Table 1. Summary of dietary concentrations (gl^{-1}) of protein (P) and carbohydrate (C) in the 29 synthetic diets differing in protein-to-carbohydrate (P:C) ratios and in protein plus carbohydrate (P+C) concentration. The eight diets used in the Experiment 1 are highlighted in bold type and those 28 diets used in the Experiment 2 are underscored.

Source	**DF**	**MS**	***F***	***P***
Sex	1	1.62×10^{-1}	519	<0.001
Species	1	1.35×10^{-1}	432.35	<0.001
Dietary P:C ratio	7	1.04×10^{-2}	33.3	<0.001
Sex × Species	1	8.13×10^{-3}	26.1	<0.001
Sex × Dietary P:C ratio	7	6.96×10^{-4}	2.23	0.031
Species × Dietary P:C ratio	7	3.25×10^{-3}	10.43	<0.001
Sex × Species × Dietary P:C ratio	7	4.59×10^{-4}	1.47	0.176
Error	321	3.10×10^{-4}		

3. Experiment 1: Comparing the nutritional reaction norms

Experimental design and setup

This experiment was designed to compare the effect of dietary P:C ratio on key life-history traits in two sibling species in the genus *Drosophila, D. melanogaster* and *D. simulans*. To this end, I employed a full factorial experimental design, with two *Drosophila* sibling species and eight dietary P:C ratios being fully crossed to yield a total of 16 species-by-diet combinations. For each species, four preadult traits (egg-to-adult viability, development time, body mass, lipid content) and two adult traits (lifespan, egg production rate) were quantified from flies assigned to one of eight diets differing in P:C ratio (1:16, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, or 8:1). These diets contained the same P+C concentration of 120 g l^{-1} . Since protein and carbohydrate yield similar amounts of calories per gram (4 kcal g^{-1}), these eight experimental diets were considered to be near isocaloric. All experiments were carried in an incubator set at 23℃ under a 12 h: 12 h light:dark photoregime and 70% relative humidity.

Protocol

A large number of newly laid eggs were used for the determination of preadult traits for both *D. melanogaster* and *D. simulans.* To obtain these eggs, approximately 1000 freshly emerged male and female adults were released into

the plastic egg laying cage (21 cm × 41 cm × 21 cm) and allowed to lay eggs on molasses-agar plates (see above) supplied with yeast paste (Jang and Lee, 2018) for each species. Eggs laid on this oviposition substrate were washed with 1 × phosphate buffered saline and subsequently harvested by filtering the resulting egg suspension through a fine nylon mesh (70 μm). Using a fine brush, collected eggs were carefully placed inside a square grid (3 mm×3 mm) printed on the strip of overhead projector (OHP) film (8 mm × 24 mm) and arranged in a single layer. These eggs fit in each square were then photographed with a high-resolution DSLR camera (Canon EOS 600D; Canon Inc, Tokyo, Japan). Each film strip loaded with ca. 50 eggs was randomly transferred to each 20-ml fly vial containing 7 ml of one of eight experimental diets. The exact number of eggs seeded to each vial was counted from the photographed images of the eggs placed on each film strip. There were eight replicate vials per diet treatment for each species, resulting in a total of 128 replicate vials being used in this experiment. For each replicate vial, the newly eclosed adults were collected every 4 h and the time of their emergence was recorded. Egg-to-adult viability or preadult survivorship was calculated as the percentage of eggs that successfully reached the adult stage for each replicate vial. Development time was determined as the time (h) taken from egg to adult eclosion for individual flies emerged from eight replicate vials per diet treatment for each species. Files collected from these replicate vials were pooled and killed by freezing at -20℃. Carcasses of these

freeze-killed flies were sexed by inspecting their sex comb and then flies of each sex were randomly divided into 12 cohorts of five flies per diet treatment. Cohorts were dried in an oven set at 65 °C for 48 h and then weighed to the nearest 1 μg using a BM-22 analytical balance (A & D Co. Ltd, Tokyo, Japan). The body mass of individual flies was calculated by dividing the mass of each cohort by five. To extract lipids from dried carcasses, dried cohorts were sealed in polyethylene tea bags and soaked in 10 ml of diethyl ether for 24 h. Lipid-extracted cohorts were re-dried and re-weighed. The difference in dry body mass before and after lipid extraction was taken as the lipid content of each cohort. The proportion of lipid stored in each cohort was computed as the fraction of lipid content in the dry body mass of each cohort.

The measurement of adult lifespan was carried out using several hundred adult flies derived from the outbred natural population of each species (see above). These flies were reared throughout their larval stage on the standard rearing diet at a consistent rearing density of 200-250 per bottle for at least three generations. Newly ecolosed adult flies of each species were collected within 24 h of adult eclosion and immediately transferred into 150-ml fly bottles containing 25 ml of the standard rearing diet at a density of 100-120 flies per bottle. There, flies were allowed to mate for 48 h. For each species, mated male and female flies were separated under mild CO_2 anesthesia and grouped as cohorts of 50 same-sex individual flies. Cohorts were housed in plastic fly demography cages (7 cm × 7

cm × 10 cm) that had one side-arm inlet and a breathable mesh (4-cm diameter) on the lid. The inlet accommodates a 20-ml fly vial containing 4 ml of one of eight experimental diets. To supply water sources, a piece of cotton soaked with distilled water was placed on the lid of the demography cage. Experimental diets and water sources were refreshed every 2 days. Dead flies were counted and removed daily until no flies remained alive. There were three replicate cages per diet treatment for each species, resulting in a total of 4,800 flies used in this lifespan assay. These demography cages were rotated within the incubator twice a day to eliminate any undesired effects of microclimate.

For each species, egg production rate was measured from triads of one female and two male individual flies housed in 20-ml fly vials containing 4 ml of one of eight experimental diets. These flies were collected immediately upon adult eclosion from the outbred population, as described above. Each fly triad was transferred into a fresh vial daily and the number of eggs produced by each triad per day was counted for the first 10 days of adult life (days 3-13). The early-life egg production rate was computed as the average number of eggs produced over 10 days. There were 20 replicate vials per treatment for each species, resulting in a total of 320 vials used in this fecundity assay.

Statistical analysis

The effects of species, dietary P:C ratio, and their interaction on egg-to-adult viability were analyzed using the generalized linear model (PROC GENMOD) with a logic link function and a binomial distribution. The generalized linear mixed model (PROC GLIMMIX) with an identify link and a Gaussian distribution was used to analyze the effects of species, dietary P:C ratio, and their interaction on preadult development time. In this model, the main factors were designated as the fixed effects and the replicate vials nested within diet treatment were included as the random effect. I used the general linear model (PROC GLM) to analyze the effects of species, dietary P:C ratio, and their interaction on body mass, lipid content, lifespan, and egg production rate. In case of those traits that were measured from males and females (body mass, lipid content, lifespan), I first performed three-way ANOVA including all possible two- or three-way interactions between sex, species, and dietary P:C ratio. If I found any significant sex effect or the interactions between sex and other main factors, two-way ANOVAs testing the effects of species, dietary P:C ratio, and their interaction were conducted separately for males and females. To better illustrate the effect of dietary P:C ratio on each measured life-history trait, locally weighted scatterplot smoothing technique (PROC LOESS in SAS) was applied to fit smoothing lines for each trait across dietary P:C ratios. The smoothing parameter used for fitting these lines was 0.6 for all traits. All statistical analyses used in Experiment 1 were conducted using SAS v 9.12 (SAS Institute, Cary, NC, USA).

4. Experiment 2: Comparing the nutritional landscapes

Experimental design and setup

While the main focus of Experiment 1 was the effect of dietary P:C balance on the life-history traits of these two sibling species of *Drosophila*, it is important to remind that the nutritional environments encountered by the animals in nature vary not only in the relative composition of macronutrients but also in the concentration of total macronutrients. To fully understand how two sibling species of *Drosophila* differ in their responses to dietary variation in protein and carbohydrate occurring in nature, it is necessary to construct the nutritional landscapes for key traits related to fitness and to compare their topography between the two species. To map nutritional landscapes, I quantified four preadult traits (egg-to-adult viability, development time, body weight, lipid content) and the two measures of fitness (the net reproductive rate, the intrinsic rate of population increase) from *D. melanogaster* and *D. simulans* flies assigned to one of 28 chemically defined diets. These 28 diets used in this experiment consisted of all possible combinations of seven P:C ratios (1:16, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1) and four P+C concentrations (60, 120, 180, 240 g l^{-1}).

Protocol

The same procedure described in Experiment 1 was followed to measure preadult traits expressed across 28 diet treatments in this experiment. Newly laid eggs by each species were transferred into 10 replicate vials per diet treatment, resulting in a total of 560 vials being used in this assay. Egg-to-adult viability was recorded from all replicate vials while other preadult traits (development time, body mass, lipid proportion) were recorded from individuals assigned to five randomly chosen vials per diet treatment.

Fitness was measured from triads of two males and one female housed in 20-ml fly vials containing 7 mL of one of 28 experimental diets. For each diet, I set up 10 replicate triads by randomly grouping newly eclosed two males and one female flies. Each triad was transferred to fresh vials every other day and this process continued for 44 days. Eggs laid over two days in each vial were allowed to develop into adult at 23℃ under a 12 h: 12 h light:dark photoregime and 70% relative humidity. For each vial, all offspring were collected within 24 h of adult emergence, sexed, and counted. The net reproduction rate (R_0) was calculated as the total number of female offspring produced by a triad over the first 44 days of adulthood. In addition to R_0, I calculated the intrinsic rate of population growth or Euler's *r* (Gotelli, 2001; Wigby and Chapman, 2005) using the Euler equation:

$$\sum_{x=0}^{max\ age} e^{-rx} l_x m_x \approx 1$$

where x is age, l_x is age-specific survival, and m_x is age-specific fecundity. A total of 162,552 offspring flies (*D. melanogaster*: 59,315 flies; *D. simulans*: 103,237 flies) were harvested, sexed, and counted in this fitness assay.

Statistical analysis

Non-parametric thin-plate splines methodology was implemented to map the nutritional landscapes describing how each trait was expressed over a grid of dietary protein and carbohydrate using the Fields package (Nychka et al., 2017) in R v 4.0.2. (R Development Core Team, 2012). The smoothing parameter (λ) that minimized the generalized cross-validation score (GCV) was used to plot these landscapes for each trait. The major advantage of this method is that it does not constrain the shape of the surface (Blows and Brooks, 2003). For each nutritional landscape, the position at which the trait in question was maximized (global maximum) or minimized (global minimum) was estimated using the OptimaRegion package (del Castillo et al., 2016) in R v 4.0.2.

To estimate the linear and nonlinear (i.e., quadratic and correlational) effects of protein and carbohydrate concentration in the diet on each measured traits, I performed polynomial multiple regression using the general linear model (PROC GLM in SAS v 9.2). As recommended by Lande and Arnold (1983), I first ran a model containing only the linear terms of protein (P) and carbohydrate (C)

concentration as fixed factors. From this model, the linear effects of these nutrients were estimated. I next ran a second model in which the quadratic (P^2, C^2) and correlational or cross-product (P × C) terms of both macronutrients were added to the first model. The gradients representing the quadratic and correlational effects of these nutrients were estimated from the second model. The topography of the nutritional landscapes fitted for each trait was compared between species using partial *F*-tests, which tested the significance of pairwise differences in the overall effects of dietary component on nutritional landscapes (Chenoweth and Blows 2005). Prior to performing partial *F*-tests, response variables in question were standardized. All statistical analyses except the thin-plate splines were performed using SAS v 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

1. Experiment 1: Comparing the nutritional reaction norms

1.1. *Preadult life-history traits*

Across all diet treatments, *D. melanogaster* exhibited a higher egg-to-adult viability than *D. simulans* (mean±SE, *D. melanogaster*: 0.61±0.02; *D. simulans*: 0.50±0.03), as indicated by a significant effect of species (χ^2= 94.85, df= 1, P<0.001; Fig. 1). Egg-to-adult viability was significantly affected by dietary P:C ratio (χ^2= 904.52, df= 7, P<0.001). For both species, this measure of preadult survivorship was maintained high (>60%) at the P:C ratios higher than 1:4, but dropped progressively rapidly as the P:C ratio fell below 1:4. The interaction between species and dietary P:C ratio was significant (χ^2= 54.94, df= 7, P<0.001) although the nature of the effect of dietary P:C ratio on egg-to-adult viability was more or less conserved in the two species.

Two species also differed significantly in their development time ($F_{1,102}$=6.78, P=0.011), with *D. melanogaster* taking ca. 21 h longer to reach their adult stage than *D. simulans* (*D. melanogaster*: 342.9±1.44 h; *D. simulans*: 321.8±1.33 h; Fig. 2). Dietary P:C ratio also significantly affected development time ($F_{7,102}$=212.25, P<0.001). For both species, development time was prolonged as a function of decreasing dietary P:C ratio. The extent to which development

time increased in response to low P:C ratio was steeper in *D. melanogaster* as compared to *D. simulans*, as indicated by a significant interaction between species and dietary P:C ratio ($F_{7,102}$=2.42, P=0.025).

Females exhibited ca. 27% and 20% significantly heavier body mass at adult eclosion than males in *D. melanogaster* and *D. simulans*, respectively, as indicated by a significant effect due to sex detected in the three-way ANOVA where species, sex, dietary P:C ratio were the main factors (Table 2; Fig. 3). In this analysis, significant two-way interactions between all combinations of species, sex, and dietary P:C ratio were detected (Table 2), leading me to analyze the effects of species, dietary P:C ratio, and their interaction separately for each sex. For both sexes, the body mass recorded at adult emergence was heavier for *D. melanogaster* versus *D. simulans* (19% for males and 26% for females; Fig. 3A, B), as indicated by a significant effect due to species (male: $F_{1,160}$=173.33, P<0.001; female: $F_{1,161}$=270.37, P<0.001). The interaction term between species and dietary P:C ratio was also found to be highly significant for both sexes (male: $F_{7,160}$=7.21, P<0.001; female: $F_{7,161}$=5.81, P<0.001). This significant interaction was largely attributed to the fundamental differences in the way in which each species qualitatively responded to dietary variation in P:C ratio. As illustrated in Fig. 3 for both males and females, the body mass of *D. melanogaster* was associated with dietary P:C ratio in a nonlinear manner, displaying the peak at the intermediate P:C ratios of 1:2. This convex relationship between body mass and

dietary P:C ratio in *D. melanogaster* was more pronounced in females than in males. In marked contrast, the body mass of *D. simulans* was generally much lighter than that of *D. melanogaster* and remained largely insensitive to the dietary variation in P:C ratio.

A significant three-way interaction between species, sex, and dietary P:C ratio was detected in three-way ANOVA for lipid proportion (Table 3), which led me to analyze the effects of species, dietary P:C ratio, and their interaction separately for each sex. For both sexes, *D. melanogaster* had higher lipid proportion than *D. simulans* across dietary P:C ratios (Fig. 4A, B), as indicated by a significant effect of species (male: $F_{1,160}$=53.11, P<0.001; female: $F_{1,161}$=63.78, P<0.001). Dietary P:C ratio had significant effect on lipid proportion for both sexes (male: $F_{7,160}$=6.71, P<0.001; female: $F_{7,161}$=10.8, P<0.001). Regardless of sex and species, lipid proportion increased gradually as dietary P:C ratio decreased (Fig. 4A, B). The interaction between species and dietary P:C ratio on lipid proportion was not significant for males ($F_{7,160}$=0.69, P=0.682), suggesting that the rate at which lipid content increased as a function of decreasing dietary P:C ratio paralleled between two species in males. This interaction between these two main factors was however significant for females ($F_{7,161}$=2.55, P=0.016), which suggests that the rate at which lipid proportion increased in response to decreasing P:C ratio was steeper in *D. melanogaster* than in *D. simulans* in females.

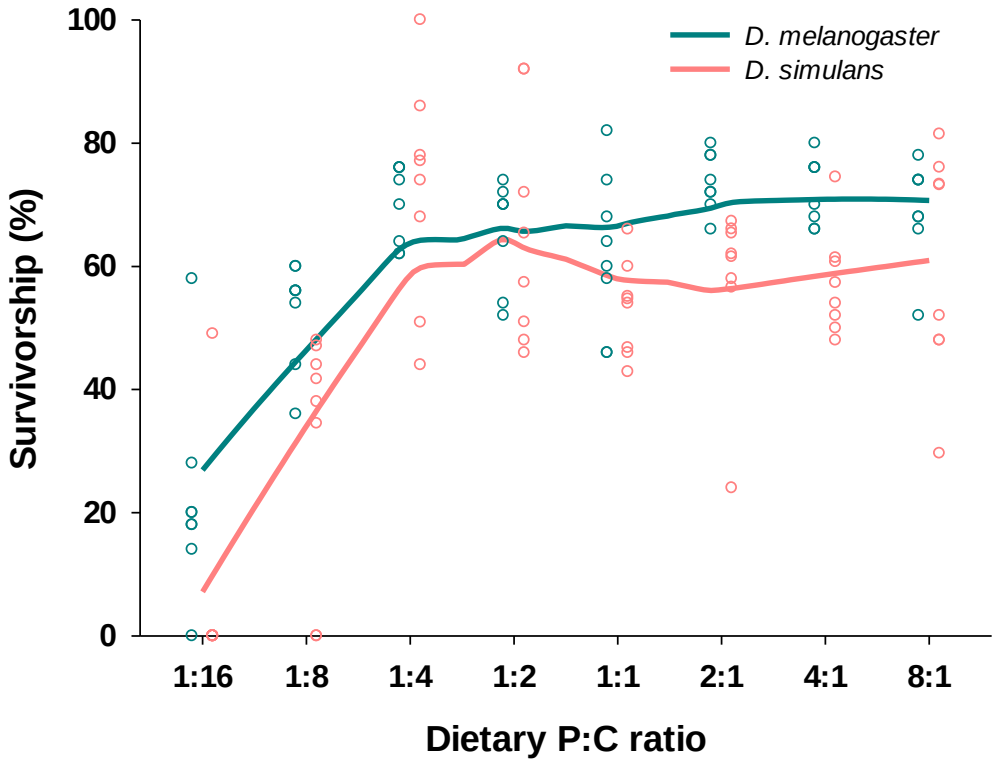

Figure 1. Effects of dietary P:C ratio on preadult survivorship (egg-to-adult viability) of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

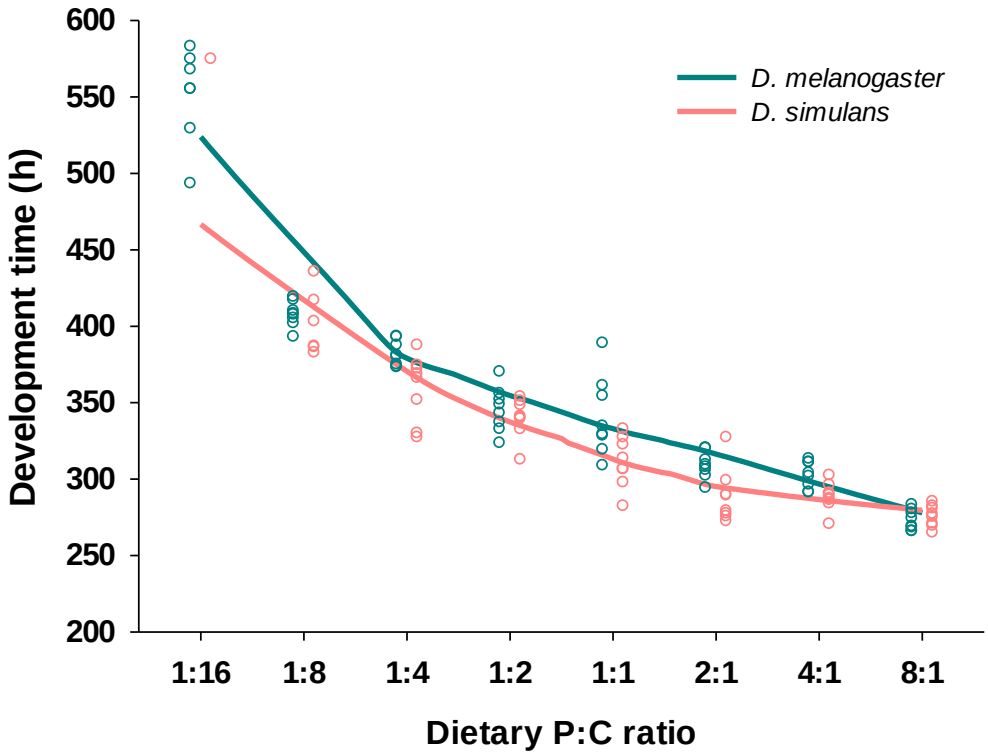

Figure 2. Effects of dietary P:C ratio on preadult development time of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

Table 2. Results of general linear model (GLM) testing the effects of sex, species and dietary P:C ratio on adult body mass at emergence in the two sibling species of *Drosophila*.

Source	**DF**	**MS**	***F***	***P***
Sex	1	1.62×10^{-1}	519	<0.001
Species	1	1.35×10^{-1}	432.35	<0.001
Dietary P:C ratio	7	1.04×10^{-2}	33.3	<0.001
Sex × Species	1	8.13×10^{-3}	26.1	<0.001
Sex × Dietary P:C ratio	7	6.96×10^{-4}	2.23	0.031
Species × Dietary P:C ratio	7	3.25×10^{-3}	10.43	<0.001
Sex × Species × Dietary P:C ratio	7	4.59×10^{-4}	1.47	0.176
Error	321	3.10×10^{-4}		

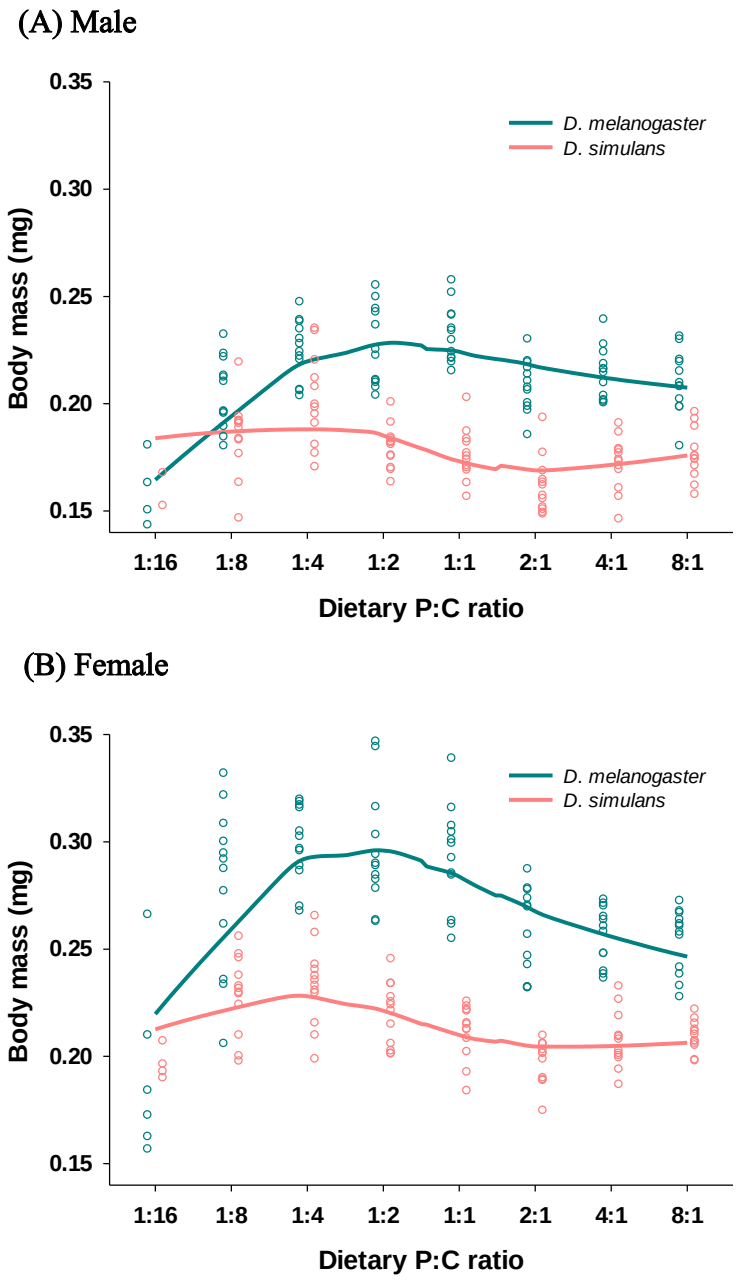

Figure 3. Effects of dietary P:C ratio on (A) male and (B) female body mass at adult emergence of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

Table 3. Results of general linear model (GLM) testing the effects of sex, species and dietary P:C ratio on adult lipid proportion in the two sibling species of *Drosophila*.

Source	**DF**	**MS**	***F***	***P***
Sex	1	5.50×10^{-3}	26.26	<0.001
Species	1	3.87×10^{-2}	184.8	<0.001
Dietary P:C ratio	7	3.09×10^{-3}	14.75	<0.001
Sex × Species	1	6.61×10^{-4}	3.16	0.077
Sex × Dietary P:C ratio	7	4.93×10^{-4}	2.35	0.024
Species × Dietary P:C ratio	7	2.76×10^{-4}	1.32	0.241
Sex × Species × Dietary P:C ratio	7	3.44×10^{-4}	1.64	0.122
Error	321	2.09×10^{-4}		

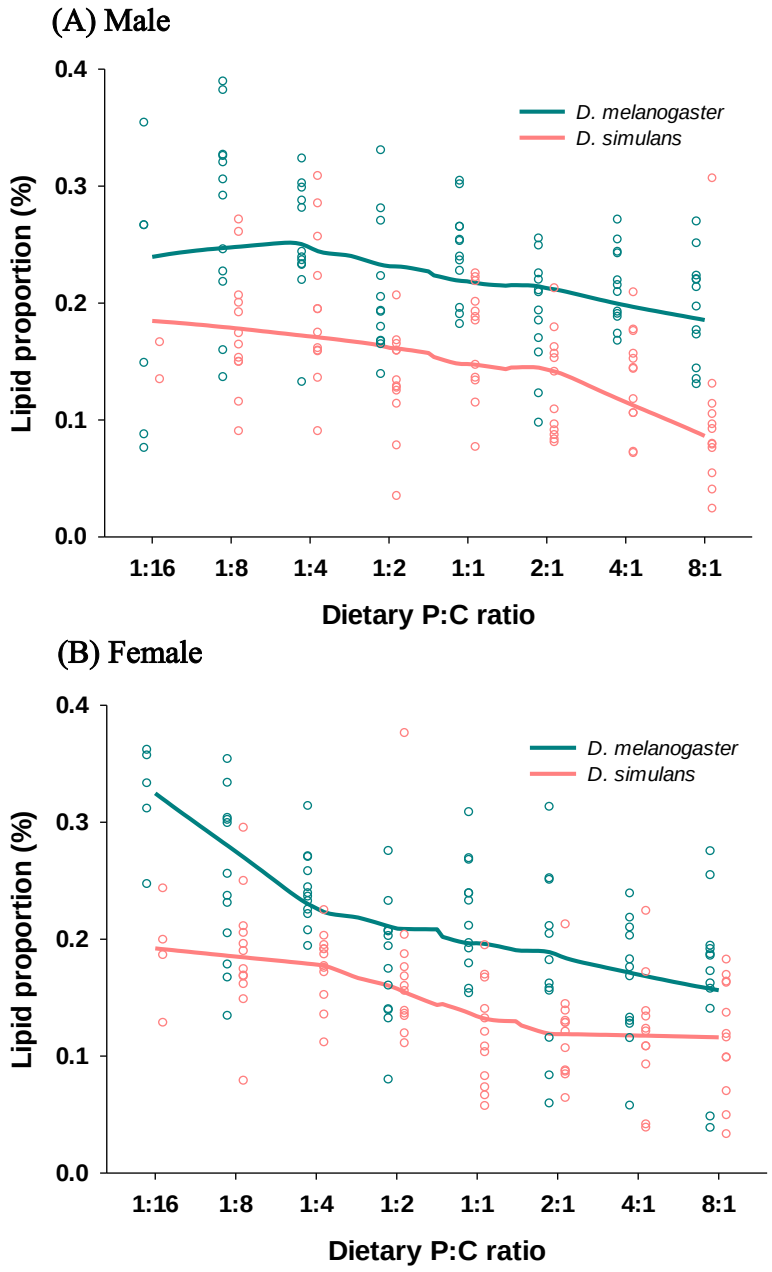

Figure 4. Effects of dietary P:C ratio on (A) male and (B) female lipid proportion at adult emergence of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

1.2. *Adult life-history traits*

Three-way ANOVA results indicated that there were significant two or three-way interactions between species, sex, and dietary P:C ratio for lifespan (Table 4). The effects of species, dietary P:C ratio and their interaction were thus analyzed separately for each sex using two-way ANOVA. Regardless of sex, *D. melanogaster* outlived *D. simulans*, as indicated by a significant effect of species (male: $F_{1,1879}$=224.42, P<0.001; female: $F_{1,2077}$=1009.99, P<0.001). It is important to note that there was a significant interaction between species and dietary P:C ratio (male: $F_{7,1879}$=36.71, P<0.001; female: $F_{7,2077}$=68.6, P<0.001), indicating that the way in which lifespan responded to dietary P:C ratio differed significantly between the two species (Fig. 5A, B). The lifespan of male *D. melanogaster* was maintained high (ca. 60 days) at P:C ratios ranging between 1:16 and 1:2 and shortened as the P:C ratio increased above 1:2 (Fig. 5A). However, the mean lifespan of male *D. simulans* demonstrated rather reversed pattern, showing a marginal increase in lifespan as the P:C ratio increased. As an outcome of this interaction, male *D. melanogaster* lived ca. 24 days longer than male *D. simulans* at P:C ratios ranging between 1:16 and 1:2, but there was no significant species difference in male lifespan between the two species at P:C ratios higher than 1:2 (Fig. 5A). In a manner similar to their male conspecifics, the lifespan of female *D. melanogaster* was maintained high (ca. 90 days) at P:C ratios ranging between 1:16 and 1:2 and fell rapidly as the P:C ratio increased above 1:2 (Fig. 5B).

However, the lifespan of female *D. simulans* remained constantly low (ca. 52 days) and insensitive to dietary P:C ratios. As a consequence of this different lifespan responses between the two species, female *D. melanogaster* lived ca. 38 days longer than female *D. simulans* at P:C ratios ranging between 1:16 and 1:2, However, no species difference in female lifespan was found at P:C ratios higher than 1:2 (Fig. 5B)..

For both species, the rate of egg production over the first 10 days of adulthood increased as a function of increasing dietary P:C ratio (Fig. 6), as indicated by a significant effect of dietary P:C ratio ($F_{7,304}$=139.6, P<0.001). Importantly, there was a significant interaction between species and dietary P:C ratio ($F_{7,304}$=19.52, P<0.001), indicating that the extent to which egg production rate increases with increasing dietary P:C ratio was significantly greater for *D. simulans* as compared to *D. melanogaster* (Fig.6). The two species showed similar egg production rate at P:C ratios ranging between 1:16 and 1:2, but *D. simulans* produced more eggs per day than *D. melanogaster* at P:C ratios higher than 1:1. The sex difference in egg production rate was most pronounced at the highest P:C ratio of 8:1. On average, *D. simulans* produced ca. 44% more eggs per day than *D. melanogaster* over the first 10 days of their adulthood (*D. melanogaster*: 13.37±0.66 eggs per day; *D. simulans*: 19.31±1.02 eggs per day), as indicated by a significant effect of species ($F_{1,304}$=106.44, P<0.001).

Table 4. Results of general linear model (GLM) testing the effects of sex, species and dietary P:C ratio on lifespan in the two sibling species of *Drosophila*.

Source	**DF**	**MS**	***F***	***P***
Sex	1	156590.31	531.26	<0.001
Species	1	309571.19	1050.27	<0.001
Dietary P:C ratio	7	16327.51	55.39	<0.001
Sex × Species	1	28917.39	98.11	<0.001
Sex × Dietary P:C ratio	7	14174.69	48.09	<0.001
Species × Dietary P:C ratio	7	28158.24	95.53	<0.001
Sex × Species × Dietary P:C ratio	7	1807.97	6.13	<0.001
Error	3956	294.75		

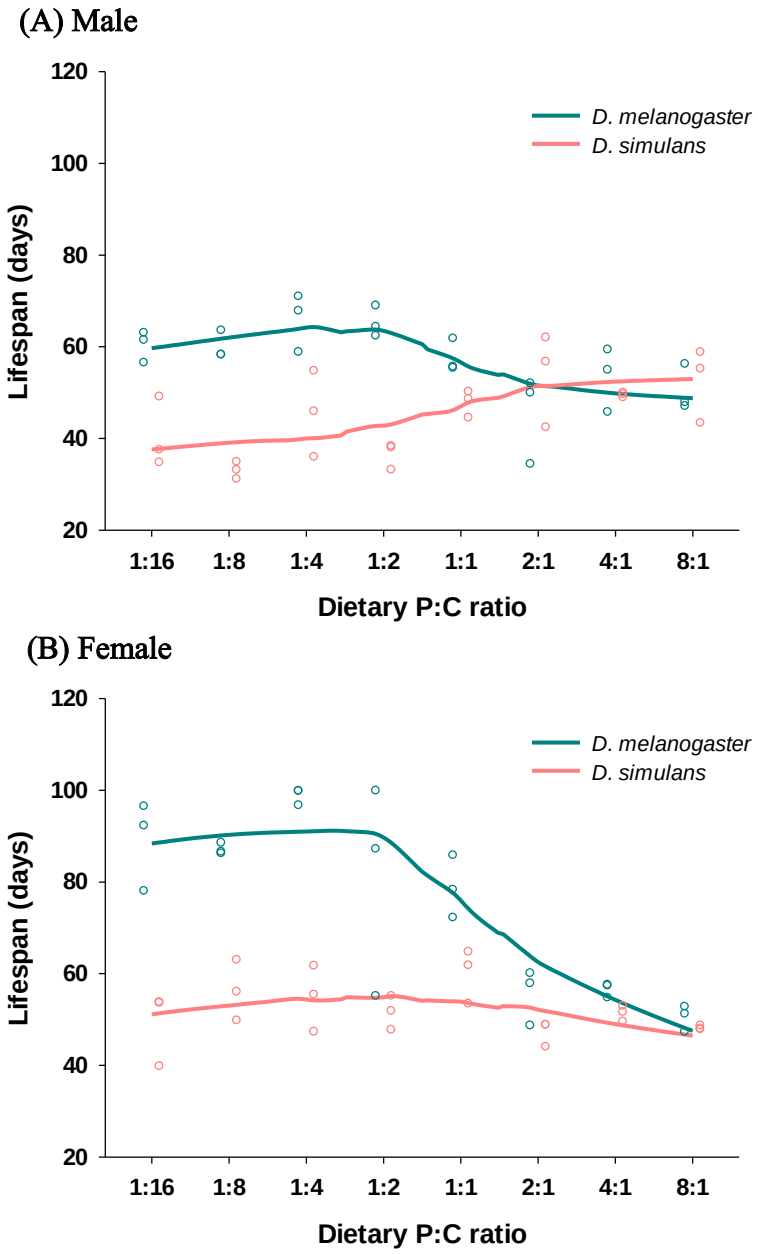

Figure 5. Effects of dietary P:C ratio on (A) male and (B) female lifespan of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

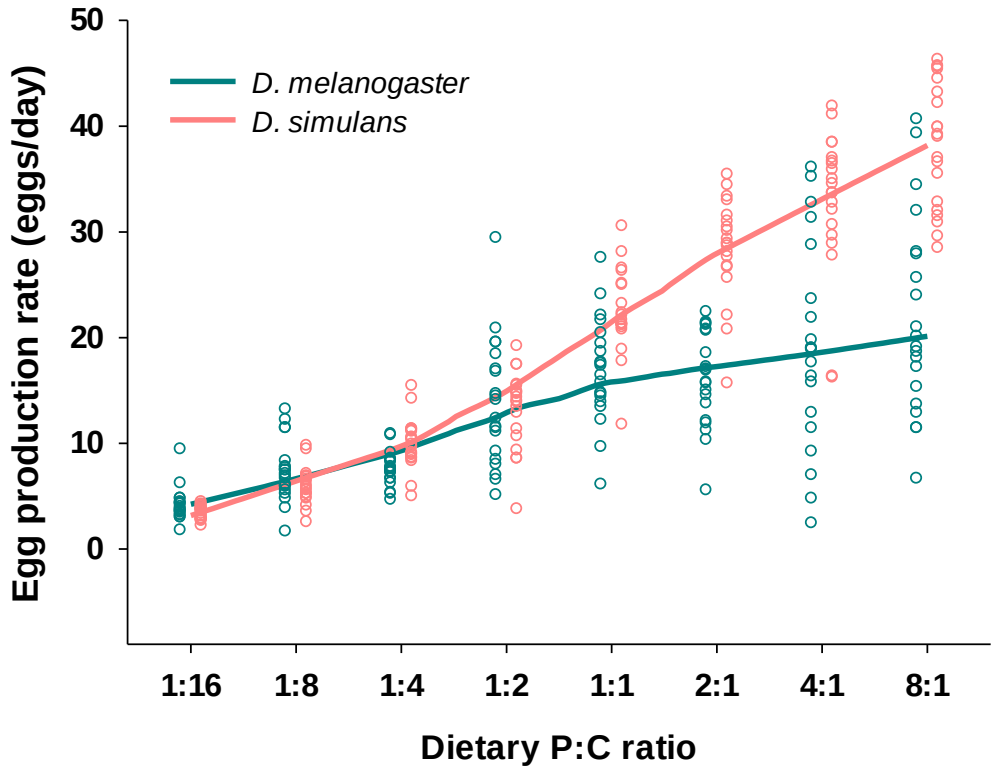

Figure 6. Effects of dietary P:C ratio on egg production rate of the two sibling species of *Drosophila*. *D. melanogaster* and *D. simulans* are represented as dark green and dark orange, respectively.

2. Experiment 2: Comparing the nutritional landscapes

2.1. *Preadult life-history traits*

Across all diet treatment, the proportion of eggs successfully reached the adult stage differed signficanlty between the two species (*D. melanogaster*: 31.85±3.76%, *D. simulans*: 26.57±4.24%; paired t test: t27=2.275, P=0.031), with *D. melanogaster* having a higher survivorship than *D. simulans*. There was a marginal species difference in the shape of nutritional landscape plotted for this trait (partial F test; F5,548=2.26, P=0.047; Fig. 7). The global maximum of preadult survivorship was identified at the P:C ratio of 1.7:1 (P= 52.29 g l-1, C= 30.64 g l-1) for *D. melanogaster* and 1:1.4 (P= 43.40 g l-1, C= 61.58 g l-1) for *D. simulans*. The preadult survivorship of both species was significantly affected by a negative quadratic gradient of protein concentration, which is suggested by the fact that preadult survivorship decreased as the protein concentration in the diet either increased or decreased from the global maximum (Table 5). The preadult survivorships of both species were also significantly affected by the linear gradient of carbohydrate concentration (Table 5), exhibiting a gradual decrease as carbohydrate concentration increased (Fig. 7). For both species, there was a significant negative cross-product gradient of protein and carbohydrate concentration, indicating that preadult survivorships decreased as protein concentration increased and carbohydrate concentration decreased (Table 5).

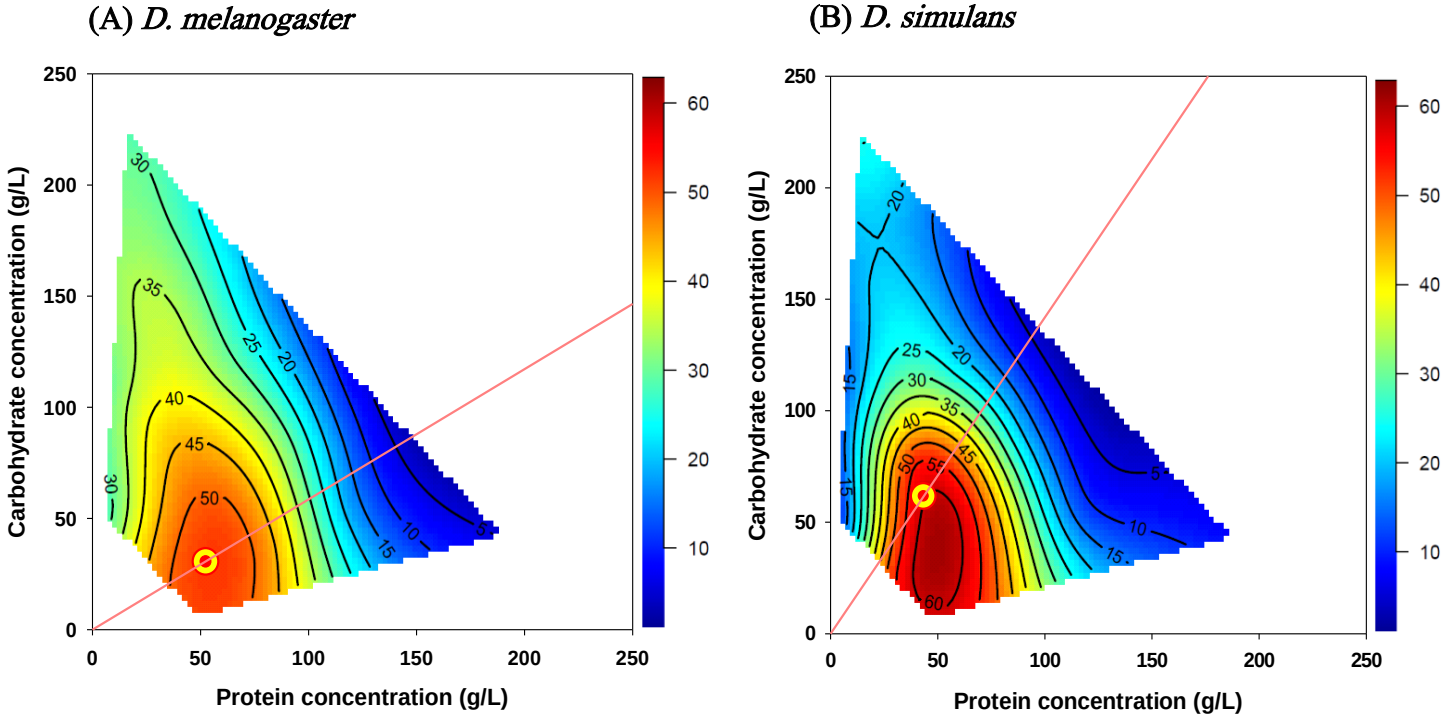

Figure 7. Nutritional landscapes for preadult survivorship (egg-to-adult viability) expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinate where the trait is maximized is represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 5. Results of the second-order polynomial multiple regressions on preadult survivorship (egg-to-adult viability) expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each species.

Species	Larval survivorship	Linear gradients		Quadratic gradients		Cross product gradients
		P	C	P^2	C^2	P × C
D. melanogaster	Gradient ± SE	-2.06×10^{-1} ± 2.57×10^{-2}	-1.07×10^{-1} ± 2.15×10^{-2}	-3.39×10^{-3} ± 4.86×10^{-4}	1.1×10^{-5} ± 3.81×10^{-4}	-3.31×10^{-3} ± 7.63×10^{-4}
	t_{279}	-8.01	-4.99	-6.99	0.03	-4.34
	P	<0.001	<0.001	<0.001	0.977	<0.001
D. simulnas	Gradient ± SE	-1.49×10^{-1} ± 3.24×10^{-2}	-1.59×10^{-1} ± 2.72×10^{-2}	-3.86×10^{-3} ± 6.1×10^{-4}	1.11×10^{-3} ± 4.78×10^{-4}	-2.93×10^{-3} ± 9.59×10^{-4}
	t_{279}	-4.59	-5.86	-6.33	2.32	-3.05
	P	<0.001	<0.001	<0.001	0.021	0.003

D. melanogaster took ca. 17 h more to complete their preadult development than *D. simulans* across all diet treatments (*D. melanogaster*: 377.8 ± 13.45 h; *D. simulans*: 385.3 ± 15.41 h), but the two species did not differ significantly in their mean development time (t_{26}=1.352, P=0.188). The topography of the nutritional landscapes differed between the two species ($F_{5,5675}$=16.45, P<0.001; Fig. 8). The shortest preadult development time occurred at the P:C ratio of 1.4:1 (P= 64.13 g l^{-1}, C= 45.49 g l^{-1}) for *D. melanogaster* and 1.8:1 (P= 60.59 g l^{-1}, C= 34.17 g l^{-1}) for *D. simulans.* For both species, preadult development time was significantly affected by a positive quadratic gradient of P concentration (Table 6), suggesting that preadult development increased as protein concentration either increased or decreased from the global minimum (Fig. 8). It is important to note that this concave relationship was asymmetrical, showing that the extent to which development time was extended was far greater when protein concentration in the diet fell below 25 g l^{-1} than when it rose above 100 g l^{-1}. Preadult development time increased with increasing carbohydrate concentration especially when protein concentration in the diet ranged between 25 and 100 gl^{-1} (Fig. 8), as indicated by a significant negative quadratic gradient of carbohydrate concentration (Table 6). There was a significant negative cross-product of protein and carbohydrate for preadult development time (Table 6), indicating that preadult development time increased when dietary protein concentration was low and dietary carbohydrate concentration was high.

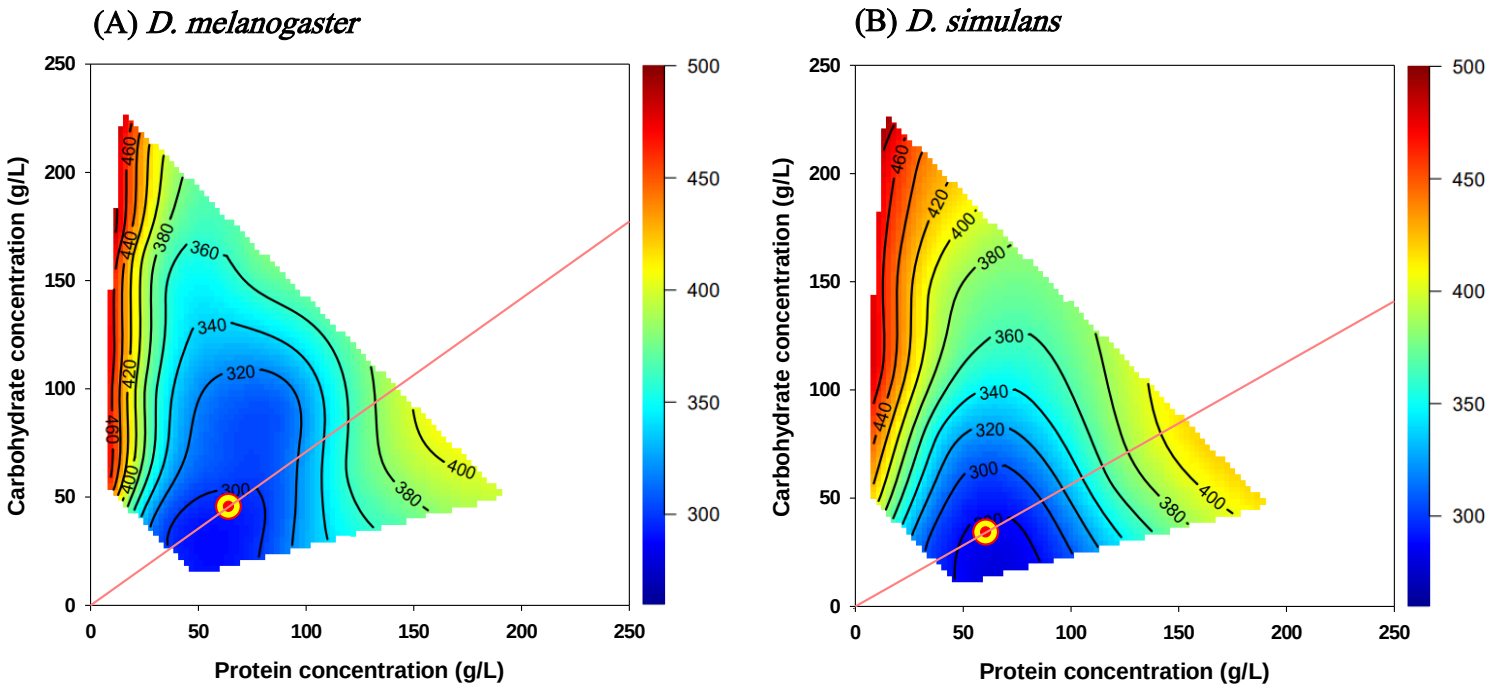

Figure 8. Nutritional landscapes for preadult development time expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinate where the trait is minimized is represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 6. Results of the second-order polynomial multiple regressions on preadult development time expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each species.

Species	Larval development time	Linear gradients		Quadratic gradients		Cross product gradients
		P	C	P^2	C^2	P × C
D. melanogaster	Gradient ± SE	-6.13×10^{-1} ± 3.66×10^{-2}	5.34×10^{-1} ± 1.98×10^{-2}	1.87×10^{-2} ± 5.65×10^{-4}	-1.51×10^{-3} ± 3.02×10^{-4}	-4.67×10^{-3} ± 8.42×10^{-4}
	t_{2942}	-16.75	26.97	33.06	-5.01	-5.55
	P	<0.001	<0.001	<0.001	<0.001	<0.001
D. simulnas	Gradient ± SE	-2.31×10^{-1} ± 3.88×10^{-2}	9.07×10^{-1} ± 2.10×10^{-2}	1.81×10^{-2} ± 6.03×10^{-4}	-2.99×10^{-3} ± 3.18×10^{-4}	-5.02×10^{-3} ± 1.01×10^{-3}
	t_{2745}	-5.95	43.18	30	-9.41	-4.98
	P	<0.001	<0.001	<0.001	<0.001	<0.001

The body mass of male adults at emergence was significantly different between the two species (t_{26}=11.33, P<0.001), with *D. melanogaster* exhibiting ca. 37% heavier male body mass than *D. simulans* (*D. melanogaster*: 0.202±5.77×10^{-3} mg; *D. simulans*: 0.147±6.34×10^{-3} mg). The overall shape of the nutritional landscapes fitted for male body mass also differed significantly between the two species ($F_{5,442}$=14.68, P<0.001; Fig. 9). The most apparent difference between the two species was found in the position of their global maximum. The P:C ratio at which male body mass was maximized was 1:1.2 (P=53.51 g l^{-1}, C= 66.13 g l^{-1}) for *D. melanogaster* but was 1.8:1 for *D. simulans* (P=51.55 g l^{-1}, C= 28.13 g l^{-1}). In *D. melanogaster,* male body mass decreased as the concentration of both protein and carbohydrate in the diet either increased or decreased from the optimal protein and carbohydrate concentration (Fig. 9), as indicated by a significant negative quadratic gradient of both protein and carbohydrate concentration (Table 7). In *D. simulans*, male body mass also had a convex association with dietary protein concentration, as indicated by a significant negative quadratic gradient of protein concentration (Table 7). The male body mass of *D. simulans* decreased steadily as carbohydrate content in the diet increased from the optimal carbohydrate concentration (Fig. 9), as indicated by a negative linear gradient for carbohydrate concentration (Table 7). The cross-product gradient of protein and carbohydrate concentration for male body mass was significantly negative in *D. melanogaster*, but was not significant in *D. simulnas* (Table 7).

Similar to male body mass, *D. melanogaster* had ca. 42% heavier female body mass at adult emergence than *D. simulans* (*D. melanogaster*: $0.256 \pm 9.76 \times 10^{-3}$ mg, *D. simulans*: $0.180 \pm 8.92 \times 10^{-3}$ mg; $t_{26}=9.29$, $P<0.001$). The shape of nutritional landscapes also differed significantly between the two species ($F_{5,433}=13.66$, $P<0.001$; Fig. 10). The P:C ratio at which female body mass was maximized was 1:1.1 (P=56.11 g l^{-1}, C= 61.12 g l^{-1}) for *D. melanogaster* and 1.4:1 (P=44.63 g l^{-1}, C= 32.91 g l^{-1}) for *D. simulans*. The way in which the concentration of protein and carbohydrate influenced the shape of nutritional landscape for female body mass was the identical to that found for male body mass for each species (Table 7; Fig. 10).

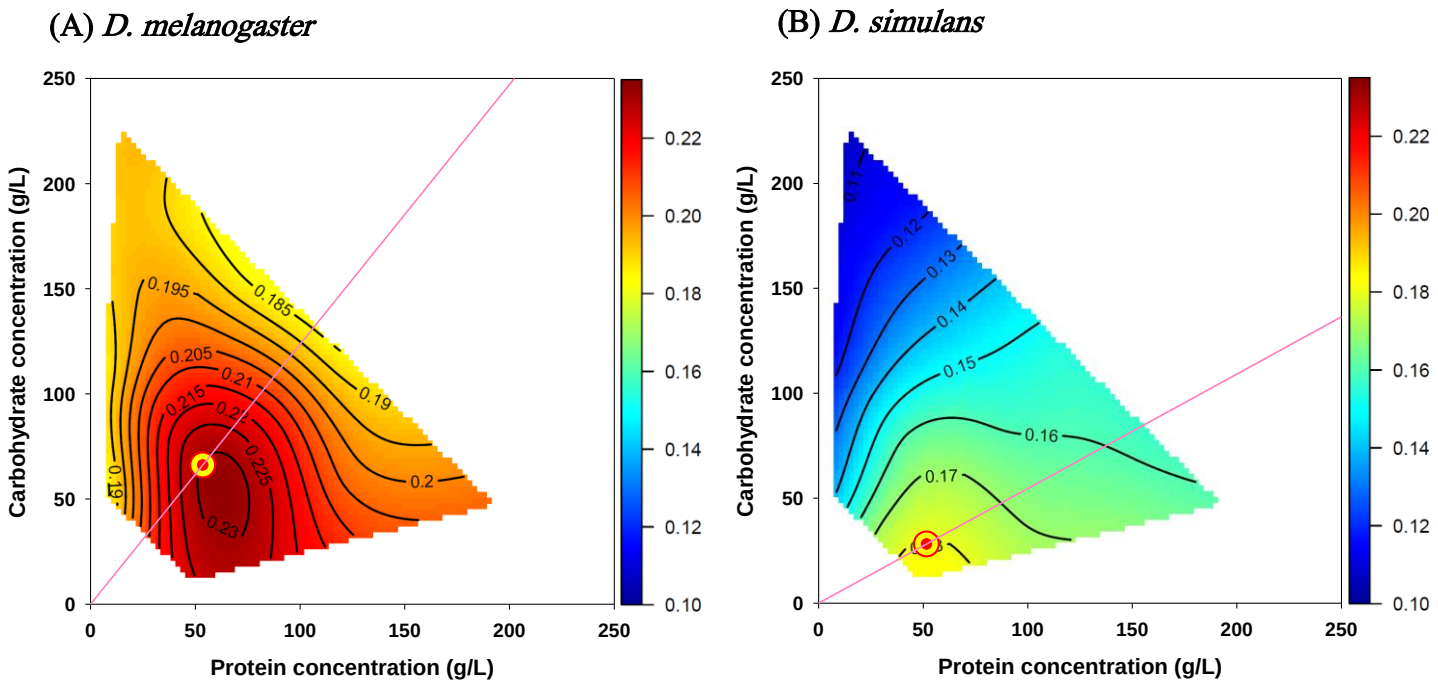

Figure 9. Nutritional landscapes for male body weight expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

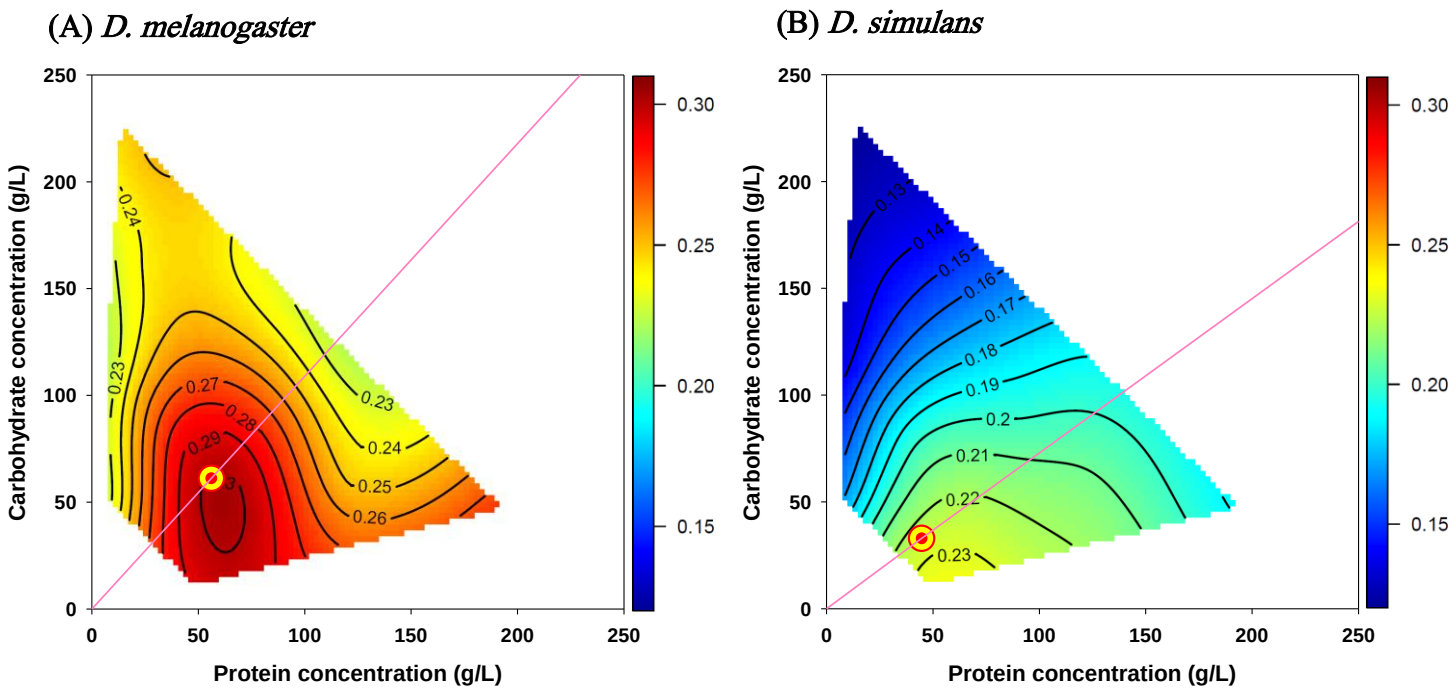

Figure 10. Nutritional landscapes for female body weight expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 7. Results of the second-order polynomial multiple regressions on adult body mass expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each sex and species.

Sex	Species	Adult body mass	Linear gradients		Quadratic gradients		Cross product gradients
			P	C	P^2	C^2	P × C
Male	*D. melanogaster*	Gradient ± SE	1.48×10^{-4} ± 5.94×10^{-5}	-9.2×10^{-5} ± 3.41×10^{-5}	-7.9×10^{-6} ± 1×10^{-6}	-1.1×10^{-6} ± 5.7×10^{-7}	-7.4×10^{-6} ± 1.56×10^{-6}
		t_{237}	2.5	-2.7	-7.86	-1.88	-4.71
		P	0.013	0.007	<0.001	0.061	<0.001
	D. simulnas	Gradient ± SE	-1.6×10^{-5} ± 4.5×10^{-5}	-3.8×10^{-4} ± 2.67×10^{-5}	1.9×10^{-6} ± 8.2×10^{-7}	1.17×10^{-6} ± 4.7×10^{-7}	1.95×10^{-6} ± 1.37×10^{-6}
		t_{211}	-0.35	-14.39	-2.34	2.48	1.42
		P	0.729	<0.001	0.020	0.014	0.158
Female	*D. melanogaster*	Gradient ± SE	2.61×10^{-4} ± 9.33×10^{-5}	-1.5×10^{-4} ± 5.14×10^{-5}	-1.2×10^{-5} ± 1.68×10^{-6}	-1.6×10^{-7} ± 8.8×10^{-7}	-5.8×10^{-6} ± 2.41×10^{-6}
		t_{235}	2.8	-2.88	-6.98	-0.19	-2.4
		P	0.006	0.004	<0.001	0.851	0.017
	D. simulans	Gradient ± SE	-2.1×10^{-5} ± 5.81×10^{-5}	-5.5×10^{-4} ± 3.35×10^{-5}	-3.8×10^{-6} ± 1.03×10^{-6}	1.66×10^{-6} ± 5.8×10^{-7}	2.75×10^{-6} ±1.68×10^{-6}
		t_{204}	-0.36	-16.55	-3.68	2.87	1.63
		P	0.718	<0.001	<0.001	0.005	0.104

The lipid proportion of male adults at emergence was significantly different between the two species (t_{26}=2.18, P=0.039), with *D. melanogaster* exhibiting ca. 24% higher male lipid proportion than *D. simulans* (*D. melanogaster*: 0.290±1.55×10^{-2}; *D. simulans*: 0.234±1.80×10^{-2}). The overall shape of the nutritional landscapes fitted for male lipid proportion also differed significantly between the two species ($F_{5,442}$=8.75, P<0.001; Fig. 11). The P:C ratio at which male lipid proportion was maximized was 1:6 (P=26.04 g l^{-1}, C= 157.37 g l^{-1}) for *D. melanogaster* but was 1:1.4 for *D. simulans* (P=130.99 g l^{-1}, C= 93.76 g l^{-1}). In *D. melanogaster,* male lipid proportion decreased as the concentration carbohydrate in the diet either increased or decreased from the optimal carbohydrate concentration (Fig. 11), as indicated by a significant negative quadratic gradient of carbohydrate concentration (Table 8). In *D. simulans*, male lipid proportion decreased linearly as protein content in the diet decreased from the optimal protein concentration (Fig. 11), as indicated by a positive linear gradient for protein concentration (Table 8). The sign of the significant cross-product gradient of protein and carbohydrate concentration for male lipid proportion was negative in *D. melanogaster*, but was positive in *D. simulans*. (Table 8).

D. melanogaster had ca. 49% higher female lipid proportion than *D. simulas* (*D. melanogaster*: 0.296±1.21×10^{-2}, *D. simulans*: 0.199±2.66×10^{-2}; t_{26}=6.12, P<0.001). The shape of nutritional landscapes also differed significantly

between the two species ($F_{5,433}$=7.53, P<0.001; Fig. 12). The P:C ratio at which female lipid proportion was maximized was 1:1 (P=96.18 g l^{-1}, C= 100.76 g l^{-1}) for *D. melanogaster* and 1:2.3 (P=43.84 g l^{-1}, C= 102.76 g l^{-1}) for *D. simulans*. In *D. melanogaster*, female lipid proportion had a convex association with dietary carbohydrate concentration (Fig. 12), as indicated by a significant negative quadratic gradient of carbohydrate concentration (Table 8). In *D. simulans*, female body mass decreased as the concentration of both protein and carbohydrate in the diet either increased or decreased from the optimal protein and carbohydrate concentration (Fig. 12), as indicated by a significant negative quadratic gradient of both protein and carbohydrate concentration (Table 8).

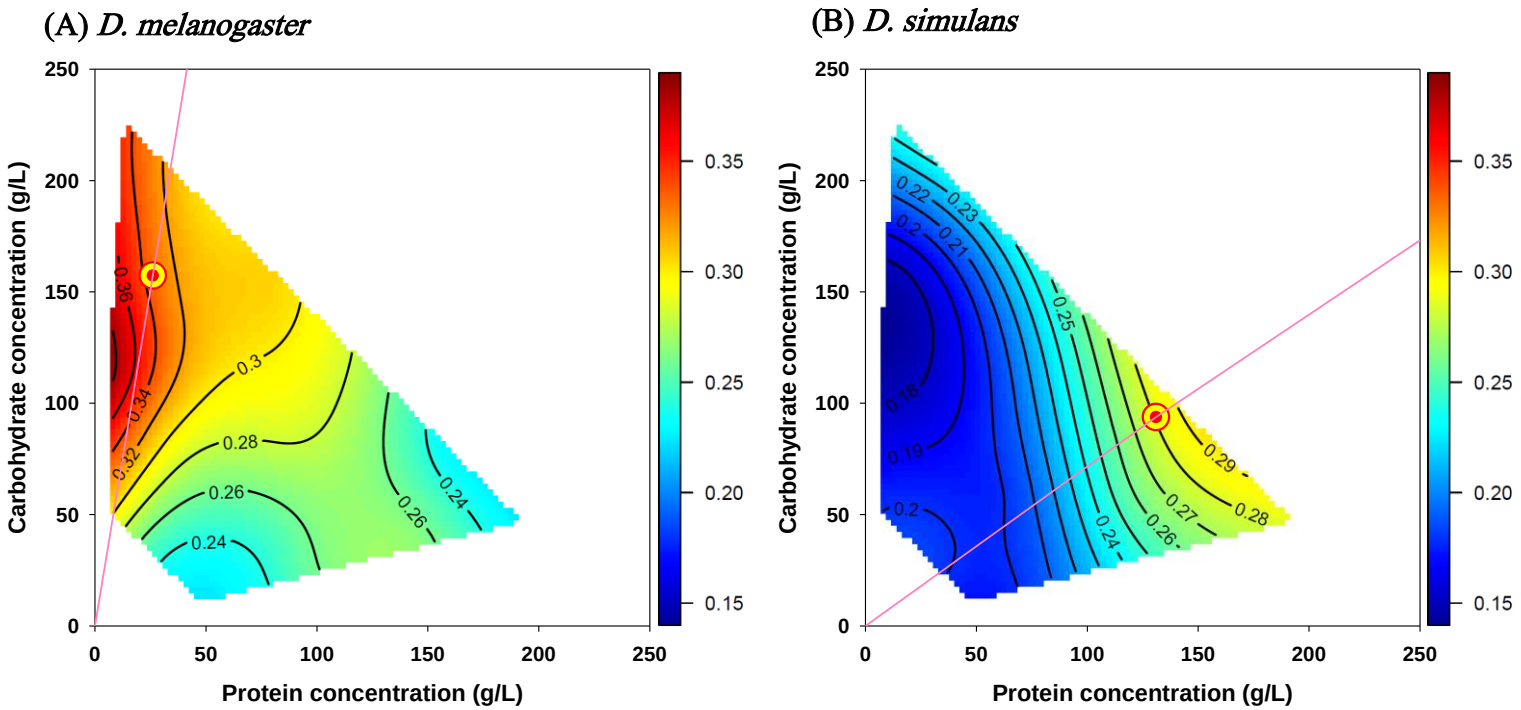

Figure 11. Nutritional landscapes for male lipid proportion expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

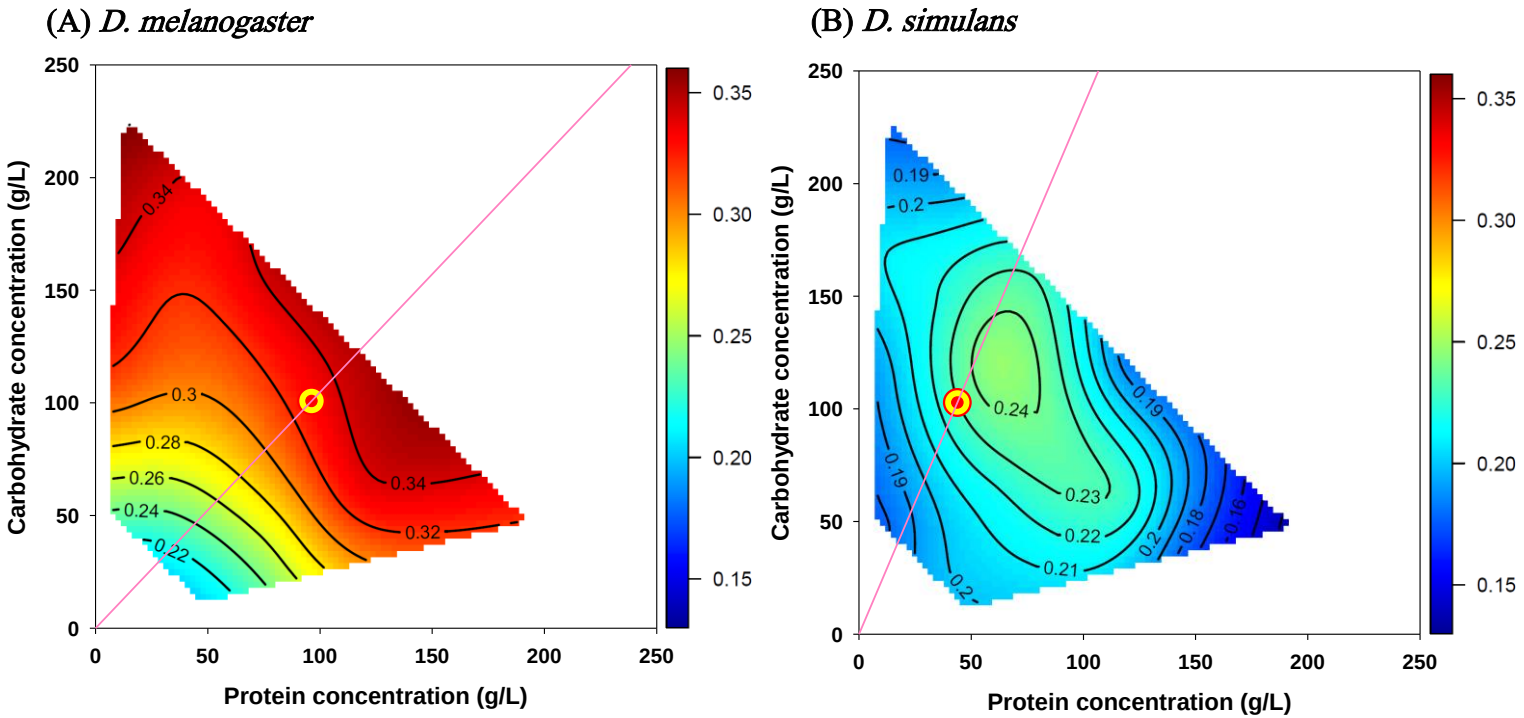

Figure 12. Nutritional landscapes for female lipid proportion expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 8. Results of the second-order polynomial multiple regressions on adult lipid content expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each sex and species.

Sex	Species	Adult lipid proportion	Linear gradients		Quadratic gradients		Cross product gradients
			P	C	P^2	C^2	P × C
Male	*D. melanogaster*	Gradient ± SE	-3.5×10^{-5} ± 1.61×10^{-4}	5.02×10^{-4} ± 9.23×10^{-5}	2.97×10^{-6} ± 2.98×10^{-6}	-5.1×10^{-6} ± 1.7×10^{-6}	-9.2×10^{-6} ± 4.64×10^{-6}
		t_{237}	-0.22	5.43	0.99	-2.97	-1.97
		P	0.826	<0.001	0.321	0.003	0.050
	D. simulnas	Gradient ± SE	5.79×10^{-4} ± 2.04×10^{-4}	1.87×10^{-4} ± 1.21×10^{-4}	6.97×10^{-6} ± 3.73×10^{-6}	7.95×10^{-6} ± 2.14×10^{-6}	2.15×10^{-5} ± 6.23×10^{-6}
		t_{211}	2.83	1.54	1.87	3.71	3.45
		P	0.005	0.125	0.064	<0.001	<0.001
Female	*D. melanogaster*	Gradient ± SE	5.59×10^{-4} ± 1.56×10^{-4}	8.26×10^{-4} ± 8.57×10^{-5}	1.18×10^{-6} ± 3.03×10^{-6}	-4.6×10^{-6} ± 1.58×10^{-6}	-9.8×10^{-6} ± 4.34×10^{-6}
		t_{235}	3.59	9.64	0.39	-2.92	-2.27
		P	<0.001	<0.001	0.698	0.004	0.024
	D. simulans	Gradient ± SE	-1.2×10^{-4} ± 1.86×10^{-4}	-2.2×10^{-5} ± 1.07×10^{-4}	-7.3×10^{-6} ± 3.43×10^{-6}	-4×10^{-6} ± 1.94×10^{-6}	1.85×10^{-8} ±5.63×10^{-6}
		t_{204}	-0.62	-0.21	-2.14	-2.06	0
		P	0.534	0.837	0.038	0.041	0.997

2.2. *Fitness*

The net reproductive rate (R_0), which is the total number of female offspring produced by a triad of one female and two male files, was ca. 66% higher for *D. simulans* as compared to *D. melongaster* across all diet treatments (*D. melanogaster*: 100.10±12.11 eggs, *D. simulans*: 166.32±23.42 eggs), as indicated by a significant effect of species (t_{28}=5.08. P<0.001). The shape of nutritional landscapes fitted for this measure of fitness was significantly different between the species ($F_{5,531}$=3.10, P=0.009; Fig. 13). Despite such significant overall differences between the two species, the global maximum for the net reproductive rate was located at the similar P:C ratio, with the optimal P:C ratio being 2:1 (P=76.05 g l^{-1}, C= 38.19 g l^{-1}) for *D. melanogaster* and 1.9:1 (P=105.69 g l^{-1}, C= 54.76 g l^{-1}) for *D. simulans*. Furthermore, the net reproductive rate of both species was significantly affected by a negative quadratic gradient of protein and a negative linear gradient of carbohydrate concentration (Table 9), indicating that the net reproductive rate was associated with protein concentration in a convex manner and decreased gradually as carbohydrate concentration increased.

The intrinsic rate of population increase (r) was also significantly different between the two species (t_{28}=4.56, P<0.001). *D. simulans* exhibited ca. 14% higher intrinsic population growth rate than *D. melanogaster* (*D. melanogaster*: 0.452±3.13×10^{-2}, *D. simulans*: 0.514±4.02×10^{-2}). However, the shape of nutritional landscape fitted for this measure of fitness was not

significantly different between the two species ($F_{5,531}$=2.04, P=0.071; Fig. 14), with the optimal P:C ratio of this measure of fitness being 2:1 (P=87.81 g l^{-1}, C= 48.21 g l^{-1}) for *D. melangoster* and 1.9:1 (P=76.22 g l^{-1}, C= 51.12 g l^{-1}) for *D. simulans*. For both species, the intrinsic rate of population increase was maintained high (*D. melanogaster*: >0.5, *D. simulans*: >0.6) as long as protein concentrations were higher than 25 g l^{-1} and fell rapidly at protein concentrations lower than 25 g l^{-1} (Fig. 14), as indicated by a significant negative quadratic gradient and positive linear gradient of protein concentration (Table 10).

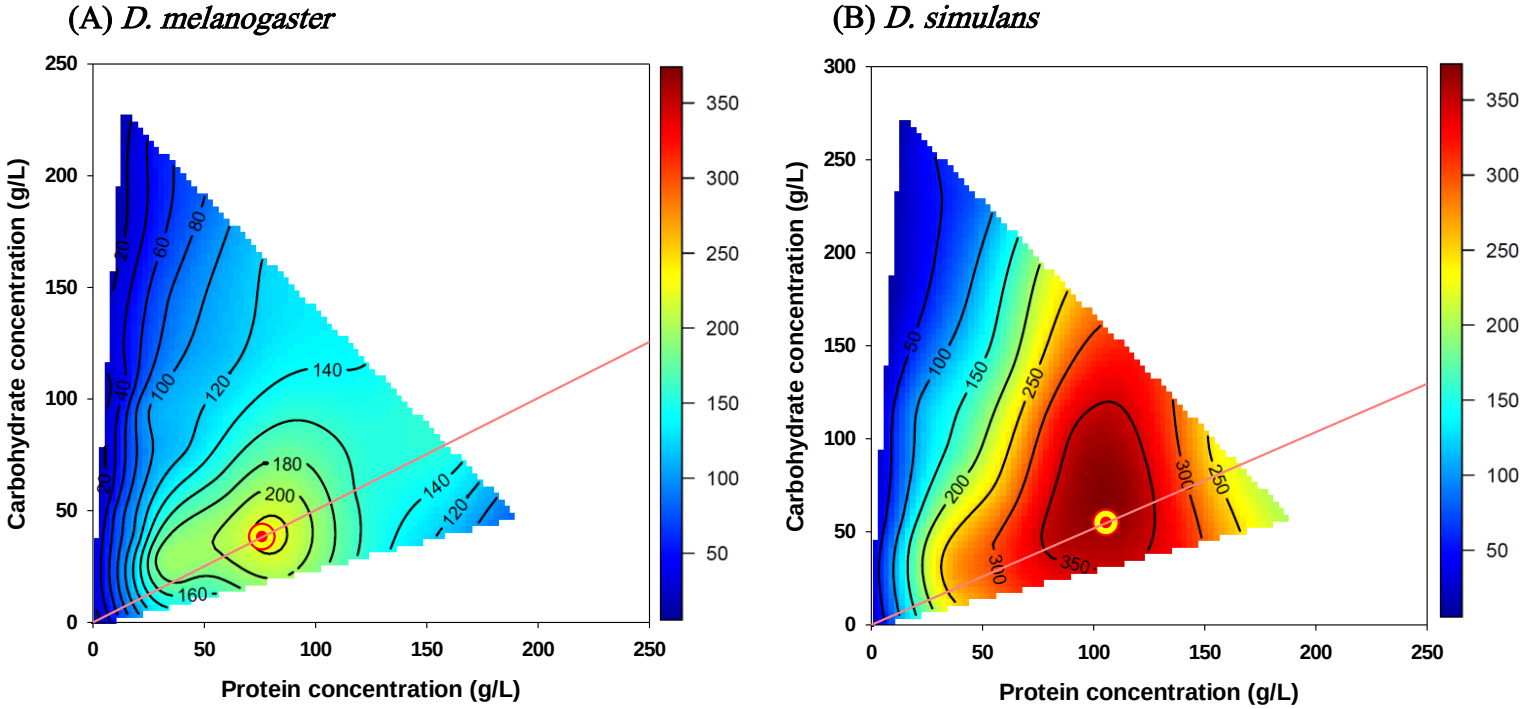

Figure 13. Nutritional landscapes for the net reproductive rate (R_0) expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 9. Results of the second-order polynomial multiple regressions on the net reproductive rate (R_0) expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each species.

Species	R_0	Linear gradients		Quadratic gradients		Cross product gradients
		P	C	P^2	C^2	P × C
D. melanogaster	Gradient ± SE	4.82×10^{-1} ± 1.02×10^{-1}	-4.83×10^{-1} ± 8.55×10^{-2}	-1.46×10^{-2} ± 1.85×10^{-3}	2.10×10^{-3} ± 1.45×10^{-3}	1.29×10^{-3} ± 2.91×10^{-3}
	t_{265}	4.72	-5.65	-7.87	1.45	0.44
	P	<0.001	<0.001	<0.001	0.149	0.657
D. simulnas	Gradient ± SE	1.44 ± 1.34×10^{-1}	-8.64×10^{-1} ± 1.11×10^{-2}	-2.53×10^{-2} ± 2.22×10^{-3}	2.44×10^{-3} ± 1.71×10^{-3}	-1.02×10^{-3} ± 3.43×10^{-3}
	t_{276}	10.69	-7.75	-11.4	1.42	-0.3
	P	<0.001	<0.001	<0.001	0.156	0.767

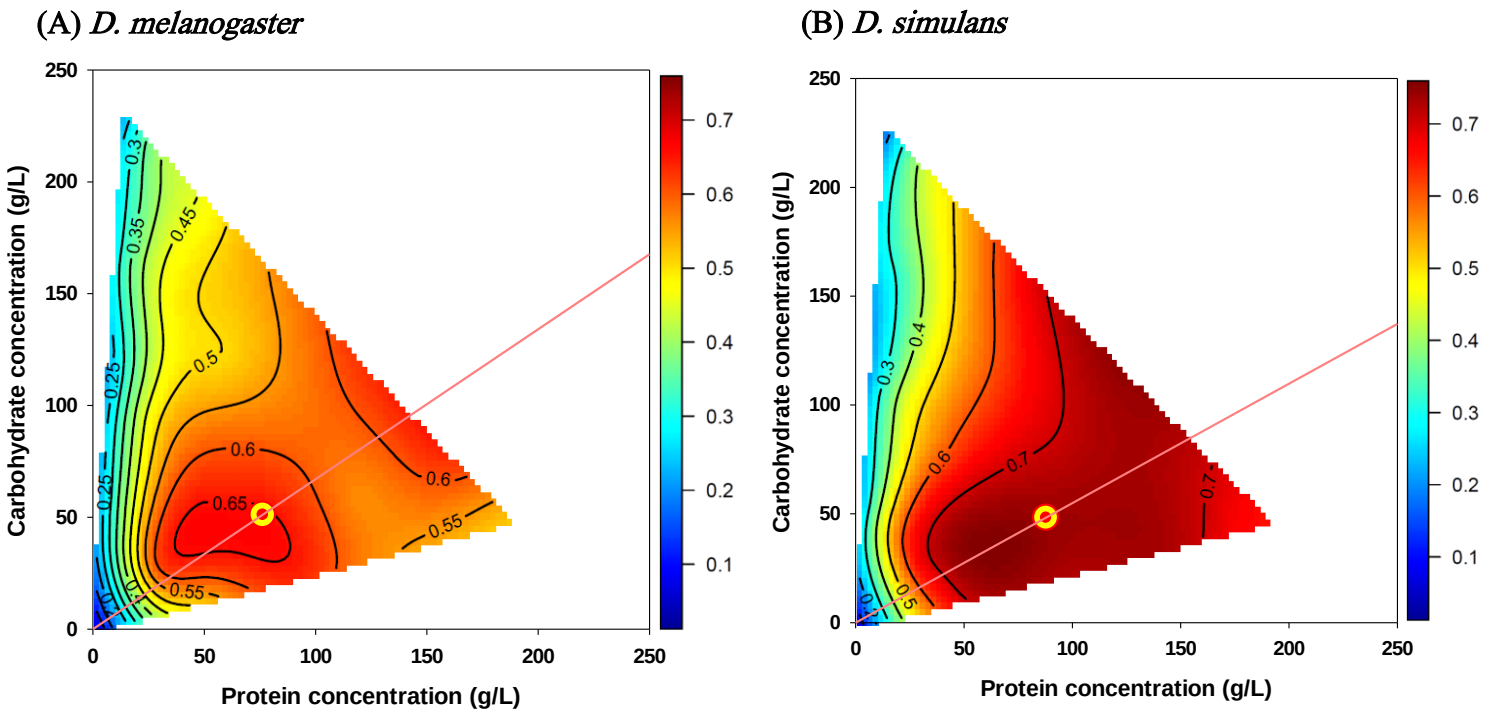

Figure 14. Nutritional landscapes for the intrinsic rate of population increase (*r*) expressed across 28 chemically defined diets in (A) *D. melanogaster* and (B) *D. simulans*. For each landscape, the regions where the trait is expressed at the highest and lowest level are represented by dark red and blue, respectively. The nutritional coordinates where traits optimized were represented by bull's-eyes. The slope of pink line that passed the origin and the nutritional optimum represents the optimal P:C ratio for the trait.

Table 10. Results of the second-order polynomial multiple regressions on the intrinsic rate of population increase (*r*) expressed across 28 chemically defined diets. Linear, quadratic, and cross product gradients fitted for dietary protein (P) and carbohydrate (C) concentration are summarized for each species.

Species	*r*	Linear gradients		Quadratic gradients		Cross product gradients
		P	C	P^2	C^2	P × C
D. melanogaster	Gradient ± SE	1.65×10^{-3} ± 1.89×10^{-4}	-6.5×10^{-4} ± 1.58×10^{-4}	-2.8×10^{-5} ± 3.3×10^{-6}	1.81×10^{-6} ± 2.58×10^{-6}	8.33×10^{-6} ± 5.18×10^{-6}
	t_{265}	8.76	-4.14	-8.59	0.7	1.61
	P	<0.001	<0.001	<0.001	0.484	0.109
D. simulnas	Gradient ± SE	2.38×10^{-3} ± 1.88×10^{-4}	-1.18×10^{-3} ± 1.56×10^{-4}	-3.3×10^{-5} ± 2.86×10^{-6}	6.42×10^{-6} ± 2.21×10^{-6}	1.8×10^{-5} ± 4.43×10^{-6}
	t_{276}	12.65	-7.57	-11.47	2.91	4.06
	P	<0.001	<0.001	<0.001	0.004	<0.001

DISCUSSION

D. melanogaster and *D. simulans* have long been used as the key model organisms for studying ecological adaptation and the process of speciation (Parsons, 1975; 1983; Parsons and Stanley, 1981; David et al., 1983; 2004; Gilbert et al., 2004). In this thesis, I was able to experimentally compare how these two sibling species of *Drosophila* responded to dietary variation in protein and carbohydrate by mapping nutritional landscapes for various traits related to evolutionary fitness in these two species. The results gained from this thesis have provided a valuable raw data for understanding how the two species have evolved through their adaptations to nutritional environments.

In Experiment 1, I focused on comparing the effect of dietary P:C ratio on key life-history traits between *D. melanogaster* and *D. simulans*. Compared to *D. melanogaster, D. simulans* took approximately one day earlier to reach their adult stage, but exhibited lower preadult survivorship and lighter body mass at adult emergence. These results are largely consistent with those of the previous studies which compared various preadult life-history traits between these two sibling species (David et al., 2004; Gilbert et al., 2004). It has been demonstrated from multiple studies that the consumption of low-protein, high-carbohydrate diets often leads to reduced preadult survivorship, extended development time, and

decreased body size in many insects including *Drosophila* (Lee et al., 2002; Rodrigues et al., 2015; Matavelli et al., 2015; Gray et al., 2018; Jang and Lee, 2018; Kim et al., 2020). This general pattern was also followed by two *Drosophila* species in the present study, with the two species displaying almost paralleling reaction norms across the range of dietary P:C ratio for all preadult traits except body mass. The body mass of *D. melanogaster* peaked at the P:C ratio of 1:4 and decreased as the ratio either increased or decreased from this optimal P:C ratio. In marked contrast, the body mass of *D. simulans* was largely insensitive to dietary P:C ratio. Why the body mass of two species differed in their responses to macronutrient balance remains unclear.

Numerous studies have investigated the effects of dietary P:C ratio on adult lifespan and reproduction in a wide variety of insects, including *Drosophila* flies, *Bactocerca* flies, crickets, etc (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Bruce et al., 2013; Lee, 2015; Jensen et al., 2015; Jang and Lee, 2018). It has been repeatedly demonstrated from these previous studies that an increase in dietary P:C ratio generally leads to increased female egg production and decreased adult longevity, suggesting that the balance of protein and carbohydrate has opposing effects on these key fitness components (Lee et al., 2008; Simpson and Raubenheimer, 2012). Consistent with these previous studies, *D. melanogaster* used in this study showed significantly shortened lifespan at P:C ratios higher than 1:2. Although its exact mechanism remains to be elucidated, the

lifespan-shortening effect of consuming protein-excess diets is likely to be caused by the toxicity of nitrogenous waste products, increased mitochondrial generation of radical oxygen species, altered nutrient signaling pathways (e.g., insulin/insulin-like growth factor-1, target of rapamycin signaling pathways), and/or reduced immune response (Sanz et al., 2004; Kapahi et al., 2004; Mirzaei et al., 2014; Le Couteur et al., 2016; Simpson et al., 2017). Unlike its sibling species and many insects studied to date, *D. simulans* did not follow this seemingly universal ifespan responses to dietary P:C ratio. Strikingly, the lifespan of *D. simulans* remained similar across dietary P:C ratio. During the lifespan assay, I observed that colorless liquid was formed on the surface of chemically defined diets presented to *D. simulans* after 24 h. This liquid was never formed when the exactly the same diets were given to *D. melanogaster*. It is unclear why this transparent liquid appears only in *D. simulans* but not in *D. melanogaster*, but the appearance of this unexpected liquid on the surface of diets presented to *D. simulans* raises the possibility that the peculiar lifespan responses found in *D. simulans* may be the outcome of an experimental artifact. Interestingly, similar to what I have seen in this study, Watada et al. (2020) also observed the formation of liquid in the fly vials specifically maintaining *D. simulans* flies when assaying their longevity. Because of this unexpected liquid formation and high fly mortality caused by drowning, Watada et al. (2020) gave up recording the lifespan of *D. simulans*.

As has been described from *Drosophila* and other insects (Lee et al., 2008; Lee, 2015; Jensen et al., 2015; Jang and Lee, 2018), our results showed that the rate of egg production increased as the P:C ratio in the diet increased in both *D. melanogaster* and *D. simulans*. This result is explicable by the fact that protein is the raw material for producing eggs and also stimulates endocrine cascades regulating oogenesis in insects (Wheeler, 1996; Mirth et al., 2019). A particularly intriguing aspect of our data was that the extent to which the rate of egg production increased with increasing dietary P:C ratio was significantly greater for *D. simulans* as compared to *D. melanogaster.* This species-specific differences were indicated by the presence of a significant interaction between species and dietary P:C ratio. In consequence, *D. simulans* exhibited a significantly greater egg production rate than *D. melanogaster* especially when the P:C ratio of the diet they fed was higher than 1:2. These results are largely consistent with the results of a recent study by Watada et al. (2020) who found higher female fecundity in *D. simulans* than in *D. melanogaster* using Japanese populations of these two species. However, our results and those of Watada et al. (2020) strongly contradicted the results of earlier studies by Boulétreau-Merle and Sillans (1996) and also by David et al.(2005) who compared the egg production capabilities of the two species derived from natural populations in France. These earlier studies using French populations showed that *D. melanogaster* was reproductively superior to *D. simulans*, with the former having not only more ovarioles numbers

(Boulétreau-Merle and Sillans, 1996) but also higher egg production rate per ovariole (David et al., 2005) than the latter. The discrepancies found between the results of these studies suggest that the inter-species differences in reproductive performance and possible other life-history and ecophysiological traits are not always consistent and may vary geographically.

In Experiment 1, I focused on investigating how two sibling species of *Drosophila* differed in their responses to dietary P:C ratio using a simplistic nutritional reaction norm. However, in reality, the abundance and distribution of macronutrients in nature are highly variable and complex (Simpson and Raubenheimer, 2012), implying that macronutrients not only vary in their relative balance but also in their individual and summed concentrations. To have an integrated overview of these complex nutritional effects, I decided to fit nutritional landscapes for various fitness-related traits expressed over the wide range of dietary protein and carbohydrate content in Experiment 2. The results of preadult life-history traits found in Experiment 2 were largely consistent with those shown in Experiment 1, with *D. melanogaster* exhibiting consistently heavier body mass and longer preadult development time compared to *D. simulans*. The preadult survivorship of both *D. melanogaster* and *D. simulans* demonstrated a convex relationship with dietary protein concentration, with the peak being centered around the intermediate protein concentrations. Reduced preadult survivorship at low protein concentration is most likely due to protein

limitation (Mattson, 1980; Scriber and Slansky, 1981), as has been previously described (Jang and Lee, 2018). For both species, preadult survivorship also decreased as protein concentration in the diet exceeded the optimal protein concentration in the nutritional landscape. Such adverse effect of excessive protein consumption on preadult survivorship has been documented from a number of insects including *Drosophila* (Rodrigues et al., 2015; Matavelli et al., 2015; Gray et al., 2018; Jang and Lee, 2018). Although the exact mechanism underlying this effect remains to be elucidated, it is possible that high energetic costs of metabolizing and excreting excessive protein and toxicity caused by increased nitrogenous wastes could be responsible (Lee et al., 2002, Simpson and Raubenheimer, 2012). As expected from previous studies (Jang and Lee, 2018), the duration of preadult development of the two sibling species was significantly delayed when protein content in the diet decreased. Preadult development time also increased to some degree in those insects that managed to survived diets containing excessively high protein concentrations, also indicating the presence of metabolic cost associated with processing protein surplus. Overall, the two sibling species demonstrated qualitatively similar responses to dietary variation in protein and carbohydrate, with all measured traits except body mass having similar nutritional optima. In case of body mass at adult emergence, the optimal P:C ratio was higher than 1:1 in *D.simulans* (1.8:1 for male, 1.2:1 for female) but was lower than 1:1 in *D.melanogaster* (1:1.2 for both sexes).

One of the most important contributions of this study was the direct measurement of evolutionary fitness of these two sibling species across a wide spectrum of dietary protein and carbohydrate. This measurement of fitness enabled me to estimate the nutritional niche of these two species with unprecedented accuracy. In Experiment 2, fitness was determined by calculating the two following parameters, the net reproductive rate (R_0) and intrinsic rate of population increase (r) (Birch, 1948; Charlesworth, 1994; Gotelli, 2001). The latter was considered to be more reliable measure for characterizing the fundamental niche, which is defined as the combinations of conditions allowing above zero population growth rate. By comparing the fitness landscapes of both species, I found evidence that the fundamental nutritional niche of these two species largely overlapped. In a manner similar to the results of egg production rate in Experiment 1, these two parameters of fitness also increased as a function of increasing dietary protein concentration for both species. Furthermore, the extent to which fitness parameters increased with increasing protein concentration was more pronounced in *D. simulans* compared to *D. melnoagster.* Despite its lower preadult survivorship, *D. simulans* had a significantly higher fitness than *D. melanogaster*, which is most likely to be contributed by its high egg laying capacity.

Under natural conditions, *D. melanogaster* and *D. simulans* are known to coexist, leading to the prediction that the competition between these two species is

likely to occur intensively. The finding that *D. simulans* had a higher fitness than *D. melanogaster* raises the strong possibility that the former is more likely to outcompete the latter. If this is the case, then, how could the coexistence of these two species be maintained in nature? Several possible explanations come to mind.

First, the coexistence between the two species may arise due to a possible trade-off between reproduction and survivorship. There are ample studies reporting that *D. melanogaster* is more resistant to various environmental stressors, such as starvation, desiccation, alcohol, acetic acid, and CO_2, than *D. simulans* (see literature cited in David et al., 2004), indicating that the former is more likely to have survival advantageous under natural conditions. In particular, it is widely held that starvation resistance is considered to one of the most powerful natural selective pressures that determines life or death of an organism. The capacity of an organism to resist this starvation stress is positively correlated with the amount of lipid reserves stored in the body of many organisms, including these two sibling species (Rion and Kawechi, 2007; Ballard et al., 2008; McCue, 2010; Lee and Jang, 2014; Jang and Lee, 2015). Although starvation resistance was not quantified in this study, the fact that *D. melanogaster* deposited more lipids in their body than *D. simulnas* leads me to predict that *D. melanogaster* may have a better chance of surviving the prolonged periods of food deprivation than *D. simulans*. Collectively, it is conceivable that the higher likelihood of surviving environmental stressors in *D. melanogaster* might have offset its reproductive

disadvantage relative to *D. simulans*, thereby contributing to the coexistence between the two species.

Second, the difference in thermal responses between *D. simulans* and *D. melanogaster* could provide another possible mechanism underlying the coexistence of these two species. In the current study, all experiments were conducted at a fixed temperature of 23℃. However, in reality, the temperature encountered by insects in nature varies substantially. According to the data recorded by Korean Meteorological Administration, the daily temperature of the Mt. Gwanak where the natural populations of two species used in this study were collected, fluctuates by ± ca. 3℃ from the mean. It is generally considered that *D. melanogaster* is more adapted to relatively cooler thermal environments than *D. simulans* (see literature cited in David et al., 2004; Gilbert et al., 2004). If the rank of interspecific difference in fitness measured at 23℃ is reversed at any lower temperatures (for 19℃), it is predictable that the coexistence of the two species can be maintained by variation in temperature. This interesting possibility needs to be tested in future studies.

Last, the coexistence between the two *Drosophila* species can be maintained by resource partitioning. Many sympatric species are known to partition resources by differentially foraging and selecting food (Robinson and Wilson, 1998; De León et al., 2014; Kent and Sherry, 2020). For example, Kent and Sherry (2020) showed that the North American warblers preferred different

food sources from one another so as to avoid the overlap of dietary niche. More recently, using NG, Behmer and Joern (2008) revealed that seven coexisting species of grasshoppers found in North America occupied different nutritional niches by selecting different ratios and amounts of protein and carbohydrate. A similar approach can be taken to compare the nutrient selection and preference between *D. melanogastor* and *D. simulans*. Two species are known to differ in the pattern of colonization, with *D. simulans* adults arriving and ovipositing to fruits earlier than *D. melanosgaster* adults (Nunney, 1990). Since the nutritional composition of fruit is known to change dynamically as fruit ripens and with successional changes in yeast or microbial communities (Morais et al., 1995; Tournas and Kasoudas, 2005), these two species are likely to encounter very different nutritional environment in nature. It is possible that their adaptations to different nutritional environments may lead these two species to have different nutritional preferences, leading to the partitioning of dietary and nutritional niches.

In this thesis, I have carried out a comprehensive analysis on the impact of dietary protein and carbohydrate composition on key life-history traits and fitness in *D. melanogaster* and *D. simulans*. In particular, I have compared the nutritional landscapes fitted over a gradient of dietary protein and carbohydrate content between the two species in order to test whether the nutritional niche of these two coexisting species overlaps. The present study clearly showed that *D. simulans* exhibited lower preadult survivorship, smaller body size, and less energy

storage than *D. melanogaster*. However, *D. simulans* developed faster and produced more offspring than its siblings, which would seem to have overridden aforementioned disadvantages. Taking all into account, the fitness was significantly higher for *D. simulans* than *D. melanogaster* and this species difference was most pronounced in the protein-rich environments. It is hoped that the results reported in this thesis will shed lights on the the important yet neglected role played by nutrition in mediating the ecological interactions between these two sibling species and also provide valuable raw data for unveiling the mechanisms explaining the evolutionary relationship between them.

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ABSTRACT IN KOREAN

음식물의 거대영양소 조성이 자매종인 노랑초파리(*Drosophila melanogaster*)와 어리노랑초파리(*D. simulans*)의 생활사 형질에 미치는 영향에 관한 비교연구

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이혜진

최신 영양생태학 연구에 따르면 음식물에 존재하는 단백질과 탄수화물 등 거대영양소의 균형이 곤충의 진화적 적응도(evolutionary fitness)를 결정하는 가장 중요한 요인임을 시사한다. 형태적으로 유사한 자매종인 노랑초파리(*Drosophila melanogaster*)와 어리노랑초파리(*D. simulans*)는 수많은 생태 및 진화 연구의 모델 생물로 오랫동안 사용되어 왔다. 이 두 자매종은 약 2백만 년 전 한 조상으로부터 분화하였으며, 한국을 포함한 전 세계에 공존하는 것으로 알려져 있다. 계통적으로는 가깝지만, 두 종은 생물학적으로 많은 부분에서 크게 다른 것으로 보고되고 있다. 특히 두 종이 어떻게 주변온도에 반응하는지를 비교하는 연구는 그 동안 많이 수행되었지만, 식이 거대영양소가 이들의 발육, 생존, 생식과 같은 적응도 관련 형질에 미치는 영향을 비교·조사한 연구는 거의 알려진 바 없다.

본 학위논문의 핵심목표는 음식물의 단백질과 탄수화물 조성이 자매종인 *D. melanogaster*와 *D. simulans*의 여러 생활사 형질과 진화적 적응도에 미치는 영향을 비교·분석하는 것이다. 본 논문에서는 이 두 종의 자연개체군을 이용하여 두 가지 실험을 차례로 수행했다. 실험 1에서는 *D. melanogaster*와 *D. simulans*에서 유충 및 성충 단계에서 발현되는 주요 생활사 형질에 미치는 음식물의 단백질 대 탄수화물의 비율의 효과를 규명하는 것을 중심으로 진행되었다. 이 실험에서 *D. melanogaster*와 *D. simulans*는 단백질:탄수화물 비율이 8가지(1:16, 1:8, 1:4, 1:1, 1:1, 2:1, 4:1 또는 8:1)로 다르지만 두 거대영양소의 총합은 120 gl^{-1}로 동일한 순합성 인공사료를 제공받았다. 실험결과, *D. melanogaster*는 *D. simulans*보다 성충까지의 발달 시간이 더 오래 걸렸지만 성충으로 우화한 후에는 더 높은 생존율과 무거운 체중을 보였다. 두 종 모두 음식물의 단백질:탄수화물 비율이 증가할수록 유충 생존율이 향상되고 체중이 증가하며, 발육이 빨라지는 것으로 나타났다. *D. melanogaster*의 체중은 단백질:탄수화물 비율 1:4 에서 정점에 달했고, 이 최적 비율보다 증가하거나 감소함에 따라 감소하였다. 이와 대조적으로, *D. simulans*의 체중은 단백질:탄수화물 비율에 따라 큰 차이를 보이지 않았다. 수명은 두 종 모두, 암컷이 수컷보다 길었다. 성별에 관계없이, *D. melanogaster*는 저단백질, 고탄수화물 식단에서 더 오래 살았지만 단백질:탄수화물 비율이 1:2 이상으로 높아지면 수명이 크게 단축되었다. 반면, *D. simulans*의 경우, 단백질:탄수화물 비율이 수명에 미치는 영향은 뚜렷하게 나타나지 않았다. 두 종 모두 단백질:탄수화물 비율이 증가할수록 알 생산량도 증가했지만, *D. melanogaster*보다 *D. simulans*에서 증가의 정도가 더 두드러졌다. 즉 단백질:탄수화물 비율이 1:2 이상의 경우*D. simulans*가 *D. melanogaster*보다 높은 생식력을 보였다.

실험 1은 식이 P: C 비율의 영향만을 검증하기 위해 설계되었기 때문에 서로 다른 거대영양소의 단독효과와 및 이들 간의 상호작용을 정확히 검정할 수 없었다. 이러한 한계를 극복하기 위해 실험 2에서는 최근 개발된 영양기하학기법(nutritional geometry)을 활용하여 *D. melanogaster*와 *D.*

*simulans*의 다양한 생활사 형질과 적응도에 대한 영양 경관도(nutritional performance landscape)를 적합하였다. 이 실험의 경우, 두 자매종은 음식물의 단백질:탄수화물 비율(1:16, 1:8, 1:4, 1:2.1.1, 2:1, 4:1)과 단백질과 탄수화물 총 농도(60, 120, 180, 240gl^{-1})이 다른 총 28가지 순합성 인공사료 중 하나를 제공받았다. 실험 1과 유사하게, *D. melanogaster*가 *D. simulans*보다 생존률이 더 높고, 발달 시간이 더 길며 체중이 더 무거운 것으로 나타났다. 전체적인 영양 경관도의 형태는 두 종에서 크게 다르지 않았으며, 이는 단백질과 탄수화물이 적응도 형질에 미치는 효과의 성격은 두 종에서 유사한 것으로 나타났다. 또한 본 연구에서는 두 자매 종의 적응도가 단백질과 탄수화물 조성에 따라 어떻게 발현되는 지를 직접 측정하였는데, 이는 순생식률(R_0)과 내재적 개체군 성장율(r)을 정량화함으로써 가능하였다. 두 종 모두 음식물 내 단백질 함량이 높아질수록 적응도가 점진적으로 향상되었다. 그러나 단백질 농도 증가에 따라 적응도가 증가하는 정도는 *D. melanogaster*보다 *D. simulans*에서 더 두드러졌다. 성충 이전의 생존율이 낮음에도 불구하고, *D. simulans*는 *D. melanogaster*보다 훨씬 높은 적응도를 가지는 것으로 분석되었다.

이 학위논문에서 본인은 음식물의 단백질과 탄수화물 함량이 어떻게 두 *Drosophila* 자매종의 생활사 형질과 진화적 적응도에 영향을 미치는지를 비교했다. 특히, 이 두 종에 대한 영양 경관도를 적합함으로써, 본 연구는 이 두 자매종이 서로 비슷한 영양적 지위(nutritional niche)를 가지는 것으로 확인했다. 이렇게 이 두 종의 영양적 지위가 크게 겹침에도 불구하고, 자연계에서 이 두 종이 어떻게 공존하는 지에 대한 메커니즘에서 대한 설명으로는, 생식과 생존의 트레이드 오프(trade-off), 환경 변동(environmental heterogeneity), 자원 분할(resource partitioning) 등이 있다. 결론적으로 본 학위논문의 연구결과는 이 두 자매종의 진화과정과 생태적 상호작용과 관련하여 영양이 담당하는 중요한 역할에 대해 많은 점을 시사한다.

주요어 : 발달, 적응도, 노랑초파리, 어리노랑초파리, 수명, 영양 기하학, 생식, 영양적 지위, 공존

학번 : 2019-27355