The effect of dietary protein and carbohydrate availability throughout development and adulthood on life history and sexually selected traits in male Jamaican field crickets (*Gryllus assimilis*)

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in

partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

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Abstract

Protein and carbohydrate availability can profoundly influence development speed, size, and reproduction. To date, most researchers investigating nutritional effects have only examined one nutrient at a time. Furthermore, when researchers have examined multiple nutrients, their studies have focused on adults, ignoring juveniles. I quantified how dietary nutrient balance (protein:carbohydrate) during development and adulthood impacted fitness conferring traits using male Jamaican field crickets (*Gryllus assimilis*). I also examined juvenile and adult dietary nutrient preferences. My findings revealed males grow larger and faster, and begin signalling earlier when reared on high protein diets, but switching diets in adulthood did not impact signalling. Additionally, when given a choice, males consumed greater amounts of carbohydrates relative to proteins. Given larger males signal more often and produce more attractive signals, and females preferentially mate with larger males, the ability to locate and consume high protein nutrients during development profoundly impacts male fitness.

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Introduction

Selection should erode variation in traits that confer fitness but extensive variation in fitness conferring traits persists (comprehensive comparison of over 200 animal population studies: Houle, 1992; expansive literature review of 30 species: Pomiankowski & Møller 1995). What maintains that variation? Some of the variation in traits that confer fitness may be maintained by variation in the ability to locate, uptake, and retain nutrients required for growth, development, energetically demanding activities, maintenance, and longevity (Fox & Macauley, 1977; Mattson, 1980; geese: Sedinger *et al.*, 1995; zooplankton: Sterner & Schulz, 1998; caterpillars: Perkins *et al.*, 2004; planthoppers: Huberty & Denno, 2006; butterflies: Saastamoinen & Rantala, 2013; crickets: Clark *et al.*, 2015; moths: Evenden *et al.*, 2015; beetles: Schwab & Moczek, 2016). For example, juvenile European house crickets, *Acheta domesticus*, reared on high phosphorus diets gained more weight, were in better condition at adulthood, and showed a trend for becoming larger adults in general (Visanuvimol & Bertram, 2011). As phosphorus availability is extremely low in the environment (oftentimes 10-20 times lower than the dietary needs of invertebrate herbivores), locating this nutrient is highly important for individuals (Visanuvimol & Bertram, 2011). It is therefore important to understand how nutrient availability influences life history and sexually selected traits.

Much research on how nutrition influences life history trait variation has focused on food quantity (spiders: Uetz *et al.*, 2002; crickets: Holzer *et al.*, 2003; Scheuber *et al.*, 2003a; Scheuber *et al.*, 2003b; Hedrick, 2005; Whattam & Bertram, 2011; fruit flies: Shelly & Kennelly, 2003). However, diet quality is also very important. For example, when using beetles, *Tribolium castaneum*, to test the influence of diet quality

[microcrystalline cellulose mixed with wheat flour in a low (1:4), medium (1:2), and high (4:1) quality diets] on life history traits, males developed faster and their body size was larger on the high-quality diet (Ming & Cheng, 2012). Males also exhibited significant correlations between developmental traits such that larval mass positively correlated with pupal mass and adult body mass, but development time negatively correlated with all three of these male traits (Ming & Cheng, 2012).

Food quality can also impact sexually selected trait variation (house finches: Hill, 1992; beetles: Emlen, 1997; fruit flies: Aluja *et al.*, 2001; Shelly *et al.*, 2002; crickets: Bertram *et al.*, 2009). For example, male black field crickets (*Teleogryllus commodus*) reared on high-protein diets invest more energy in signalling during early adulthood (Hunt *et al.*, 2004); male bush-crickets, *Requena verticalis*, fed high-protein diets experience more mating attempts (Schatral, 1993); and male neotropical fruit flies (*Anastrepha serpentina*, *A. striata*, and *A. obliqua*) fed high-protein diets signal significantly more frequently for mates (Aluja *et al.*, 2001). Diet quality was crudely manipulated throughout these aforementioned studies, as the combinations of proteins, lipids, carbohydrates, sucrose, and calcium were also often altered with no indication of how the diets differed in these other essential nutrients. Therefore, while these studies highlight the importance of protein to components of fitness, it is difficult to ascertain how important protein is relative to other unintentionally manipulated nutrients.

Further, only a handful of studies have investigated the relative importance of protein content to carbohydrate content (Maklakov *et al.*, 2008; South *et al.*, 2011; Harrison *et al.*, 2014). Maklakov *et al.* (2008) revealed that the availability of carbohydrates relative to protein in the diet strongly influences male sexual signalling effort as male crickets fed

low protein:carbohydrate (P:C) (carbohydrate-rich) diets signalled with significantly higher efforts than males fed high protein:carbohydrate (protein-rich) diets. Similarly, South *et al.* (2011) showed that carbohydrate (not protein) intake influences male sex pheromone expression and attractiveness in male cockroaches, *Nauphoeta cinerea*, as the expression of all three components of the male sexual pheromone was maximized on diets containing a low P:C ratio (carbohydrate-rich). More recently, Harrison *et al.* (2014) demonstrated that adult weight gain and acoustic mate signalling are maximized on diets with different protein:carbohydrate (P:C) ratios. Using the spring-field cricket, *G. veletis*, Harrison *et al.* (2014) also revealed that crickets preferred diets with low P:C ratios, and, with regards to mate signalling parameters specifically, most increased (indicative of improved quality) with increasing total P and C intake, regardless of the nutrient ratio. In these studies, the level of protein to carbohydrates was explicitly known. Moreover, the levels of all other nutrients in the diets (lipids, vitamins, minerals) were kept constant allowing the researchers to clearly determine whether the observed effects on fitness components were due to changes in protein or carbohydrate availability. To date, these results suggest that carbohydrate availability may be more important for adult mate attraction signalling than protein availability.

Many of the aforementioned studies investigating the relative importance of protein content to carbohydrate content only manipulated these essential nutrients during the adult life stage. However, protein availability may be more important than carbohydrate availability during development, given that protein availability corresponds to greater growth in a host of different species (caterpillars: Roeder & Behmer, 2014; nematodes: Hansen *et al.*, 2015; spiders: Mayntz & Toft, 2001; tadpoles: Venesky *et al.*, 2012; fish:

Kpundeh *et al.*, 2015; birds: Searcy *et al.*, 2004; Adeyemo *et al.*, 2012). For example, in Hunt *et al*'s (2004) study that crudely manipulated dietary protein content throughout *T. commodus* development, protein availability was positively associated with faster development, greater nymph survival to adult eclosion, and larger body size at adulthood (Hunt *et al.*, 2004).

Similarly, greater protein during development may allow individuals to gain weight, possibly increasing their overall condition. Residual mass is often used to indicate overall body condition (Féret *et al.*, 2005) such that low condition males are characterized by a low residual mass (Harrison *et al.*, 2013). While using residual mass as a measure of condition can be problematic as it ignores the fact that one unit of residual mass is relatively different for individuals that vary in size, resulting in the general problem of relative growth, as different organs scale differently with body size (Kotiaho, 1999). Regardless of what condition measure is used, greater protein availability during development seems to impact the ability to gain weight during development and sexual signalling in adulthood. For example, Holzer *et al.* (2003) revealed that protein availability during development affects condition (body mass), resulting in the condition-dependent trait (call frequency), which ultimately impacts performance (attractiveness to females). Performance is revealed through the condition-dependent trait although there is no real causal link present between the trait and the performance (Hill, 2011).

Given the importance of examining potential nutrient interactions on traits that confer fitness in both juveniles and adults, I quantified how dietary nutrient balance (relative amounts of protein and carbohydrate) during juvenile development and adulthood impacted life history and sexually selected traits.

Sexual selection is a form of natural selection that occurs when individuals vary in their ability to compete with others for mates (intrasexual selection) or to attract members of the opposite sex (intersexual selection; Darwin, 1871). Attracting members of the opposite sex inevitably involves mate choice. Mate choice is non-random mating that occurs when behavioural or morphological traits increase an individual's chances of being chosen as a mate (Halliday, 1983; Andersson, 1994). Darwin (1871) noted that while both sexes can exercise mate choice, female mate choice is more common. Female mating preferences can influence the evolution of male traits, as males with preferred traits typically experience higher mating success and, as a result, higher fitness (Andersson, 1994).

Males in a variety of taxa exhibit extensive variation in mate attraction signalling behaviours (fish: Candolin, 2000; Wong & Svensson, 2009; baboons: Kitchen *et al.*, 2003; Fischer *et al.*, 2004; spiders: Delaney *et al.*, 2007; crickets: Bertram *et al.*, 2011; 2013; Fitzsimmons & Bertram, 2011; frogs: Crothers & Cummings, 2013; katydids: Hartbauer *et al.*, 2015). For example, some male crickets signal extensively (spending over 50% of their adult lives attempting to attract females) whilst, at the opposing end of the spectrum, others signal rarely (Bertram *et al.*, 2011). Some of this variation in signalling can be explained by variation in body size. For example, three-spined stickleback (*Gasterosteus aculeatus*) males change their red nuptial colouration over the season depending on their body size at the start of breeding (Candolin, 2000). Additionally, in the curve-tailed bush katydid, *Scudderia curvicauda*, males exhibit extensive variation in the number of syllables per phrase, variation that is significantly related to male body size (pronotum length) (Tuckerman *et al.*, 1993). Similarly, Gray

(1997) showed that in house crickets, *A. domesticus*, male size correlates with the number of pulses per chirp.

Body condition also explains variation in male signalling behaviour. Ketola & Kotiaho (2010) showed that body mass had a negative effect on both first call bout duration and overall mean call bout duration in the decorated cricket, *Gryllodes sigillatus*. Moreover, Holzer *et al.* (2003) found that in the field, male *G. campestris* fed supplemental food not only showed a significant increase in body condition (body mass) but also called more frequently than control males (who received less food). Likewise, residual mass sometimes influences signalling, suggesting the ability of an individual to put on weight during development and in adulthood can also impact signalling (Uetz *et al.*, 2002; Whattam & Bertram, 2011). For example, Whattam & Bertram (2011) determined that interpulse duration, pulse rate and chirp duration honestly signalled juvenile condition, whilst dominant frequency signalled adult condition in *G. assimilis* male Jamaican field crickets. These relationships between variation in signalling and morphology underscore the importance of investigating the interplay between tradeoffs between sexually selected traits and diet during different developmental stages.

Elaborate male behavioural displays and morphological traits used in mate attraction can be costly to produce and maintain, and a male's ability to bear such costs may indicate his genetic quality and thereby the potential for him to provide direct or indirect fitness benefits to females (Zahavi, 1975; 1977). As recently shown by Kuhelj *et al.* (2015) the cost of mate attraction displays can require 12.4 times the energy as the resting state in the leafhopper, *Aphrodes makarovi*. The idea that male mate attraction displays require much more energy than the resting state of animals is mirrored across several

different taxa (katydids: Stevens & Josephson, 1977; cicadas: MacNally & Young, 1981; tree crickets: Prestwich & Walker, 1981; frogs: Taigen & Wells, 1985; Prestwich *et al.*, 1989; birds: Eberhardt, 1994; house crickets: Hack, 1997; spiders: Kotiaho *et al.*, 1998). Moreover, Stoddard & Salazar (2010) noted that the proportion of the energy budget allocated to signal production typically ranged from relatively nothing for many signalling organisms (e.g., beetles: Lighton, 1987; fireflies: Woods Jr. *et al.*, 2007; weakly electric fish: Salazar & Stoddard, 2008) to an excess of 50% for acoustic signals in sexually selected species with short lifespans (e.g., tree crickets: Prestwich & Walker, 1981; wax moths: Reinhold *et al.*, 1998).

Importantly, the size of the sound-producing structure (swim bladder of fish: Alexander, 1966; harp and plectrum wing structures of crickets: Bennet-Clark, 1989; syrinx of birds: Suthers & Zollinger, 2004) can also play a significant role in determining the extent of the energetic costs across species (frogs: Gans, 1973; Ryan, 1985; domestic fowls: Brackenbury, 1977; bats: Speakman *et al.*, 1989; crickets: Bailey *et al.*, 1993; Symes *et al.*, 2015; spiders: Kotiaho *et al.*, 1998). For example, the cost of acoustic signalling is ~8 times higher than the resting metabolic rate in ectotherms, compared to the smaller (but significant) value of ~2 times higher in birds (Ophir *et al.*, 2010). In this study, the calling muscles of the frogs and insects averaged 10% and 4% of their total body mass respectively, whilst the bird syrinx was only 0.2% of its total body mass (Ophir *et al.*, 2010). Alternatively, within species, this suggests that the energetic costs for larger males may outweigh the energetic costs for smaller males. This trade-off in energy expenditure somewhat compensates for the disadvantages smaller males incur due to their size. However, it also begs the question of whether smaller individuals have

reduced energy expenditure because they have less available energy to use stemming from a lack of nutrients (reduced body condition) or whether they are actively allocating less energy to sexual displays in general (Olson & Owens, 1998; Cotton *et al.*, 2004). As males allocate more nutrients and energy to sexual displays over other life history traits, it is possible that males of higher quality (in good body condition and possessing attractive calls) may obtain more nutrients, fueling this extra investment in both body condition and signal production. Under such a circumstance, there may be a correlation between body condition and signal structure which means that small males are physically incapable of sounding like large rivals (Brown & Gwynne, 1997) because the acoustic spectrum of a call is determined by the size of the sound-producing structures (Sales & Pye, 1974; Scheuber *et al.*, 2003a).

Female mate choice is often influenced by male body size, visual ornaments, pheromones, material resource offerings, and/or behavioural displays, such as acoustic signals (Andersson & Iwasa, 1996). Several studies have shown that females prefer to mate with males who produce more energetically costly behavioural displays (reviewed by Ryan & Keddy-Hector, 1992; e.g., spiders: Kotiaho *et al.*, 1998; frogs: Welch *et al.*, 1998; bats: Voigt & von Helversen, 1999; crickets: Wagner & Reiser, 2000; Holzer *et al.*, 2003; fireflies: Cratsley & Lewis, 2003; guppies: Karino *et al.*, 2011). For example, in fireflies (*Photinus ignites*) females preferentially respond to male displays that are longer in duration (Cratsley & Lewis, 2003). In guppies (*Poecilia reticulata)* females prefer longer male dorsal fins which harbor energetic costs as they are associated with reduced swim performance (Karino *et al.*, 2011). In animals that use acoustic cues to attract potential mates, females often prefer males that signal at higher rates (willow warblers:

Radesäter *et al.*, 1987; house crickets: Stout & McGhee, 1988; gray tree frogs: Gerhardt & Klump, 1987; Gerhardt *et al.*, 1996; field crickets: Hunt *et al.*, 2005; Wagner Jr. & Basolo, 2007), for longer durations (spring peepers: Doherty & Gerhardt, 1984; great reed warblers: Catchpole *et al.*, 1986; field crickets: Hedrick, 1986), and louder (higher sound pressure levels; wax moths: Limousin, 2009; zebra finches: Ritschard *et al.*, 2010; crickets: Pacheco & Bertram, 2014). These elevated signalling traits are energetically more expensive for males to produce (Prestwich, 1994; Bertram *et al.*, 2011; Thomson *et al.*, 2014). Given that female choice is acting more strongly on certain aspects of signalling, it is surprising that a great deal of variation persists. What maintains that variation? Given signalling energetics is often related to nutrition during development and into adulthoood, the variation that exists in signalling may be due to individual variation in nutrition or energy stores (Thomson *et al.*, 2014).

I therefore examined how dietary nutrient balance (relative amounts of protein and carbohydrate) during juvenile development and adulthood impacted life history and sexually selected traits using Jamaican field crickets (*Gryllus assimilis*) as a model organism. Male crickets fight for access to calling territories (Alexander, 1961; Rillich *et al.*, 2009) and then produce acoustic mate attraction signals to attract females from a distance by raising their forewings and rubbing them together (Alexander, 1962). Sound produced by this stridulatory action is amplified by the harp area of the wing (Walker, 1957; Miyoshi *et al.*, 2007). Each closing stroke of the wing produces a pulse of sound, and pulses are concatenated into chirps (Alexander, 1962). These long-distance mate attraction signals are multicomponent as they consist of several parameters including

pulse duration, interpulse duration, pulse rate, pulses per chirp, chirp duration, interchirp duration, chirp rate, carrier frequency, and sound pressure level.

Males vary extensively in their signalling parameters and females use this variation to distinguish between potential mates (Alexander, 1962). For example, females tend to prefer larger males that signal louder and with higher effort (*G. texensis*: Cade & Cade, 1992; *G. firmus*: Crnokrak & Roff, 1995; *G. campestris*: Holzer *et al.*, 2003; *T. commodus*: Hunt *et al.*, 2004; *G. pennsylvanicus*: Judge *et al.*, 2014; *G. assimilis*: Pacheco & Bertram, 2014), at higher chirp rates (*G. lineaticeps*: Wagner Jr. & Basolo, 2007; *A. domestica*: Stout & McGhee, 1988), with long chirp durations (*G. lineaticeps*: Wagner, 1996; Wagner & Reiser, 2000), and higher pulses per chirp/trill (*G. bimaculatus*: Popov & Shuvalov, 1977; *G. texensis*: Wagner *et al.* 1995).

Acoustic mate attraction displays can be energetically costly to produce as they require movement of the signalling muscles as well as muscles functioning to support the calling muscles, particularly those associated with circulation and respiration (Prestwich, 1994). Mate attraction signals are produced aerobically and anaerobic metabolism does not seem to play a significant role in calling in insects (Prestwich, 1994). The interaction of the call parameters determines the overall energetic costs of acoustic communication (Prestwich, 1994) as the metabolic energy is coupled to acoustic energy (Ryan, 1988). Further, the availability of carbohydrates in the haemolymph appears to drive signalling effort (*T. commodus*: Maklakov *et al.*, 2008; *G. assimilis* & *G. texensis*: Thomson *et al.*, 2014). Females may thus be selecting males that are the biggest and best at signalling: males that can 1) locate enough protein during development to grow large and produce large sound-producing structures, and 2) locate enough carbohydrates in adulthood to fuel

the energetic demands of moving their sound-producing structures in the manner required to produce the most attractive acoustic mating signals.

Focus of Research and Predictions

I asked three questions. Question 1: How does the availability of protein versus carbohydrates in the diet impact male life history traits throughout development and male sexually selected traits in adulthood? I hypothesized that the ratio of carbohydrates to protein in the diet during development will influence development time, nymph survival to adult eclosion, body size at eclosion, and residual mass at eclosion (Hypothesis 1). I predicted that juvenile males that developed on high P:C diets (more protein) will be larger and relatively heavier than males that developed on low P:C diets (more carbohydrates) (Prediction 1a). Further, males that developed on high P:C diets will moult to adulthood faster and have higher juvenile to adult moulting success (Prediction 1b).

Question 2: How does juvenile and adult diet and their interaction influence adult male acoustic mate attraction signalling. I hypothesized that the ratio of carbohydrates to protein in the diet during development and adulthood will influence male acoustic mate attraction signalling (Hypothesis 2). I predicted that juveniles fed high P:C diets (more protein in their diet) during development will start producing acoustic mate attraction signalling traits earlier in adulthood (Prediction 2a). I also predicted that adult males fed low P:C diet (more carbohydrates in their diet) in adulthood will signal more often, with higher effort signals (louder chirps produced at higher rates) than adult males on high P:C diet (Prediction 2b). I was unsure how switching diets between juvenile and adult life stages would interact to impact signalling, so I did not make any clear predictions about

this interaction.

Question 3: Do males switch their diet preference between juvenile and adult stages as their trajectory switches from growth to reproduction and survival? I hypothesized that juvenile crickets will make dietary choices that will minimize their development time, maximize their survival to eclosion, and maximize their body size and residual mass at eclosion (Hypothesis 3a). I predicted that juvenile males will prefer a high P:C diet (more protein) over low P:C diets (more carbohydrates) because males need to build tissue and tissue building is predominantly fueled by protein stores (Prediction 3a). I also hypothesized that adult crickets will make dietary choices that will maximize the attractiveness of their acoustic mate attraction displays (Hypothesis 3b). I predicted that adult males will prefer low P:C diets (more carbohydrates) over high P:C diets (more protein) because signalling is energetically expensive and is predominantly fueled by carbohydrate stores (Prediction 3b).

Methods

Study Species

My study was conducted in accordance with the guidelines of the Canadian Council on Animal Care. I conducted all my research using the Jamaican field cricket, *Gryllus assimilis*. My *G. assimilis* crickets were descendants of a population that were collected near the Stengl Lost Pines Biological Station at the University of Texas at Austin (Bastrop County, TX, U.S.A; latitude ~30°17′N, longitude ~97°46′W, elevation ~145 m) in September 2008. No field-collecting permits were required to make this collection. Further, no import permits were required to bring *Gryllus assimilis* into Canada. *G. assimilis* crickets were housed in a Plant Pest Containment (PPC) facility (Greenhouse Bay 1 in the Nesbitt Biology Building at Carleton University in Ottawa, Ontario, Canada) in accordance with PPC Level 1 Guidelines (PPC1: PC-2014-053). The crickets I used in my experiments had been reared in the laboratory for over 20 generations. I placed juvenile crickets in communal groups in plastic bins (64 x 40 x 42 cm L x W x H) with pieces of cardboard egg carton for the crickets to use as shelter, *ad libitum* water and food (powdered Harlan Teklad Rodent diet #8604, Harlan Laboratories, Indianapolis, IN, USA). This powdered diet was comprised of 24.3% protein, 40.2% carbohydrate, 1.1% phosphorus, and 12.4% indigestible fiber. At the penultimate instar, I transferred each male cricket into its own clear plastic container with a screened lid, and provided it with shelter, *ad libitum* water and its experimental food(s), described below. Crickets were housed in the greenhouse throughout their experimental trials, under the same temperature and lighting conditions they experienced during earlier development.

Body Size

Male crickets were weighed prior to diet trials using an Ohaus PA214 Pioneer

Analytical Balance (Model: Adventurer SL AS64; d = 0.0001g) (Ohaus Corporation,

Pine Brook, NJ, USA), and then photographed in a dorsal position using a Lumix camera

(Panasonic Lumix ZS40, Mississauga, ON, Canada). The head and pronotum were

photographed alongside a printed scale (mm) using an EmCal Mycro USB Microscope

(EmCal Scientific Inc., CA, USA) and MicroCapture software (Celestron LLC, CA,

USA). Pronotum width, pronotum height, and eye width were measured from the

photographs using ImageJ v.1.48 software.

Experimental Food Ingredients

I created all diets for Experiments 1 and 2 using a protein (P) source that consisted of

a 3:1:1 mixture of vitamin-free casein, bacteriological peptone, and egg albumen. My

carbohydrate (C) source consisted of a 1:1 mixture of powdered sucrose and dextrin. I

ensured that all diets contained 2.2% calcium phosphate, 0.127% choline chloride,

0.053% vitamin mix (carbohydrate deficient), 1.4% mineral mix (deficient in phosphorus

and calcium), 0.55% cholesterol, 0.55% linoleic acid, and 0.275% ascorbic acid.

Experiment 1: Influence of Dietary Nutrient Balance on Fitness Conferring Traits
(Questions 1&2)

Experimental foods were created following established protocols (Simpson &

Abisgold, 1985). Foods consisted of 1300g of two different P:C ratios ($3P:1C$ - high P,

and $1P:3C$ - high C). The range of dietary P:C ratios was chosen following a study that

mapped life-history and fitness conferring traits across a wide range of P:C ratios (Maklakov *et al.*, 2008). I based my specific ratios on a study that determined which dietary ratios maximized fitness conferring traits (Harrison *et al.*, 2014).

I checked the communal bins every 48 h, and, if males had reached the wing-bud stage, they were added to the experiment (housed in individual 520 ml plastic containers). At the start of diet trials, each juvenile male cricket (N=140) was weighed and photographed for later morphological analysis and then randomly assigned to one of two dietary treatments (high P or high C). Crickets were provided with fresh food in food dishes (50 x 9 mm Petri dishes with upturned 15 x 15 mm plastic lids glued at the centre) weekly.

Crickets were checked daily to determine date of adult eclosion, and upon adult eclosion the time required to develop from wing-bud stage to adulthood was quantified. At adult eclosion, crickets were rephotographed for later morphological analysis following the aforementioned protocol. The newly eclosed adult cricket's diet was then reassessed. Each male was either maintained on its previous diet or switched to the opposite diet resulting in four dietary treatments. Overall, 37 males remained on the high P diet throughout the experiment, 33 males were switched from the high P to the high C diet at adult eclosion, 35 individuals were maintained on the high C diet throughout the experiment, and 35 individuals were switched from the high C to the high P diet at adult eclosion.

After providing each male with his adult diet, I monitored male acoustic signalling continuously, beginning on day 1 of adult eclosion and continuing until day 28 of adulthood. I monitored each male's acoustic mate attraction signals using the Electronic

Acoustic Recording System II (EARS II). The EARS II consists of 96 Styrofoam

enclosures, each containing 5.1 cm thick walls that are fully lined with a 2.5 cm thick

acoustically-isolating foam. The cricket was transferred into the Styrofoam enclosure

inside his own clear plastic container with a screened lid, that contained its shelter, *ad

libitum* water and its experimental food. I maintained all crickets in the EARS II on a

14:10 h light/dark cycle by an individual LED light-bulb with a microphone attached at

its apex. The microphone enabled me to electronically monitor each male's acoustic mate

attraction signals in real-time using the EARS II CricketSong software (Cambridge

Electronic Design Ltd., Unit 4, Science Park, UK). CricketSong software filtered out

background noise while recording and analyzing 10 signalling parameters: pulse duration

(ms), interpulse duration (ms), pulse rate (# pulses/sec), chirp duration (ms), interchirp

duration (ms), number of pulses per chirp, chirp rate (# chirps/min), carrier frequency

(Hz), amplitude (dB), and time spent calling (min/24 h period) (Figure 1). Daily I paused

the EARS II for one 30 min period to allow time for food and water replenishment.
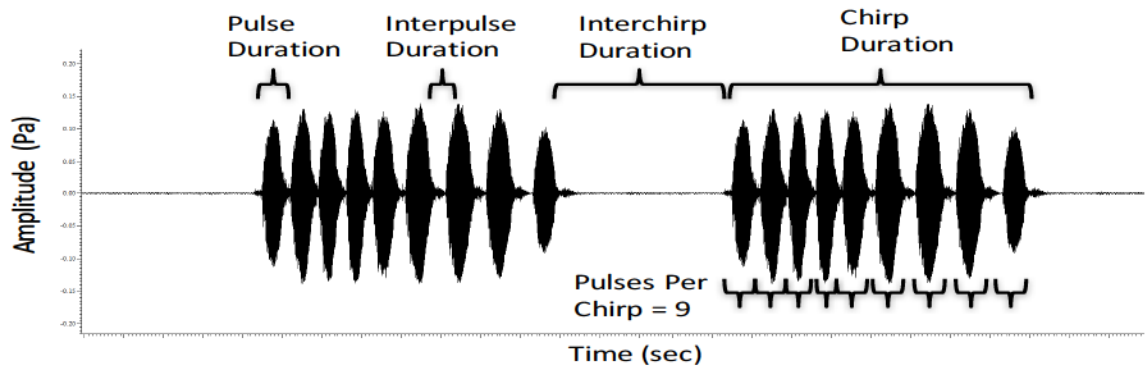

Figure 1. Waveform of *G. assimilis* long-distance mate attraction call showing some of

the parameters measured by the EARS II: pulse duration (PD), interpulse duration (IPD),

chirp duration (CD), interchirp duration (ICD), pulses per chirp (PPC), and amplitude.

I determined the day each cricket started signalling (signalling onset). Each male that survived to 28 days post-adult eclosion (N=132, 94% of males) was weighed following established protocols and subsequently euthanized via freezing. If a male died prior to the end of the experiment (28 days) I recorded his death date.

Statistical Analysis

I analyzed all my data using JMP, v.12.0.0 (SAS Institute, Cary, NC, U.S.A.). Because of strong correlations between morphological traits, I ran a principal component analysis (PCA) on pronotum length, pronotum width, and head width to obtain a composite measure of overall male body size. PC1size accounted for 96% (eigenvalue = 2.8661) of the observed variation in juvenile male body size, and 98% (eigenvalue = 2.9313) of the observed variation in male imaginal moult body size. All body size measures were loaded equally on these PC1's (eigenvector range: 0.575 - 0.580).

To determine how the ratio of P:C in the diet during development influenced development time, nymph survival to adult eclosion, body size at eclosion, residual mass at eclosion (Question 1), and onset of signalling (Question 2), I used one-way ANOVAs, with juvenile diet as the independent variable.

To determine how the ratio of P:C in the diet during development influenced lifetime signalling parameters (averaged over the 28 days of adulthood; Question 2), I used: (a) linear models provided the residuals were normally distributed (pulse duration, pulses per chirp, chirp duration, interchirp duration, amplitude, and pulse rate); and (b) generalized linear models (log link function) when the residuals were skewed (time spent signalling, interpulse duration, carrier frequency, call duration, and chirp rate). The independent variables were juvenile diet, adult diet, and the interaction between juvenile diet and adult

diet for both these linear and generalized linear model types. I also explored: (a) how juvenile and adult diet influenced the probability that males would signal or not over time, and (b) for those that sang, how diet during development and into adulthood impacted daily time spent signalling. To do this I used linear models: (a) with proportion of individuals signalling as the dependent variable and the independent variables were day in EARS, juvenile diet, adult diet, the interaction between juvenile diet and adult diet, the interaction between juvenile diet and day in EARS, and the interaction between adult diet and day in EARS; and (b) with time spent signalling as the dependent variable and the independent variables were day in EARS, juvenile diet, adult diet, the interaction between juvenile diet and adult diet, adult size PC1, the interaction between juvenile diet and day in EARS, and the interaction between adult diet and day in EARS.

Experiment 2: Diet Preference during Juvenile and Adult Stages (Question 3)

Using the methods I outlined above, I created three foods for Experiment 2: a pure P diet ($46_P:0_C$), a pure carbohydrate diet ($0_P:46_C$), and a diet consisting of equal amounts of P:C ($23_P:23_C$). I checked communal bins every 48 h, and, if males had reached the wing-bud stage, weighed them, housed them in individual 540 ml plastic containers, and added them to my experiment.

All foods were pre-dried and weighed (as outlined below) before use. I simultaneously provided each juvenile male cricket with one pure protein and one pure carbohydrate food (N=35) or two identical foods of equal amounts of P:C as an experimental control (N=32). I provided crickets with fresh diets weekly and also changed the position (alternated) of the paired food types within each container. Diet trials lasted from wing-bud stage to 21 days post-adult eclosion. Crickets were checked

daily to determine date of death and adult eclosion. At adult eclosion, crickets were again weighed following established protocols.

Weekly protein and carbohydrate intakes were calculated from total food intake (difference in weight of food dish before and after consumption). Food dishes (same as Experiment 1) containing food were dried in a Fisher isotemp drying oven (200 Series, Model 230G) (Fisher Scientific International, Inc., Pittsburgh, Pa, USA) for 48 h at 30^0 C prior to weighing and were weighed using an Ohaus PA214 Pioneer Analytical Balance after removing faeces with fine forceps. Control food containers (consisting of pairs of diets without crickets) were housed in 520 ml plastic containers with water and were dried with experimental food containers. If control food containers increased in weight, experimental food containers were dried for an additional 24 h. At 21 days post-adult eclosion, all males were weighed following established protocols and subsequently euthanized via freezing.

Statistical Analysis

All data was analysed using JMP, v.12.0.0 (SAS Institute, Cary, NC, U.S.A.). To determine whether crickets preferentially consumed more of one food type within each treatment pair, generalized linear mixed models (GLMMs) were used (Question 3). Total intake of each food type was the dependent variable, individual identity was added as a random effect, initial body mass, and body size at adulthood PC1 were used as covariates, and food type was a fixed effect. Controls (containers with no cricket inside) were not used in mean calculations as these pairs did not allow the expression of diet choice.

Results

Experiment 1: Influence of Dietary Nutrient Balance on Fitness Conferring Traits

    a) Does juvenile diet influence development and morphology (Question 1; Hypothesis 1)?

    Protein and carbohydrate intake during development influenced development time (Prediction 1b). Males who had access to protein-rich food reached adulthood faster (Table 1, Figure 2a). Males reared on the protein-rich food also tended to have higher juvenile to adult moulting success (Prediction 1b), but this result was not significant (8 total: 2 on high protein diets and 6 on high carbohydrate diets; $\chi^2 = 2.155$, p = 0.1422, DF = 1,139). Protein and carbohydrate intake during development influenced body size at adult eclosion (Prediction 1a). Males who had access to protein-rich food as juveniles were larger at adulthood, even after I controlled for variation in initial body size (Figure 2b; Table 1). There was a non-significant trend for males reared on the high protein diet to have higher residual mass (Figure 2c; Table 1).
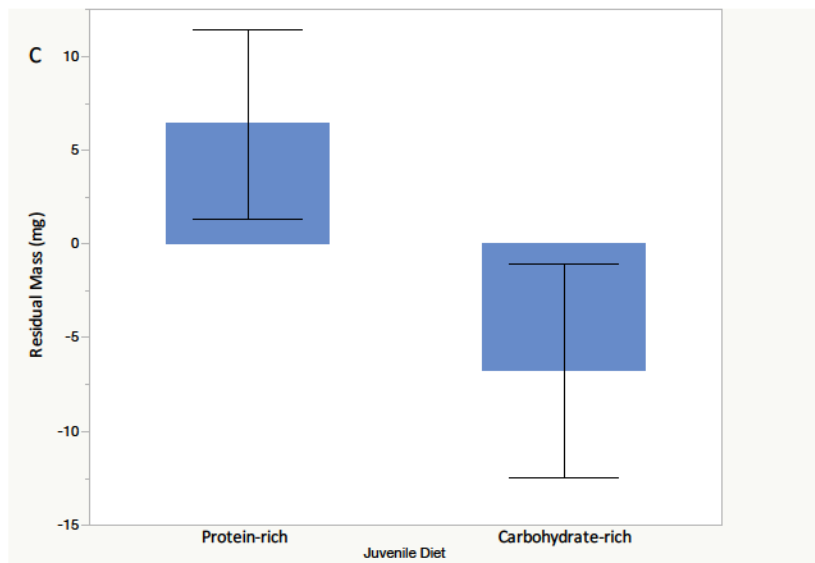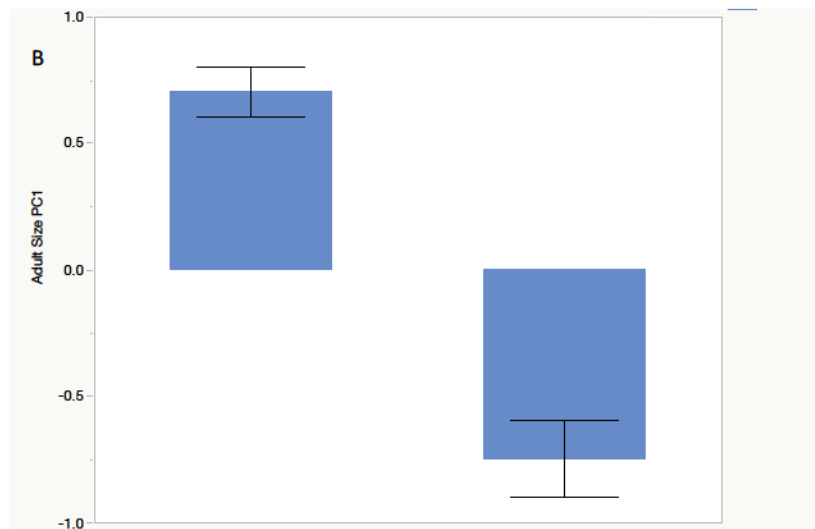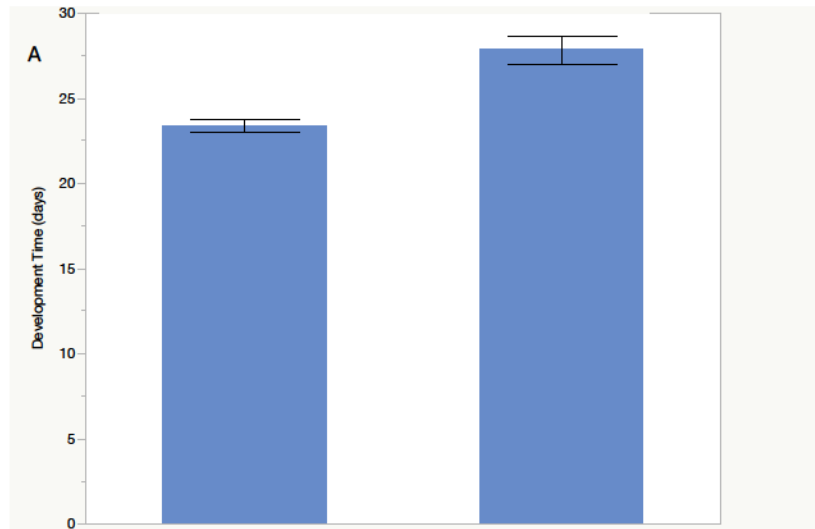
Figure 2. How development and morphology at adult eclosion are affected using male juvenile crickets consuming high protein and high carbohydrate diets. (a) the influence of diet on development time, (b) the influence of diet on body size at adulthood, and (c) the influence of diet on residual mass. Statistics are shown in Table 1.

Table 1. General linear models (simplified models) exploring how juvenile diet influences development and morphology at adult eclosion. Significant *P*-values are in boldface.

Trait	Protein-rich x̄±SE	Carbohydrate-rich x̄±SE	DF	F	P	R^2_{Adj}
Development Time	23.36±0.60	27.83±0.62	1,132	26.47	**<0.0001**	0.17
Body Size	0.70±0.12	-0.75±0.13	1,132	65.06	**<0.0001**	0.33
Residual Mass	6.39±5.31	-6.78±5.47	1,132	2.98	0.0866	0.02

b) How does diet influence mate attraction signalling (Question 2)?

Protein and carbohydrate intake during development influenced the timing of signalling onset (Prediction 2a). Males who were fed protein-rich food as juveniles began to signal at a younger adult age than males who were fed food rich in carbohydrates (6 days versus 8 days post-adult eclosion; Figure 3a; Table 2). Variation in time spent signalling was impacted by juvenile diet (Prediction 2b). Males reared on diets rich in carbohydrates had higher average daily time spent signalling even after controlling for variation in body size and residual mass (Figure 3b; Table 2). Protein and carbohydrate intake throughout an individual's lifetime had indirect influences on the fine-scale components of male acoustic mate attraction signalling. While juvenile and adult diet did not influence variation in any of the fine-scale signalling parameters (Prediction 2b),

variation in body size and residual mass did (Table 2; Prediction 2b). Please note, however, that because juvenile diet explains variation in adult body size and tends to explain variation in residual mass, juvenile diet appears to have indirect effects on male acoustic mate attraction signalling.
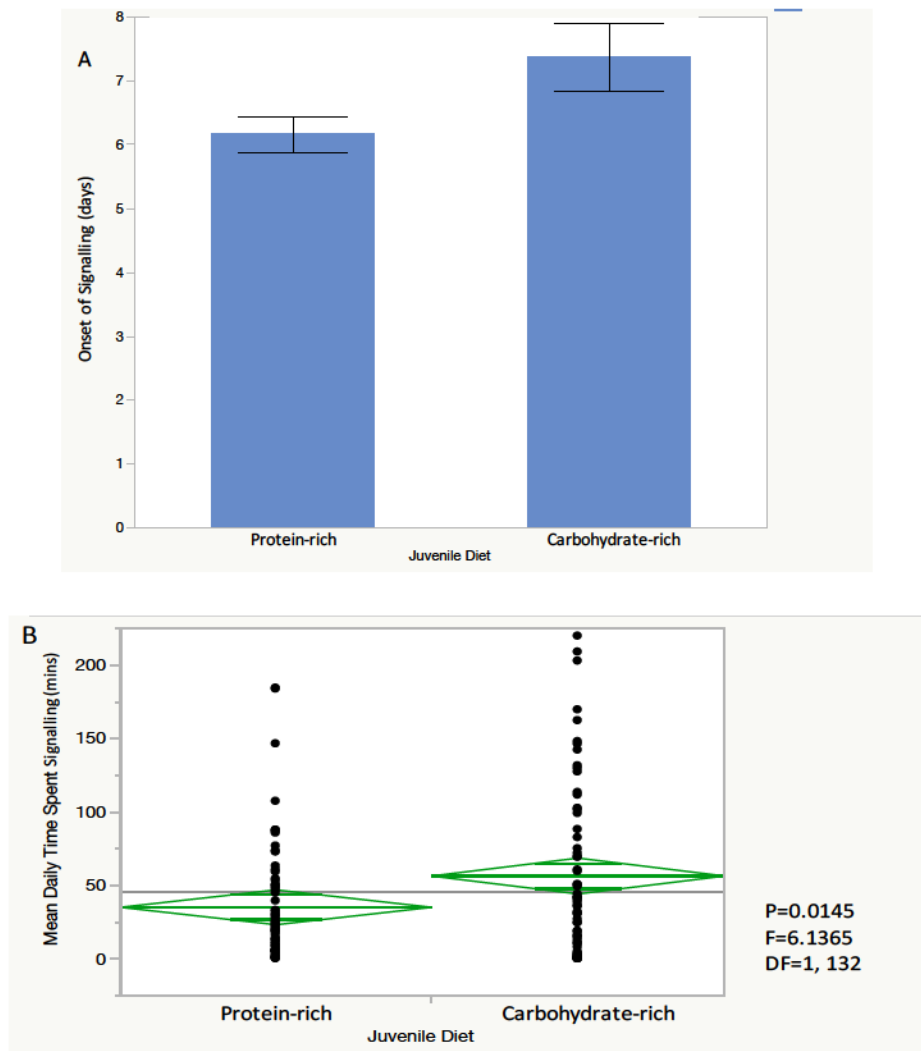
Figure 3. How acoustic mate attraction signalling is affected using male adult crickets who consumed high protein and high carbohydrate diets during development. (a) the influence of diet on signalling onset, and (b) the influence of diet on time spent signalling. Statistics are shown in Table 2.

Table 2. Results of general and generalized linear models (simplified models) showing the effects of juvenile and adult dietary intake on adult male fitness traits. Significant *P*-values are in boldface. P-values < 0.024 were considered significant following False Discovery Rate B-Y corrections.

Parameter	Term	P	F	R^2_{Adj}	χ^2
Signalling Onset	Juv. Diet	0.4062			
	Adult Diet	0.7948	2.0952	0.0336	
	Adult Size PC1	0.0617			
	Residual Mass	0.4852			
Time Spent Signalling	Juv. Diet	**0.0226**			
	Adult Diet	0.4130			6.8698
	Adult Size PC1	0.7426			
	Residual Mass	0.7358			
Pulse Duration	Juv. Diet	0.6448			
	Adult Diet	0.4990	5.7007	0.1308	
	Adult Size PC1	**< 0.0001**			
	Residual Mass	0.2692			
Interpulse Duration	Juv. Diet	0.7575			
	Adult Diet	0.1311			2.4444
	Adult Size PC1	0.5758			
	Residual Mass	0.8604			
Pulses Per Chirp	Juv. Diet	0.2038			
	Adult Diet	0.8243	1.3580	0.0112	
	Adult Size PC1	**0.0218**			
	Residual Mass	0.8201			
Chirp Duration	Juv. Diet	0.1654			
	Adult Diet	0.6916	1.3738	0.0117	
	Adult Size PC1	0.0280			
	Residual Mass	0.9277			
Interchirp Duration	Juv. Diet	0.6408			
	Adult Diet	0.7416	1.2187	0.0069	
	Adult Size PC1	0.3451			
	Residual Mass	0.0997			
Carrier Frequency	Juv. Diet	0.7405			
	Adult Diet	0.6296			16.1261
	Adult Size PC1	0.3869			
	Residual Mass	**0.0001**			

Amplitude	Juv. Diet	0.3068	12.5649	0.2685
	Adult Diet	0.5523		
	Adult Size PC1	**< 0.0001**		
	Residual Mass	0.0479		
Signal Duration	Juv. Diet	0.1844		5.9704
	Adult Diet	0.9079		
	Adult Size PC1	0.0429		
	Residual Mass	0.4338		
Pulse Rate	Juv. Diet	0.8754	2.4353	0.0436
	Adult Diet	0.6846		
	Adult Size PC1	**0.0123**		
	Residual Mass	0.5547		
Chirp Rate	Juv. Diet	0.6528		4.7701
	Adult Diet	0.9040		
	Adult Size PC1	0.2247		
	Residual Mass	0.1602		

Owing to the dietary effects on signalling onset and time spent signalling, I also investigated how diet influenced (a) whether the proportion of males signalling changed over time, and (b) how time spent signalling changed over time. The proportion of individuals signalling increased with increasing adult age. With each increasing day in the EARS, more males signalled up until day 20 post-adult eclosion. More males signalled and signalled at a younger age when they were fed protein-rich diets as juveniles and carbohydrate-rich diets as adults. Males forced onto the protein-rich diets throughout had the lowest proportion of males signalling in the population. Interestingly, the opposite occurred if males were fed carbohydrate-rich diets as juveniles (Figure 4a; Table 3).

Time spent signalling was also influenced by both age and juvenile diet. All males increased their time spent signalling as they aged, however, males who were fed carbohydrate-rich diets as juveniles had larger increases in their time spent signalling.

Further, adult males who were fed diets rich in carbohydrates also increased their time spent signalling more (Figure 4b; Table 3).
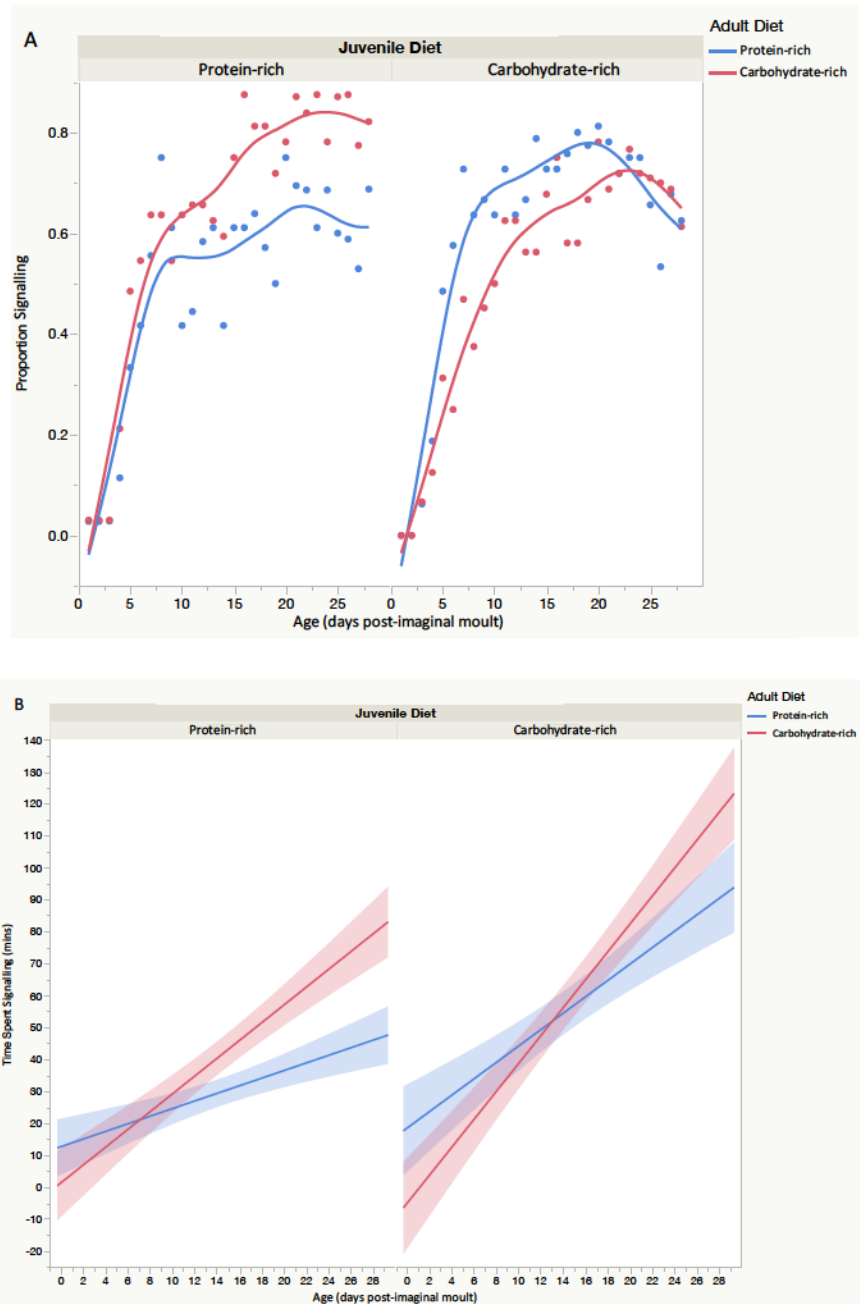

Figure 4. The impact of both juvenile and adult diet on (a) the proportion of individuals signalling across the number of days in EARS, and (b) time spent signalling as males age. Males in the left panel were reared on a protein-rich diet as juveniles, while males in the

right panel were reared on a carbohydrate-rich diet as juveniles. Within each panel, males fed a carbohydrate-rich diet as adults are shown in red, whereas males fed a protein-rich diet as adults are shown in blue.

Table 3. The impact of juvenile and adult diets on the proportion of individuals signalling as they age and time spent signalling. Significant *P*-values are in boldface.

Parameter	Term	P	F	R^2_{Adj}
Proportion Signalling	Juv. Diet	0.7922		
	Adult Diet	0.4079		
	Juv. Diet x Adult Diet	**0.0004**	28.2743	0.5958
	Age	**<0.0001**		
	Juv. Diet x Age	0.8061		
	Adult Diet x Age	0.0587		
Time Spent Signalling	Juv. Diet	**0.0137**	6.2587	
	Adult Diet	0.4456	0.5856	
	Adult Size PC1	0.5995	0.2773	0.3642
	Age	**<0.0001**	66.1948	
	Juv. Diet x Age	**0.0001**	14.5921	
	Adult Diet x Age	**<0.0001**	17.6986	

Experiment 2: Diet Preference during Juvenile and Adult Stages

a) Do crickets switch diet preference between juvenile and adult stages as their trajectory switches from growth to reproduction? (Question 3)

When given a choice, male crickets preferentially consumed carbohydrate over protein, regardless of whether they were juveniles (Prediction 3a) or adults (Prediction3b). Juvenile males ate almost twice as much carbohydrate as protein when given the choice (24 mg of carbohydrate to 13 mg of protein per day; Figure 5a; Table 4). Similarly, adults followed the same consumption rule, eating carbohydrates over protein

in an almost 2:1 ratio (21 mg of carbohydrate compared to 12 mg or protein per day;
Figure 5a; Table 4). The control males who were given two identical diets consisting of a
50:50 ratio of P:C did not discriminate between the diets (Figure 5b), indicating that the
crickets ate these diets ate random and, showing no preferential consumption.
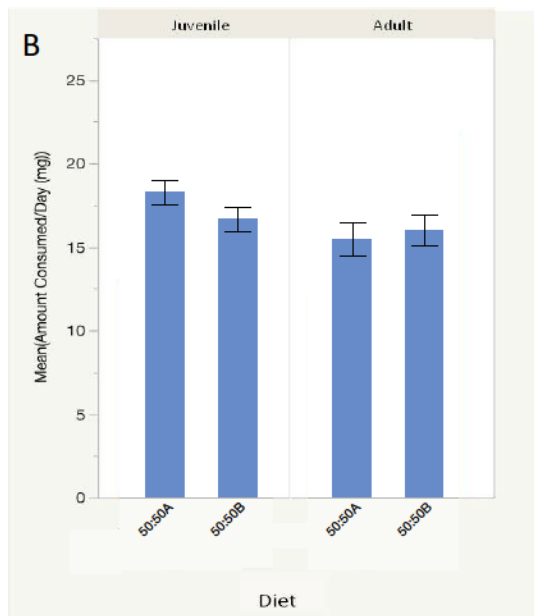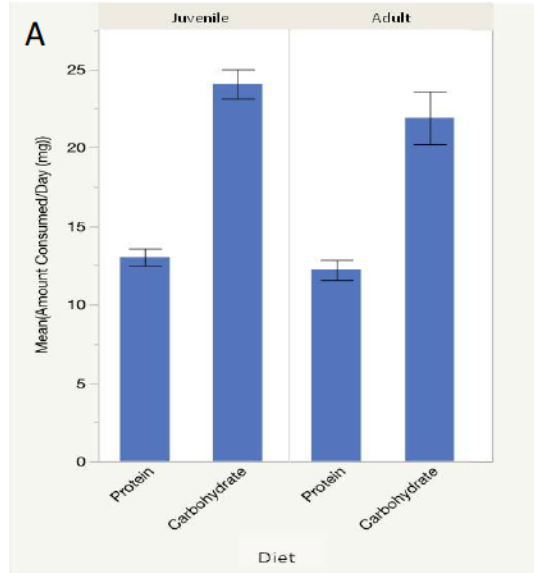
Figure 5. Mean (±SE) amount consumed per day of diets containing (a) pure protein and pure carbohydrate, and (b) equal amounts of proteins to carbohydrates. These amounts were consumed during the juvenile and the adult life stage (separated by the vertical line)

Table 4. How food consumption of pure protein and pure carbohydrate diets per day varies over a lifetime when males self-select diets throughout development and into adulthood. Significant *P*-values are in boldface.

Parameter	Term	DF	F	P	R^2_{Adj}
Amount Consumed/ Day	50:50A 50:50B Pure Carbohydrate Diet Juv./ Adult	1,259	37.6312 5.8580	0.2723 0.9075 **<0.0001** **0.0162**	0.30

b) How do juvenile and adult consumption per day vary by week during development (Question 3)?

Juvenile male crickets consistently consumed more of the pure carbohydrate diet than the pure protein diet every week during development. These individuals also significantly increased their consumption during week 3 of development (Figure 6a; Table 5). Adult male crickets also consistently consumed more of the pure carbohydrate diet than the pure protein diet every week during adulthood. Males decreased their consumption of protein every week and increased their consumption of carbohydrate in week 2 of adulthood (Figure 6b).
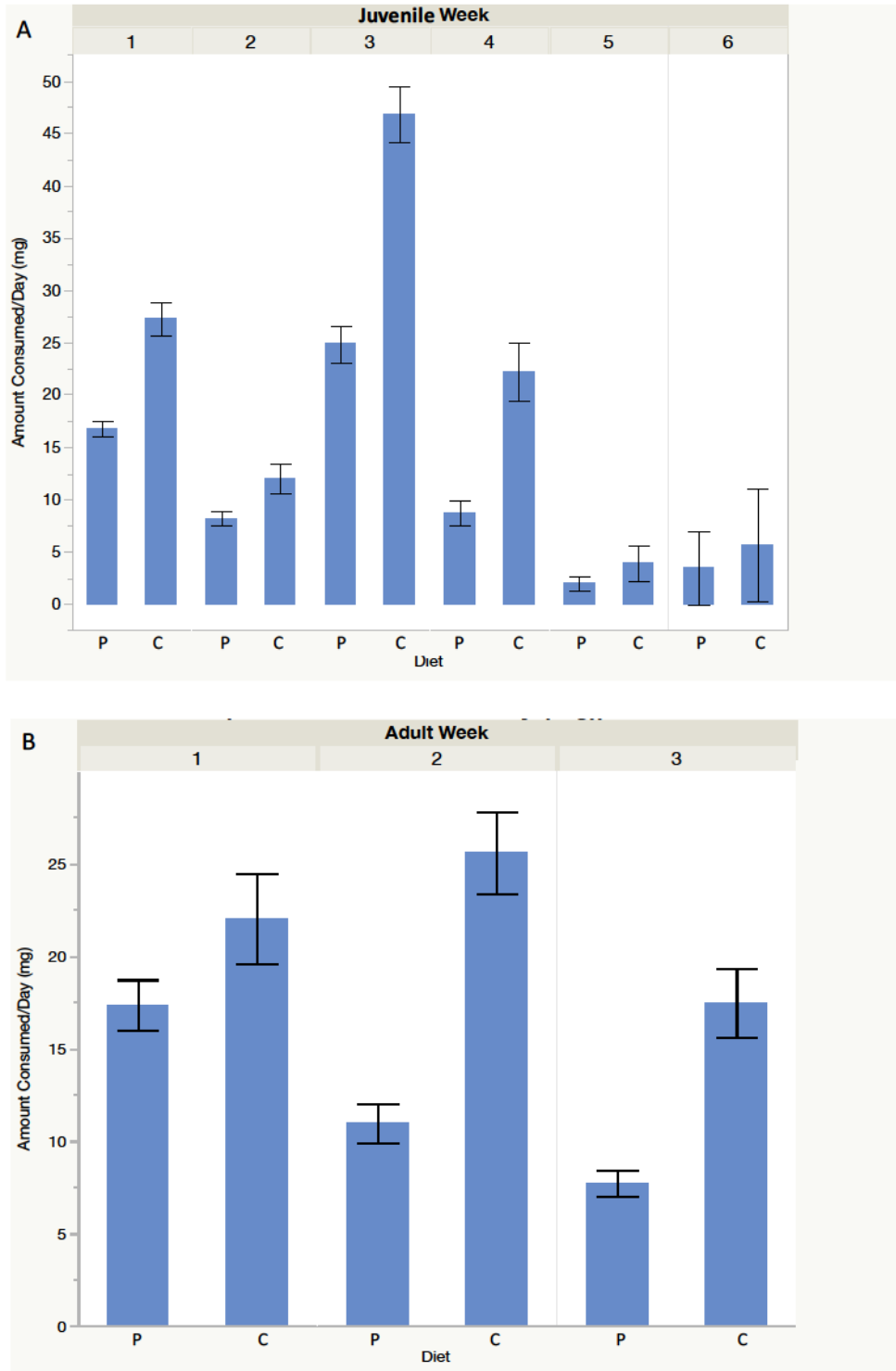
Figure 6. Mean (±SE) amount consumed per day by week of pure protein and pure carbohydrate diets by (a) juvenile male *G. assimilis* crickets, and (b) adult male *G. assimilis* crickets.

Table 5. How food consumption of pure protein and pure carbohydrate diets per day

varies by week when males self-select diets throughout development and into adulthood.

Significant *P*-values are in boldface.

Term	Pure Protein Diet x̄±SE	Pure Carbohydrate Diet x̄±SE	DF	F	P	R^2_{Adj}
Diet				45.0664	**<0.0001**	
Juvenile Week	12.76±1.15	23.65±1.15	1,316	21.5995	**<0.0001**	0.17
Juvenile Week x Diet				0.0894	0.7651	
Diet				45.2755	**<0.0001**	
Adult Week	12.14±1.01	21.76±1.01	1,198	16.2865	**<0.0001**	0.23
Adult Week x Diet				2.1601	0.1432	

Discussion

Given that nutritional demands are likely to change over time, with juveniles requiring relatively more protein for growth and adults requiring relatively more carbohydrates to fuel their energetically demanding mate attraction displays, I investigated how the relative amounts of dietary protein and carbohydrates influenced fitness conferring traits at different life stages. Specifically, I asked how juvenile diet influenced development time, body size at adult eclosion, residual mass at adult eclosion (Question 1), and the timing of signalling onset (Question 2). I also asked how juvenile and adult diet and their interaction influenced adult male acoustic mate attraction signalling (Question 2), and whether crickets changed their dietary preferences as they developed (Question 3). My results revealed that protein availability influenced juvenile development time, adult body size at eclosion, and the timing of mate attraction signalling onset, but it failed to influence adult residual mass at eclosion (Questions 1&2). Specifically, juvenile crickets who had access to higher amounts of dietary protein during development reached adulthood faster (23 versus 28 days from wing-bud stage to adulthood; Hypothesis 1, Predication 1b), were larger at adulthood (Hypothesis 1, Prediction 1a), and started signalling earlier (6 days versus 8 days post-adult eclosion; Hypothesis 2, Prediction 2a) than those who were constrained to diets containing higher amounts of carbohydrates. Conversely, adult males with access to higher amounts of dietary carbohydrates signalled more often (Question 2; Hypothesis 2, Prediction 2b). My findings suggest that juvenile fitness proxies are maximized on a more protein based portion of the nutritional landscape while adult fitness proxies are maximized on a more carbohydrate based portion of the landscape. Given this, juvenile and adults should eat

significantly different protein to carbohydrate ratios when given the choice, such that the self-selected P:C ratio ingested reflects the ratio of nutrients required for optimal somatic and metabolic functioning. Unexpectedly, my results demonstrated that, when given the choice, the diet crickets ingested was not optimal for juvenile growth and development, as juveniles and adults both preferred a diet rich in carbohydrates (Question 3; Hypothesis 3a, Prediction 3a). Juveniles self-selecting higher carbohydrate diets may represent a compromise between the different nutritional requirements associated with juvenile growth and adult mate attraction signalling (Question 3; Hypothesis 3b, Prediction 3b).

Impact of Diet on Growth and Development (Question 1)

My results align with other studies investigating the impact of protein and carbohydrate consumption on juvenile development time. Early studies suggest that protein availability affects invertebrate growth rate. For example, protein availability sped growth rate in fifth instar larvae of Southern armyworms (*Spodoptera eridania*) (Karowe & Martin, 1989). More recently, several insect studies have confirmed this finding (predatory stinkbugs: Mahdian *et al.*, 2006; variegated grasshoppers: Idowu & Sonde, 2003; ground beetles: Saska & Honěk, 2003; crickets: Hunt *et al.*, 2004; red-flour beetles: Khattak, Azhar, & Shahid, 2004; cockroaches: Raubenheimer & Jones, 2006; green lacewing: Syed, Ashfaq, & Ahmad, 2008; crickets: Kasumovic *et al.*, 2011; pirate bugs: Bonte *et al.*, 2012; tropical sod webworm: Tofangsazi *et al.*, 2012). Lopatina *et al.* (2014) note that the insufficient protein content in the aforementioned studies resulted in both slower development and smaller body size. Similar findings have also been

observed in a wide variety of other species (humans: Marine *et al.*, 1991; trout: Dosanjh *et al.*, 1992; rats: Shi, Levy, & Lightman, 1993; birds: Searcy *et al.*, 2004; frogs: Matson *et al.*, 2010; Widyaratne & Drew, 2011; nematodes: Hansen *et al.*, 2015). The aforementioned studies typically manipulated the protein content crudely, for example by comparing insects grown on different food plants (cassava versus pawpaw versus acalypha versus a mixture of these plants) (Mahdian *et al.*, 2006). In a study that more explicitly manipulated protein availability, Roeder & Behmer (2014) found that caterpillars (*Heliothis virescens*) fed protein-biased (relative to carbohydrate) diets developed significantly faster than those raised on carbohydrate-biased (relative to protein) diets.

Murray (1960) highlights that yeast works as a feeding stimulant, and contains important growth factors for yellow mealworms, *Tenebrio molitor L.* (Fraenkel, 1950). Cregg *et al.* (2000) note that yeast is typically used as a tool to generate protein. In my experiment, casein, albumen, or peptone singly, or their combination could be acting as a feeding stimulant for male crickets. Additionally, Hansen *et al.* (2015) showed that nematodes (*Caenorhabditis elegans*) raised on a complex protein diet reached adulthood faster than those raised solely on amino acids. These researchers concluded that the complex protein diet stimulates expression and activity in a different manner than the acting amino acid diet, which ultimately promotes improved growth. In this way, male crickets in my experiment may benefit from protein-rich diets over carbohydrate-rich diets due to the processes involved in assimilating these nutrients.

My results also revealed that juvenile diet influenced body size at eclosion, as males with access to diets containing higher amounts of protein were larger at adulthood than

those individuals reared on carbohydrate-rich diets, after controlling for variation in initial body size (Hypothesis 1, Prediction 1a, Figure 2b). Numerous studies have found support for this idea when investigating the effects of diet on body size (spiders: Mayntz & Toft, 2001; variegated grasshoppers: Idowu & Sonde, 2003; birds: Searcy *et al.*, 2004; red-flour beetles: Khattak, Azhar, & Shahid, 2004; green lacewing: Syed, Ashfaq, & Ahmad, 2008; frogs: Matson *et al.*, 2010; tadpoles: Venesky *et al.*, 2012; fish: Kpundeh *et al.*, 2015). For example, farm tilapia (*Oreochromis niloticus*) showed maximum growth when fed diets with higher protein levels (Kpundeh *et al.*, 2015). Furthermore, tadpoles reared on low-protein diets were smaller and less developed than tadpoles on high-protein diets (Venesky *et al.*, 2012). Larger body size has important fitness consequences, as several studies have shown that females preferentially mate with larger males (Bateman *et al.*, 2001; Bertram *et al.*, 2016; Deb *et al.*, 2012; Loranger & Bertram, 2016; Simmons, 1986, 1995; Zuk, 1988).

While juvenile diet did not significantly influence residual mass at adult eclosion, there was a non-significant trend indicating that individuals with access to higher protein as juveniles tended to have higher residual weight compared to individuals fed carbohydrate-rich diets (Hypothesis 1, Prediction 1a, Figure 2c). In contrast, several other cricket studies show that an individual's ability to put on weight during development and in adulthood is often influenced by their diet (*R. verticalis*: Simmons *et al.*, 1992; *G. campestris*: Holzer *et al.*, 2003; Scheuber *et al.*, 2003a; *T. commodus*: Kasumovic *et al.*, 2011). For example, in nature, food-supplemented adult male *G. campestris* showed a significant increase in residual mass, whilst control males (who received less food) showed a decrease in residual mass (Holzer *et al.*, 2003). Food-supplemented males in

the Holzer *et al.* (2003) study were able to use the surplus energy towards improving body reserves, much like adult *G. campestris* males in a laboratory setting (Scheuber *et al.*, 2003a). Importantly, Scheuber *et al.* (2003a) note that under field conditions *G. campestris* males typically experience food deprivation, thus producing a trade-off between using additional energy towards enhancing body reserves or signalling behavior. Relevant to my study, storage in body reserves relates to current reproductive effort, whilst investing in signalling behavior refers to future reproductive effort. As I focused on residual mass throughout development (juvenile weight gain), and individuals were not yet signalling, juvenile males may have only been able to use the available energy for basic maintenance requirements which varied depending on their dietary constraints. Such trade-offs may thus have become more apparent only in adulthood, which I later describe in detail.

Interestingly, juvenile diet influenced the timing of the onset of mate attraction signalling (Hypothesis 2a, Prediction 2a). Males fed higher protein diets as juveniles began to signal almost two days earlier than individuals who were reared on high carbohydrate diets (e.g., 6 days versus almost 8 days post-adult eclosion; Figure 3a). Ketola & Kotiaho (2010) acknowledge that individuals (*G. sigillatus*) are sexually mature 5 days post-final moult, while Fitzsimmons & Bertram (2011) report that *G. veletis* males begin signalling around 7 days post-adult eclosion. This finding is extremely important, as the ability to signal earlier in adulthood may allow males to attract a mate at an earlier stage, potentially increasing the opportunity to attract more females through the course of their adult life, and in so doing enhancing their lifetime fitness (Kasumovic *et al.*, 2011). McNamara *et al.* (1987) suggested that variation in food availability may affect the trade-

off between investment in sexual display and other life-history traits. Body mass has a negative effect on latency to signal in *G. sigillatus* (Ketola & Kotiaho, 2010) and juvenile diet influences body size at adulthood in *G. assimilis* (Hypothesis 1a, Prediction 1a, Figure 2b), possibly highlighting the indirect influences of juvenile diet towards the latency to signal. Other cricket studies also concur that individuals fed protein-rich diets begin to signal earlier in adulthood, thus support my results on how diet effects the latency to signal (e.g., Hunt *et al.*, 2004).

Impact of Diet on Mate Attraction Signalling (Question 2)

I also asked how the diet *throughout* an individual's lifetime influences male acoustic mate attraction signalling. Juvenile diet significantly influenced the amount of time spent signalling, but did not influence any of the other signalling traits (Table 2). Surprisingly, adult diet *did not* influence any of the signalling traits I measured, including time spent signalling (Table 2). I included adult body size as a co-variate in the model, and body size explained variation in several of the parameters that are indicative of overall signalling effort; residual mass also explained variation in carrier frequency (Table 2). Carrier frequency is indicative of male body size as larger males tend to have lower carrier frequencies (*G. bimaculatus*: Popov & Shuvalov, 1977; *Tettigonia cantans*: Latimer & Schatral, 1986; Latimer & Sippel, 1987; *Oecanthus nigricornis*: Brown *et al.*, 1996; *G. campestris*: Simmons & Ritchie, 1996; Scheuber *et al.*, 2003b; *O. henryi*: Deb *et al.*, 2012; *G. pennsylvanicus*: Harrison *et al.*, 2013; *G. assimilis*: Pacheco & Bertram, 2014; Simmons & Zuk 1992; Simmons 1995). Importantly, carrier frequency is typically related to body size because frequency is constrained by the size of the sound-producing

structure (harp size) (Brown *et al.*, 1996). Moreover, as juvenile diet drives adult body size (Figure 2b) and almost drives residual mass (Figure 2c), juvenile diet (in addition to directly influencing time spent signalling) appears to indirectly influence male acoustic mate attraction signalling effort.

Female crickets typically preferentially mate with males that signal with high effort (e.g., *G. texensis*: Cade & Cade, 1992; *G. firmus*: Crnokrak & Roff, 1995; *G. campestris*: Holzer & Brinkhof, 2003; *T. commodus*: Hunt *et al.*, 2004; Bentsen *et al.*, 2006; *G. pennsylvanicus*: Judge *et al.*, 2008; 2014). Other studies also generally conclude that males obtaining high-quality diets invest more in costly signalling traits (beetles: Peschke, 1987; crabs: Backwell *et al.*, 1995; wolf-spiders: Kotiaho, 2000, Hoefler *et al.*, 2008; ambush bugs: Punzalan *et al.*, 2008; lizards: Kahrl & Cox, 2015; but see fish: Candolin, 1999). For example, wolf-spiders, *Hygrolycosa rubrofasciata*, maintained their body mass and produced their maximum drumming rate when fed high amounts of food as compared to those males who lost mass and produced the lowest drumming rates on the low-food diets. Further, Kasumovic *et al.* (2011) suggest that larval diet affects the amount of resources an individual can allocate towards trait production which ultimately affects trait expression (Emlen, 1997; Johansson *et al.*, 2001; Bonduriansky & Rowe, 2005). My finding that morphology influences signalling effort underscores the importance of obtaining the appropriate juvenile diet.

Owing to the aforementioned dietary effects on time spent signalling, I also explored the interplay between diet and adult age to ascertain whether individuals on different diets signaled differently through time. I found a direct relationship between age and the proportion of males signalling, as the proportion of males signalling each day increases

with age (Figure 4a). With each passing day in EARS, more individuals spent time signalling up until around 20 days post-adult eclosion, regardless of their dietary combinations. Hunt *et al.* (2004) determined that signalling effort in early adulthood (10-20 days) was consistently negatively correlated with longevity, but during late adulthood (25-30 days) was consistently positively correlated instead. Although I did not study longevity (as individuals were frozen 28 days post-adult eclosion), the Hunt *et al.* (2004) study predicts possible consequences of signalling effort. In the Hunt *et al.* (2004) study, crickets that consumed diets with high- and medium- protein content invested in signalling during early adulthood, whereas those that invested in signalling during late adulthood were subjected to the low-protein dietary treatment (Hunt *et al.*, 2004). Although males (*T. commodus*) fed high-protein diets died earlier, they still signalled more throughout their lifetime than low-protein fed males (Hunt *et al.*, 2004). This disproportionate investment in costly sexual traits is mirrored across various species (Kokko, 1997; 1998; Höglund & Sheldon, 1998; Kokko *et al.*, 2002), and is generally caused by sacrificing lifespan for enhanced mating success (Kokko, 1997; 2001). In contrast, long-lived *G. sigillatus* males consistently signalled more than short-lived males and reproductive effort did not depend on diet (Houslay *et al.* (2015). The idea that longer-living individuals signal more than short-lived individuals is more common throughout the literature (crickets: Fitzsimmons & Bertram, 2011; meta-analysis of 69 studies of 40 species: Jennions *et al.*, 2001).

Moreover, both juvenile diet and adult age significantly influenced time spent signalling (Figure 4b). All males increased their time spent signalling (signalled more) as they grew older, however, males that were fed high carbohydrate diets as juveniles and

adults experienced the biggest increase in the amount of time spent signalling. This finding emphasizes the importance of carbohydrate consumption to signalling. Interestingly, males consuming high carbohydrate diets either as juveniles or as adults spent relatively similar amounts of time signalling as adults. Conversely, males that consumed protein-rich diets throughout signalled least often as they aged (Figure 4b).

In my experiment, males fed protein-rich diets as juveniles who were then switched to carbohydrate-rich diets as adults were more likely to signal (greater proportion signalling) as they aged as opposed to those who remained on the protein-rich diets through adulthood. Surprisingly, individuals fed carbohydrate-rich diets as juveniles also followed this trend. Switching to protein-rich diets in adulthood did not constrain these individuals, as a larger proportion signalled as they aged compared to those who remained on the carbohydrate-rich diets through adulthood (Figure 4a). Similarly, Harrison *et al.* (2014) found that *G. veletis* males maximized mate signalling with either protein-rich or carbohydrate-rich intake. Importantly, this suggests that signalling may thus be a function of overall caloric intake as opposed to the intake of proteins or carbohydrates alone (Harrison *et al.*, 2014). Thus, the combination of being constrained to the diet switch from development into adulthood benefitted individuals in relation to the time spent signalling as it ensured they are getting access to both the protein-rich and carbohydrate–rich diets.

Dietary trade-offs between age, development time, body size, weight gain, and acoustic signalling effort can have important implications on the lifetime fitness of crickets. For example, older and/or larger males attract more females and tend to experience a higher mating success (Bateman *et al.*, 2001; Bertram *et al.*, 2016; Zuk,

1988). Females also prefer to mount more dominant males (Loranger & Bertram, 2016).

Additionally, females tend to prefer males exhibiting higher-energy signals (Hoback &

Wagner, 1997; Popov & Shuvalov, 1977. Specifically, *G. assimilis* females prefer louder

signals produced at lower carrier frequencies (Pacheco & Bertram, 2014). As higher-

energy signals are more costly to produce (Prestwich & Walker, 1981; Hoback &

Wagner, 1997; Reinhold *et al.*, 1998; Bertram *et al.*, 2011; Thomson *et al.*, 2014) and are

influenced by nutrition (Holzer *et al.*, 2003; Scheuber *et al.*, 2003 Maklakov *et al.*, 2008;

Bertram *et al.*, 2009), these signals may act as reliable indicators to females on indirect

fitness benefits of mate choice (e.g. good genes for foraging ability in offspring).

Diet Self-Selection (Question 3)

Given how important protein is to developing crickets and carbohydrates are to fuel

adult signalling, I also wanted to investigate whether juveniles and adults self-select

different amounts of protein and carbohydrates to maximize their fitness across different

life stages (Hypotheses and Predictions 3a,b). I therefore asked if juvenile crickets

preferentially consume diets rich in protein (Hypothesis 3a, Prediction 3a) and then

switch their diet preference in adulthood to a more carbohydrate based diet as their

trajectory switches from growth to reproduction and survival (Hypothesis 3b, Prediction

3b). My results clearly indicated that male crickets preferentially consumed

carbohydrates relative to proteins throughout juvenile and adult stages (Figure 5a). To my

surprise, and refuting my Hypothesis 3a, I found that juvenile males ate twice as much of

the pure carbohydrate diet over the pure protein diet daily. In accordance with the

Maklakov *et al.* (2008) study, where adult male crickets exhibited dietary preferences in

the direction that increased reproductive performance, adult males in my experiment followed this same trend as expected. These individuals consumed double the amount of the pure carbohydrate diet relative to the pure protein diet daily (Hypothesis 3b, Prediction 3b, Figure 5a).

In order to further investigate my diet preference results, I determined: (i) how juvenile consumption daily changed with weekly development, and (ii) how adult consumption daily changed with weekly development. My results indicated that juvenile male crickets consistently ate more of the pure carbohydrate diet compared to the pure protein diet regardless of the week of development, illustrating a clear preference for carbohydrates relative to proteins as juveniles (Figure 6a). During week 3 of development these individuals increased their overall food consumption significantly (Figure 6a), as the amount of pure carbohydrate diet consumed daily during this week greatly exceeded the average amount consumed daily of pure carbohydrate diet in all previous and subsequent weeks of development. Given that this increase in consumption is so large during this particular week of development, it implies that these individuals are experiencing something specific during this week that warrants such a great shift in the amount of pure carbohydrate diet (~45 mg consumed daily), compared to ~25 mg daily of the pure protein diet (Figure 6a). Kasumovic *et al.* (2012) found that diets significantly affected phenotypes during the second-last stadium though these effects were not mirrored in the last stadium of juvenile development. Perhaps, this increase in consumption during the third week of juvenile diet reflects the developmental changes that are apparent in morphology in terms of the energy gained and retained from nutrients.

Similarly, adult male crickets consistently ate more of the pure carbohydrate diet relative to the pure protein diet regardless of the week of adulthood (Figure 6b). This result suggests that males prefer carbohydrates over proteins as adults. Several studies have pointed out the benefits of carbohydrate consumption relative to proteins in adulthood (Maklakov *et al.*, 2008; South *et al.*, 2011; Harrison *et al.*, 2014). In my study, individuals decreased their daily protein consumption weekly, and consumed increasing amounts of carbohydrates daily during week 2 of adulthood specifically, suggesting age is again an important factor in food consumption, such that, individuals are experiencing a specific change during their second week of adulthood, where carbohydrates become more essential. Producing attractive acoustic signals is costly to males (Zahavi, 1975; 1977), and in order to bear these costs adult males may be consuming more carbohydrates relative to proteins during week 2 post-adult eclosion. Alternatively, Pacheco *et al.* (2013) suggest that 10-13 day old females (post-adult eclosion) are most responsive to male acoustic sexual signals. In terms of age, males may also be synching their acoustic signalling effort with female receptivity (Knowlton, 1979), as signalling for females may be more important at this age in order to maximize their chances of finding a mate and increasing their fitness, hence they need to fuel their signalling with increased consumption of carbohydrates relative to proteins.

Given how important it is for juveniles to develop quickly and grow large, and then signal as quickly as possible for mates, I found it extremely surprising that, when given a choice, males consistently ate more carbohydrates than proteins as juveniles. Similar results were found in juvenile *G. veletis* as females consistently ate more carbohydrates relative to protein at a ratio of about 1P:2.09C (Harrison & Bertram, *in prep*).

For many organisms, dietary selection changes as nutrients become more or less available (Srygley, 2017). However, as individuals were given a choice in my experiment, the role of genetics in crickets must be highlighted. It is possible that the juvenile tastes may be competing with adult tastes. Juvenile males seem to be being displaced from their optimum diet for growth (proteins), and instead are consuming more carbohydrates possibly because of constraints for optimal diets in adulthood (carbohydrates). Males are, therefore, constrained in their ability to reach their age-specific dietary optima by the biology of diet choice (possibly caused by the genes that are responsible for trait expression at the different ages). This potential for intralocus conflict also occurs between males and females (Lande, 1980; Rice & Chippindale, 2001), and intralocus sexual conflict has been described for dietary preferences a host of different species (Common fruit fly: Rice, 1996; 1998; Chippindale *et al.*, 2001; Rand *et al.*, 2001; Pischedda & Chippindale, 2006; Long & Rice, 2007; Prasad *et al.*, 2007; Collared flycatcher: Merilä *et al.*, 1997; 1998; Brommer *et al.*, 2007; Zebra finch: Price & Burley, 1993; 1994; Southern ground cricket: Mousseau & Fedorka, 2004; Red deer: Coulson *et al.*, 2007; Seed beetle: Berger *et al.*, 2014; Bank vole: Mills *et al.*, 2012; Humans: Stulp *et al.*, 2012). For example, in *T. commodus*, egg production was maximized when females are fed $1_P:1_C$ diets, compared to males who need high carbohydrate diets to maximize signalling effort (Maklakov *et al.*, 2008).

Conclusions and Future Directions

My study was the first to examine the influence of dietary nutrient ratio on fitness and life history across the lifetime of crickets. Although other studies, using the

multivariate approach, have shown that carbohydrates are more important to adult signalling (Maklakov *et al.*, 2008; South *et al.*, 2011; Harrison *et al.*, 2014), these studies were not designed to determine which nutrient is more important across the lifetime of individuals. By tracking individuals from wing-bud stage through to adulthood, I was able to ascertain the effect of protein and carbohydrate consumption on fitness conferring traits through development and into adulthood. My findings revealed that juvenile diet, by way of protein consumption, influenced development time, body size at adulthood, and signalling onset, supporting my first and second hypotheses. Given these strong effects of protein consumption on performance, it is surprising that, when given a choice, juvenile male crickets preferentially consumed more carbohydrates relative to proteins, refuting my third hypothesis. Overall, my findings highlight the importance of understanding the impact of different nutrients across various life stages in order to gain a more comprehensive understanding of nutritional ecology and its impact on fitness conferring traits. Future research should be directed at disentangling the role of genetics in the phenomenon, seeking to understand and even pinpoint genes responsible for dietary selection and/or preference. Such research could further identify any tradeoffs that might be occurring owing to factors associated with genetics. Additionally, future work should investigate how the size of the sound-producing structure is affected after diet manipulation, and in turn what role this plays in the associated signalling behaviours. Such research could help advance the knowledge on both the direct and indirect consequences of nutrient consumption in relation to acoustic signalling.

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