JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12703

Terminal investment in the gustatory appeal of nuptial food gifts in crickets

K. R. DUFFIELD*, J. HUNT†, J. RAPKIN†, B. M. SADD* & S. K. SAKALUK*

*Behavior, Ecology, Evolution & Systematics Section, School of Biological Sciences, Illinois State University, Normal, IL, USA
†Center for Ecology & Conservation, College of Life and Environmental Sciences, University of Exeter, Penryn, UK

Keywords:

life-history theory;
nuptial food gift;
reproductive effort;
spermatophylax;
terminal investment.

Abstract

Investment in current versus future reproduction represents a prominent trade-off in life-history theory and is likely dependent on an individual's life expectancy. The terminal investment hypothesis posits that a reduction in residual reproductive value (i.e. potential for future offspring) will result in increased investment in current reproduction. We tested the hypothesis that male decorated crickets (*Gryllodes sigillatus*), when cued to their impending mortality, should increase their reproductive effort by altering the composition of their nuptial food gifts (i.e. spermatophylaxes) to increase their gustatory appeal to females. Using a repeated-measures design, we analysed the amino acid composition of spermatophylaxes derived from males both before and after injection of either a saline control or a solution of heat-killed bacteria. The latter, although nonpathogenic, represents an immune challenge that may signal an impending survival threat. One principal component explaining amino acid variation in spermatophylaxes, characterized by a high loading to histidine, was significantly lower in immune-challenged versus control males. The relevance of this difference for the gustatory appeal of gifts to females was assessed by mapping spermatophylax composition onto a fitness surface derived in an earlier study identifying the amino acid composition of spermatophylaxes preferred by females. We found that immune-challenged males maintained the level of attractiveness of their gifts post-treatment, whereas control males produced significantly less attractive gifts post-injection. These results are consistent with the hypothesis that cues of a survival-threatening infection stimulate terminal investment in male decorated crickets with respect to the gustatory appeal of their nuptial food gifts.

Introduction

Life-history theory posits that natural selection should shape an individual's investment in key life-history traits (growth, survival and reproduction) in a way that maximizes fitness (Michod, 1979). Limited resources may necessitate trade-offs both between traits and within traits over time (Roff, 1992; Stearns, 1992). An especially important trade-off concerns the allocation of resources between current and future reproduction, which is likely contingent on an individual's condition and anticipated lifespan, and hence the potential to produce future offspring (Williams, 1966; Trivers, 1972; Pianka & Parker, 1975). The 'terminal investment hypothesis' proposes that individuals facing a survival threat should divert time, energy and resources away from survival, or somatic maintenance, and towards current reproduction as a means of maximizing lifetime reproductive output (Clutton-Brock, 1984). Indeed, empirical studies have found support for the terminal investment hypothesis in numerous species following a perceived survival threat, as evidenced by an increase in various components of reproductive effort (Minchella & Loverde, 1981; Bonneaud *et al.*, 2004; Velando *et al.*, 2006; Weil *et al.*, 2006; Creighton *et al.*, 2009), including increased attractiveness of plastic epigamic traits in males (Candolin, 1999; Sadd *et al.*, 2006; Cote *et al.*, 2010; Copeland & Fedorka, 2012).

Correspondence: Kristin R. Duffield, Behavior, Ecology, Evolution & Systematics Section, School of Biological Sciences, Campus Box 4120, Illinois State University, Normal, IL 61790-4120, USA. Tel.: +1 309 438 5438; fax: +1 309 438 3722; email: krduffi@ilstu.edu

In numerous insects and some spider species, males must provide females with a nuptial food gift to secure copulation. Such gifts come in numerous forms, including enlarged spermatophores, glandular secretions, prey items and even male body parts (Thornhill, 1976; Vahed, 1998, 2007; Lewis & South, 2012). Often these gifts are relatively void of nutrition, leading to extensive debate concerning their ultimate function (Quinn & Sakaluk, 1986; Parker & Simmons, 1989; Gwynne, 2008; Vahed, 2007; Lewis *et al.*, 2014). Some of these gifts appear to function as sensory traps (Sakaluk, 2000; Sakaluk *et al.*, 2006), by which males exploit the female's normal gustatory responses to seduce her to accept superfluous matings, transfer more ejaculate than is in her interest or allocate substances that influence her remating or egg-laying behaviour in favour of the male's interest (Arnqvist & Nilsson, 2000; Vahed, 2007). Selection should thus favour males that produce gifts that are sufficiently enticing to females, but need not possess any nutritional value (Sakaluk, 2000; Vahed, 2007). Consequently, terminally investing males might increase their reproductive investment through the enhanced gustatory appeal of their gifts, thereby maximizing their immediate mating or fertilization success.

During copulation in decorated crickets, *Gryllodes sigillatus*, males transfer a spermatophore consisting of a small sperm-containing ampulla surrounded by a much larger, gelatinous mass, the spermatophylax, which the female consumes after copulation as a nuptial gift. The spermatophylax is composed mostly of water, along with some accessory gland proteins and free amino acids (Warwick *et al.*, 2009; Gershman *et al.*, 2012, 2013), but is otherwise lacking in nutritional benefits (Will & Sakaluk, 1994; Ivy *et al.*, 1999; but see Ivy & Sakaluk, 2005). Immediately following mating, the female plucks off the spermatophylax with her mouth parts and feeds on it while sperm from the ampulla are evacuated into her reproductive tract. Upon the complete consumption of the spermatophylax, the female removes and eats the ampulla, thereby terminating sperm transfer; smaller spermatophylaxes require less time to consume, and consequently, males providing such gifts experience premature ampulla removal and reduced sperm transfer (Sakaluk, 1984, 1985, 1987). The number of sperm transferred, in turn, has important consequences for the fertilization success of the male (Sakaluk, 1986; Sakaluk & Eggert, 1996; Calos & Sakaluk, 1998; Eggert *et al.*, 2003) because of the sperm competition that ensues when females mate with multiple males over the course of their lifetime (Sakaluk *et al.*, 2002).

It follows from the description of mating behaviour above that males benefit most when their gifts are fully consumed, because it is under these circumstances that sperm transfer is maximized. However, females often discard the spermatophylax after mating by simply dropping it prior to its complete consumption in approximately one quarter of all cases (Sakaluk, 1984, 1987). When the female discards the spermatophylax, she typically does so within 15 min or less after mating (Gershman *et al.*, 2012), long before complete sperm transfer has occurred, and then removes the sperm ampulla shortly thereafter (Sakaluk, 1984, 1987). Recent studies have revealed that the composition of free amino acids, known phagostimulants in other insects (Calatayud *et al.*, 2002), influences the gustatory appeal of the spermatophylax and the female's willingness to continue feeding on it (Warwick *et al.*, 2009; Gershman *et al.*, 2012). Gershman *et al.* (2012) compared the amino acid profile of spermatophylaxes discarded by females with that of spermatophylaxes destined to be consumed, using multivariate selection analysis to quantify the strength and form of sexual selection acting on the composition of the gift. They identified a fitness surface with two local peaks, indicating that different combinations of amino acids promote continued feeding on the spermatophylax either because they have equivalent gustatory appeal, or because they differentially affect the gustatory appeal and texture of the spermatophylax.

Given their importance to a male's reproductive success, males should ideally customize their food gifts so that they provide sufficient gustatory appeal to promote their complete consumption and thereby maximize ejaculate transfer. It is likely that production is costly and that males are further constrained by available resources. Spermatophores represent, on average, more than two per cent of a male's body mass (Sakaluk, 1997), and male refractory periods in *G. sigillatus* are an order of magnitude longer compared to non-gift-giving species (Sakaluk, 1985). More recent studies also suggest that investment in the spermatophylax can come at substantial immunological costs (Gershman *et al.*, 2010; Kerr *et al.*, 2010). These observations suggest a trade-off between gift quality and male mating rate, such that males are constrained to invest in gifts strategically to maximize lifetime reproductive output. Indeed, the variation and plasticity found in the free amino acid composition of the spermatophylax (Gershman *et al.*, 2013) suggest that males have the potential to tailor gift quality to optimize their investment.

If spermatophylax production constitutes a significant component of male reproductive effort and lifetime investment is limited, then males should be subject to the same trade-off between current and future reproduction with respect to the composition of their food gifts as with other sexually selected signals. Specifically, a male's residual reproductive value should determine the extent of his investment in nuptial food gifts, and predicts that males whose perceived residual reproductive value has been decreased should invest more heavily in the gustatory appeal of their gifts. To test the terminal investment hypothesis as it applies to the quality of the nuptial gifts synthesized by male *G. sigillatus*,

we employed a repeated-measures design in which spermatophylaxes were collected before and after males were injected with either heat-killed *Escherichia coli* or a saline control. We predicted that if the immune challenge mediated by an exposure to nonpathogenic heat-killed bacteria signals an impending survival threat, spermatophylaxes synthesized by males injected with heat-killed bacteria should differ significantly in their amino composition from those synthesized by control males. We further predicted that this shift in amino acid composition would increase the gustatory appeal of the gifts of immune-challenged males compared with those of control males. To determine whether there are different reaction norms characterizing the level of terminal investment elicited by a specific level of immune challenge, the experiment was performed on males from several different inbred lines. We predicted that any genetic variation underlying phenotypic plasticity in a male's response to an immune challenge would be reflected in a significant line-by-environment (i.e. treatment) interaction on the amino acid composition of male food gifts.

Materials and methods

Study animals

Experimental *G. sigillatus* males used in this study were chosen from each of three genetically distinct inbred lines exhibiting significant genetic variation in the amino acid composition and gustatory appeal of the spermatophylax (Gershman *et al.*, 2013). Lines were generated by subjecting randomly assigned descendants of 500 individuals collected from Las Cruces, New Mexico, in 2001 to full-sib mating for 23 generations followed by 33–35 generations of panmixis (Ivy *et al.*, 2005). All individuals were housed in an environmental chamber at 32 °C on a 16-h:8-h light: dark cycle and given Harlan 2018CM Teklad Certified Global 18% Protein Rodent Diet meal, as well as 40-mL water vials plugged with cotton rolls *ad libitum*. Additionally, small plastic containers filled with moistened peat moss were provided as an extra water source, and egg cartons were provided to increase rearing surface area and to provide refuges. Prior to experimental trials, all crickets were held in 55-L plastic storage bins.

Experimental design

We employed a repeated-measures experimental design in which two successive spermatophylaxes were collected from each male, once before and once after experimental treatment. All experimental males were 1-week-old post-eclosion (±5 days). Males were separated from females at least 24 h prior to initial spermatophylax removal to promote their sexual receptivity. Initial spermatophylaxes were manually removed from males that had a spermatophore visible in their spermatophoric pouch by applying gentle pressure to their abdomen and then removing the spermatophylax partially extruded from the abdomen with forceps. Ampullae were also removed to promote subsequent spermatophore production by males. Immediately following initial spermatophore removal, males were randomly assigned to one of two treatments: (i) a control injection of 2 μL of Grace's insect medium (Sigma-Aldrich) or (ii) a treatment injection of 10^8 heat-killed *E. coli* suspended in 2 μL Grace's insect medium. The *E. coli* strain (DH5α) used in this study was obtained from the American Type Culture Collection (Manassas, VA, USA). These bacteria were cultured at 30 °C in medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7.2). To prepare bacterial suspensions for challenge injections, 1 mL of an overnight culture was centrifuged (850 *g*, 4 °C, 10 min) and the supernatant was discarded and replaced with sterile Grace's insect medium. This procedure was repeated three times and the concentration of bacterial cells adjusted to 10^8 mL^{-1}. The bacteria were then heat killed (90 °C, 5 min). Efficiency of the heat killing was confirmed by plating out samples of the suspension on agar.

Injected males were then housed individually in 0.5 mL plastic containers and provided with one piece of dry commercial cat food (Purina®), a water vial plugged with cotton, a small container of moistened peat moss, and egg carton. Males were then given 24–48 h to produce a subsequent spermatophore. Upon spermatophore formation, spermatophylaxes were manually extruded as described earlier. Any male that had not produced a spermatophore after 48 h post-injection was eliminated from the experiment.

Sixty-five of 84 control males (77.4%) produced a second spermatophore, whereas 61 of 77 immune-stimulated males (79.2%) produced a second spermatophore, which is not significantly different (χ^2_1 (continuity adjusted) = 0.0084; $P = 0.9271$). There was also no significant difference between treatments in the time taken to produce a second spermatophore (24 h: control = 53, immune-stimulated = 42; 48 h: control = 12, immune-stimulated = 19; χ^2_1 (continuity adjusted) = 2.089; $P = 0.1484$). All extruded spermatophylaxes were immediately stored in an airtight microcentrifuge vial, stored at −80 °C and then later lyophilized using a Labconco Freeze-dryer (Labconco, Kansas City, MO, USA) with the vacuum set at −40 °C at a pressure of 0.133 mBar.

Quantifying amino acid content in male spermatophylaxes

Freeze-dried spermatophylax samples were weighed using a microbalance (Mettler Toledo UMX2, Leicester, UK) and then ground using a pestle in an Eppendorf tube, with the addition of 150 μL of ethanol. Amino

acids were extracted from individual spermatophylaxes using an EZ:faast reagent kit for free amino acid analysis optimized for gas chromatography–mass spectrometry (GC-MS) (Phenomenex, Torrence, CA, USA). Full details of this extraction procedure are provided in the Supporting Information.

We injected 0.2 μL of the extracted amino acid sample from each spermatophylax into a GC-MS (Agilent 7890 gas chromatograph coupled with an Agilent 5975 mass spectrometer and an Agilent CTC PAL autosampler chilled to 5 °C) fitted with a ZB-AAA column (10 m × 0.255 mm internal diameter) using helium as the carrier gas. The inlet was set to 325 °C and the MS transfer line to 300 °C and the injection was performed in pulsed split-less mode. Separation of the extract into individual amino acid peaks was achieved using the optimized procedure provided with the EZ:faast kit, and full details are provided in the Supporting Information. The area under each amino acid peak was measured using CHEMSTATION software (v.E.02.00.493, Agilent Technologies, Santa Clara, CA, USA), and this was converted to an absolute quantity of each amino acid using calibration curves created for each amino acid using standard solutions provided in the EZ:faast kit. Our measure of each amino acid was therefore given in nanomoles per mL of internal standard (0.2 mM of norvaline, added during the extraction procedure, see Supporting Information).

We measured the quantity of the following 22 amino acids in each spermatophylax: alanine (ALA), glycine (GLY), a-aminobutyric acid (AAA), valine (VAL), leucine (LEU), isoleucine (ILE), threonine (THR), serine (SER), proline (PRO), asparagine (ASN), aspartic acid (ASP), methionine (MET), 4-hydroxyproline (HYP), glutamic acid (GLU), phenylalanine (PHE), glutamine (GLN), orthinine (ORN), glycyl-proline (GPR), lysine (LYS), histidine (HIS), tyrosine (TYR) and tryptophan (TRP). Amino acid peaks and the internal standard were identified according to their characteristic retention time using diagnostic qualitative ions (Table S1, Fig. S1, Supporting Information). Three of these amino acids (AAA, ORN and GPR) were not present in all spermatophylax samples and were therefore excluded from further analysis.

Statistical analysis

As the amount of amino acids contained in a spermatophylax increases with the initial size of this gift, we expressed the amount of each amino acid relative to the initial weight of the spermatophylax by dividing the amount of each amino acid by the total dry weight of the spermatophylax. Therefore, the quantities of amino acids in each spermatophylax are expressed in units of nanomoles per millilitre of an internal standard (norvaline) per gram of spermatophylax. To ensure normality, data for each amino acid were $\log_{10}$-transformed prior to analysis.

Treatment effects on spermatophylax weight and amino acid composition

We used an analysis of covariance (ANCOVA) with treatment as a main effect, inbred line as a random effect, pre-injection spermatophylax dry weight as a covariate and post-injection spermatophylax dry weight as the dependent variable to examine treatment effects on spermatophylax weight.

We employed principal component analysis (PCA) to reduce the variation in amino acids into a smaller number of dimensions. This analysis also eliminates any covariation that might occur between amino acids. Principal components (PCs) were extracted based on the correlation matrix, and we retained PCs with eigenvalues exceeding 1 for further analysis and interpret factor loadings for individual amino acids that exceed 0.45 as biologically important (Comrey, 1973). To examine treatment effects within each principal component, we employed analysis of covariance with treatment as a main effect, inbred line as a random effect, PC score of the pretreatment spermatophylax as a covariate and PC score of the post-treatment spermatophylax as the dependent variable.

Treatment effects on spermatophylax gustatory appeal

The gustatory appeal of each spermatophylax was assessed by mapping amino acid composition onto a fitness surface derived from an earlier multivariate selection analysis, showing that the free amino acid composition of the spermatophylax significantly influences whether females prematurely discard the spermatophylax (Gershman *et al.*, 2012). This approach has been previously used to measure the genetic correlation between spermatophylax feeding duration of females and the gustatory appeal of the spermatophylax (Gershman *et al.*, 2013), as well as to assess female mating preferences for male acoustic traits in waxwing moths (Jia & Greenfield, 1997). To take this approach, it was necessary to project our data in two ways. First, it was necessary to project our data into the same principal component space as used in Gershman *et al.* (2012). This was achieved by substituting the amount of each amino acid from our experiment into the linear equation (i.e. eigenvector) describing each PC used in the selection analysis of Gershman *et al.* (2012) (Table S2). Second, we used these projected PC scores to derive a measure of the multivariate attractiveness of spermatophylaxes by substituting these values into the eigenvectors describing the two significant axes of nonlinear sexual selection ($\boldsymbol{m_1}$ and $\boldsymbol{m_3}$) in Gershman *et al.* (2012) (Table S3). Thus, each spermatophylax in our data set has a measure of $\boldsymbol{m_1}$ and $\boldsymbol{m_3}$ which are known to be important axes of multivariate attractiveness in this species (Gershman *et al.*, 2012). Full details of this procedure are provided in the Supporting Information. We employed a doubly multivariate, repeated-measures MANOVA to examine treatment and inbred line effects on

m_1 and m_3, with treatment (experimental versus control) as the main effect, inbred line as a random effect and time (pre- and post-treatment) as the repeated measure.

Results

Spermatophylax dry weight was heavily influenced by individual variation, which is evident from a significant correlation between pre-injection and post-injection spermatophylax dry weights (ANCOVA, $F_{1,\ 115} = 93.27$, $P < 0.0001$). However, neither treatment (ANCOVA, $F_{1,\ 115} = 0.89$, $P = 0.3468$) nor genetic line (ANCOVA, $F_{2,\ 115} = 0.58$, $P = 0.559$) influenced spermatophylax dry weight following injection.

Principal component analysis of the free amino acids in the spermatophylax yielded three PCs with eigenvalues >1, together accounting for 67.9% of the total variation in amino acid composition (Table 1). PC1 explains 53% of the variance in amino acid content and is characterized by positive loadings of ALA, GLY, VAL, LEU, ILE, SER, PRO, ASN, ASP, GLU, PHE, GLN, LYS and TYR (Table 1). Consequently, this vector describes primarily the absolute amount of amino acids present in the male spermatophylax. PC2 accounts for an additional 9.5% of the variation in amino acid composition and describes positive loadings of THR, MET, HYP and TRP (Table 1). Finally, PC3 explains 5.4 per cent of the variation in amino acid content and is characterized by positive loadings of HIS (Table 1).

Table 1 Principal component (PC) analysis of the 19 free amino acids examined in the spermatophylaxes of *Gryllodes sigillatus*. We have retained PCs with an eigenvalue >1 in our multivariate selection analysis and we interpret factor loadings >0.45 as biologically significant (in bold).

	PC1	PC2	PC3
Eigenvalue	10.071	1.802	1.023
% variance	53.008	9.486	5.382
Amino acid			
ALA	**0.923**	−0.061	0.078
GLY	**0.878**	−0.010	0.073
VAL	**0.868**	−0.053	0.071
LEU	**0.870**	0.073	0.243
ILE	**0.925**	−0.003	0.158
THR	0.271	**0.632**	−0.275
SER	**0.896**	−0.114	−0.069
PRO	**0.907**	−0.037	−0.034
ASN	**0.796**	0.039	0.060
ASP	**0.861**	−0.012	0.000
MET	0.199	**0.788**	−0.194
HYP	0.090	**0.467**	0.216
GLU	**0.879**	−0.012	−0.050
PHE	**0.713**	0.111	0.193
GLN	**0.759**	−0.110	−0.311
LYS	**0.790**	−0.040	−0.175
HIS	**−0.406**	0.060	**0.682**
TYR	**0.557**	−0.143	0.281
TRP	−0.076	**0.697**	0.205

Gift composition

Treatment effects on the PC scores describing the variation in amino acid profiles of spermatophylaxes synthesized pre- and post-treatment are shown in Fig. 1. There was a significant effect of treatment on the post-treatment value of PC3 after controlling for the pretreatment value (ANCOVA, $F_{1,\ 115} = 8.62$, $P = 0.0040$,

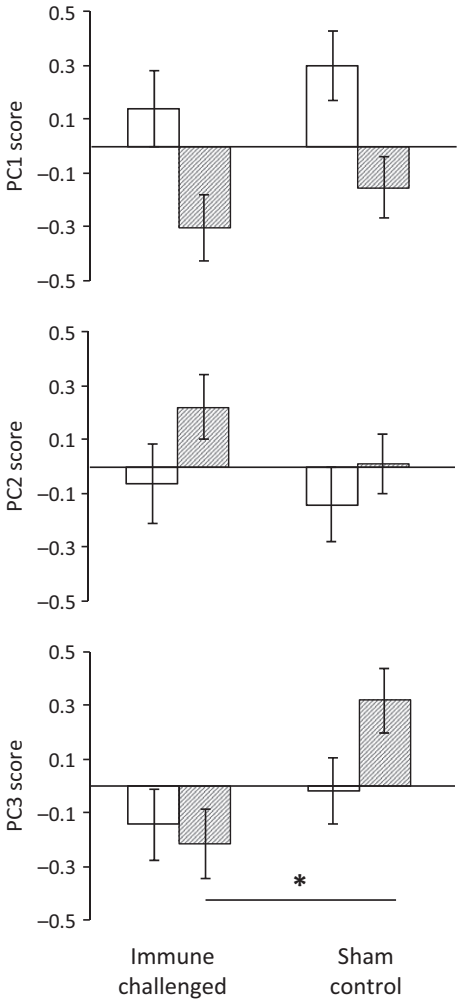

Fig. 1 Mean principal component (PC) scores for amino acid composition of spermatophylaxes derived from male *Gryllodes sigillatus* spermatophylaxes both before (empty bars) and after (filled bars) an immune challenge (injection of heat-killed *E. coli* solution) or a sham control (injection of saline). With pre-injection spermatophylax PC as a covariate, there was a significant effect of treatment in PC3 only (ANCOVA, $F_{1,\ 115} = 8.62$, $P = 0.0040$, Bonferroni corrected $\alpha = 0.0166$) with control males producing spermatophylaxes with significantly higher PC3 scores (LS mean ± SE = 0.314 ± 0.123) than immune-challenged males (LS mean ± SE = −0.216 ± 0.132) following injection. *$P < 0.01$.

Bonferroni corrected $\alpha = 0.0166$), with control males producing spermatophylaxes with significantly higher PC3 scores than immune-challenged males following injection. There were, however, no significant treatment effects on either PC1 (ANCOVA, $F_{1,\ 115} = 0.79$, $P = 0.3746$) or PC2 (ANCOVA, $F_{1,\ 115} = 1.35$, $P = 0.1965$). No effect of genetic line on gift composition was found for any of the PCs (ANCOVA, PC1 : $F_{2,\ 115} = 0.23$, $P = 0.7931$; PC2 : $F_{2,\ 115} = 0.5$, $P = 0.5763$; PC3 : $F_{2,\ 115} = 0.39$, $P = 0.6812$), and nor were any of the interactions involving line significant for any PC (all $P > 0.05$).

Gift attractiveness

Repeated-measures MANOVA of selection gradients $\boldsymbol{m}_1$ and $\boldsymbol{m}_3$ revealed a significant time-by-treatment interaction, indicating that the change in the gustatory appeal of the spermatophylax over successive spermatophores was contingent on treatment (MANOVA, Wilk's lambda: $F_{2,\ 113} = 3.34$, $P = 0.0388$, Fig. 2). Specifically, control males produced significantly less attractive second gifts, whereas immune-challenged males maintained the gustatory appeal of their spermatophylaxes. There was also a significant line effect suggesting heritable variation in the gustatory appeal of nuptial gifts produced by males of different genetic lines (MANOVA, Wilk's lambda: $F_{4,226} = 3.11$, $P = 0.0163$). Finally, the three-way interaction between time, treatment and inbred line was not significant, suggesting the absence of any genotype-by-environment interaction (MANOVA, Wilk's lambda: $F_{4,226} = 1.18$, $P = 0.3197$).

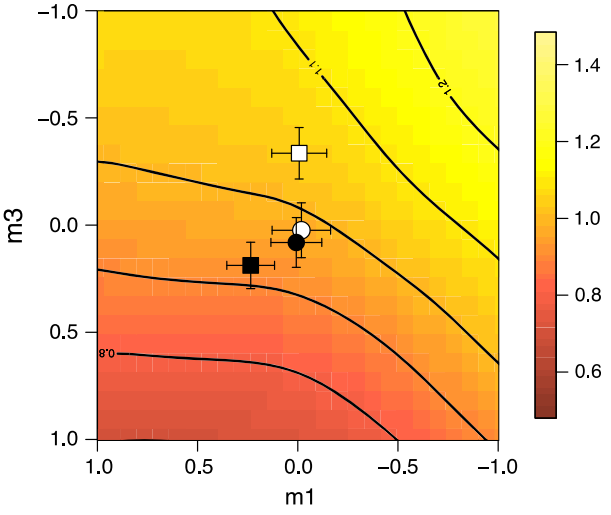

Fig. 2 Linear selection values ($\boldsymbol{m}_1$ and $\boldsymbol{m}_3$) based on eigenvectors from Gershman *et al.* (2012) of spermatophylaxes derived from male *Gryllodes sigillatus* before (white) and after (black) an immune challenge of an injection of heat-killed *E. coli* solution (circles) or a sham control injection of saline (squares) plotted on a contour fitness map generated by Gershman *et al.* (2012). Symbols represent least squares means and whiskers standard errors. Lower values on the fitness surface translate to lower values of attractiveness. We found a significant time-by-treatment interaction (MANOVA, Wilk's lambda: $F_{2,\ 113} = 3.34$, $P = 0.0388$) such that males maintain gift attractiveness following injection with heat-killed *E. coli* but decrease gift attractiveness following an injection of saline.

Discussion

Our experiment revealed significant effects of a bacteria-based immune challenge on the amino acid composition of spermatophylaxes, influencing the gustatory appeal of these nuptial gifts to females. Specifically, immune-challenged males maintained the level of gustatory appeal in their spermatophylaxes post-injection, whereas control males produced less attractive gifts. These results are consistent with the terminal investment hypothesis, as immune-challenged males, whose perceived residual reproductive value had been lowered relative to control males, invested more heavily than control males in maintaining the quality of their gifts post-injection.

Although we had not anticipated a pattern of terminal investment in which control males would actually *decrease* their investment in the gustatory appeal of the spermatophylax over successive spermatophores, this pattern is not unprecedented. Female house wrens, when immunostimulated following the experimental removal of their initial clutch, did not alter the amount of yolk allocated to eggs between pre- and post-treatment clutches, whereas control females significantly reduced their yolk mass over successive clutches (Bowers *et al.*, 2015). In a previous study of *G. sigillatus* (Kerr *et al.*, 2010), males immune challenged via injection with lipopolysaccharide (LPS), a nonpathogenic component of the cell wall derived from the common insect pathogen *Serratia marcescens*, also maintained the size of their spermatophylax over successive spermatophores, in parallel with the maintenance of the gustatory appeal of the spermatophylax documented in the present study. However, in contrast to the present study, Kerr *et al.* (2010) found that sham-injected control males actually increased the size of their spermatophylax over successive spermatophores, whereas immune-challenged males did not. We cannot explain this seeming inconsistency, except to note that the magnitude of terminal investment can vary according to the magnitude of the perceived threat (Copeland & Fedorka, 2012), and we do not know how the dose of heat-killed bacteria used in the present study compares with the concentration of lipopolysaccharides used by Kerr *et al.* (2010). In addition, the cell walls of the heat-killed bacteria used in the present study are comprised of more components than LPS alone (e.g. peptidoglycans), which might represent a stronger or distinct immune challenge that induces terminal investment.

The apparent decrease in gift attractiveness of post-injection control males could be due partly to higher

levels of histidine, as the spermatophylaxes of control males had significantly higher PC3 values, characterized by high histidine loadings, following injection than immune-challenged males. Indeed, Gershman *et al.* (2012) found that spermatophylaxes with higher levels of histidine were more likely to be prematurely discarded by female *G. sigillatus*. However, the selection surface depicting the two major axes of nonlinear selection operating on the amino acid composition of the spermatophylax reported in Gershman *et al.* (2012) reveals that gustatory appeal is influenced by a complex interplay of a number of different amino acids operating in multivariate space; this analysis cautions against attributing special significance to any one amino acid.

The fact that immune-challenged males maintained the quality of their nuptial food gifts over successive spermatophores suggests that control males could also have maintained their allocation but opted not to. This suggests that spermatophore synthesis is costly and that control males weigh the potential costs of spermatophore replenishment to future reproduction and survival against the immediate benefits that accrue to nuptial gifts of greater gustatory appeal. Indeed, a decreased investment in nuptial gifts over successive matings has been documented in other gift-giving insects, as evidenced by a reduction in spermatophore mass in fireflies (Cratsley *et al.*, 2003), butterflies (Svärd & Wiklund, 1989; Bissoondath & Wiklund, 1996), seed beetles (Savalli & Fox, 1999) and bushcrickets, who also displayed an additional decrease in the nitrogen content of the spermatophylax (Wedell & Ritchie, 2004). However, the level of investment in nuptial food gifts over successive spermatophores may depend on the interval between copulations (Hughes *et al.*, 2000; Lehmann & Lehmann, 2000). In the present study, the male's initial spermatophore was removed as soon as it had been produced, corresponding to a situation in which the refractory period between successive matings is minimized. Under normal circumstances, however, males may go several days without mating and thus may be able to acquire sufficient resources in the interim such that following a successful copulation, a male might have sufficient reserves to maintain his investment in the spermatophore without compromising future survival or reproduction. We do not know, therefore, whether the decrease in gustatory appeal of the spermatophylaxes of control males represents a long-term strategy of diminishing investment in the spermatophore over successive matings (i.e. senescence), or a short-term response to a transient demand on reproductive effort. A third possibility is that piercing of the integument of control males was sufficient to trigger wound healing and activate immunological pathways, leading to a reallocation of resources away from investment into more appealing food gifts consistent with the classical reproductive trade-off hypothesis (Sheldon & Verhulst, 1996). Ideally, we should have also included a naïve group in which males did not receive any injection to evaluate this possibility. Nevertheless, the change in spermatophylax attractiveness across treatments demonstrates a change in investment consistent with terminal investment. Males exposed to a stimulus that is consistent with a greater threat to survival (i.e. the bacteria-based challenge) exhibit higher current investment in reproductive effort relative to control males exposed to a stimulus representing a minimal threat (i.e. sterile injection). It may be that the baseline response of our controls represents decreased investment in the gustatory appeal of food gifts, whereas when the additional immunological cue and perceived threat represented by the presence of heat-killed bacteria is superimposed on this, greater investment is triggered and spermatophylax attractiveness is maintained. This scenario would suggest a potential stimulus threshold in terminal investment.

There was a genetic effect on the gustatory appeal of the spermatophylax as evidenced by a significant line effect. This is consistent with an earlier study showing significant heritable variation in the amino acid composition of the spermatophylax based on a larger sample of inbred lines (Gershman *et al.*, 2013). However, the three-way interaction between time, treatment and line was not significant, suggesting the lack of a genotype-by-environment interaction on the gustatory appeal of the spermatophylax. This suggests that reaction norms describing the level of terminal investment elicited by a specific level of immune challenge across different genotypes are relatively invariant. However, we caution that our study involved only a small number of inbred lines, and we did not vary the concentration of heat-killed bacteria used as an immune challenge, using a relatively high dose that likely exceeds any interline variation in stimulus thresholds. Genotype-by-environmental effects on male sexual signals are ubiquitous in insects (Hunt & Hosken, 2014), and with respect to at least one other sexual signal in *G. sigillatus*, the cuticular hydrocarbons (CHC) that influence mate recognition and male mating success, a significant genotype-by-environment interaction suggests that CHC expression in males is contingent on the nutritional rearing environment (Weddle *et al.*, 2012; Sakaluk *et al.*, 2014).

The terminal investment by males in the gustatory appeal of their nuptial food gifts shown here is consistent with the increased investment shown by immune-challenged males in other insect taxa for other types of male sexual signals and reproductive investments. In mealworm beetles, *Tenebrio molitor*, for example, immune-challenged males increased their investment in the pheromone signals used to attract sexually receptive females (Sadd *et al.*, 2006; Kivleniece *et al.*, 2010; Krams *et al.*, 2011; Nielsen & Holman, 2012). Immune-challenged damselflies, *Hetaerina americana*, increased their investment in territorial defence and agonistic behaviour and consequently experienced higher short-term mating

success, but this effect was contingent on male age, with older males exhibiting higher terminal investment (González-Tokman *et al*., 2013). Consistent with a pattern of terminal investment due to reduced residual reproductive value, older male moths, *Ostrinia scapulalis*, produced larger spermatophores of higher protein content than younger males (Thanda Win *et al*., 2013), from which females often derive nutritional benefits (reviewed in Lewis & South, 2012). Similar patterns of terminal investment have been shown in females of a number of insect taxa with respect to fecundity and schedules of oviposition (Adamo, 1999; Leventhal *et al*., 2014).

In conclusion, our study revealed a pattern of terminal investment based on the spermatophylax amino acid composition of male decorated crickets: immune-challenged males maintained the gustatory appeal of the spermatophylax post-treatment, whereas control males produced gifts of significantly lower gustatory appeal. These results suggest that when males are cued to their impending mortality, they increase their immediate allocation to nuptial food gifts, which results in increased gustatory appeal of the gift relative to controls. This, in turn, increases the likelihood that the female will fully consume the food gift, and thereby enhances the male's prospect of transferring a complete ejaculate to the female (Sakaluk, 1984; Gershman *et al*., 2012). Although these results are consistent with other empirical studies demonstrating terminal investment in male sexual signals following an immune challenge, they reveal that this increased reproductive effort can also be manifested in the chemical composition of an endogenously synthesized nuptial food gift, a form of mating enticement that is common across insect mating systems.

Acknowledgments

This work was supported by grants from the National Science Foundation to S.K.S. and grants from the Beta Lambda Chapter of Phi Sigma and the Graduate Student Association of Illinois State University to K.R.D. J.H. was funded by a University Royal Society Fellowship and a Royal Society Equipment Grant. The warmest thanks to Colleen McGrath and Sarah Kluk for help with laboratory work and animal husbandry. Thanks also to three anonymous reviewers for helpful comments on the manuscript.

References

Adamo, S. 1999. Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Anim. Behav.* **57**: 117–124.

Arnqvist, G. & Nilsson, T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Anim. Behav.* **60**: 145–164.

Bissoondath, C. J. & Wiklund, C. 1996. Effect of male mating history and body size on ejaculate size and quality in two polyandrous butterflies, *Pieris napi* and *Pieris rapae* (Lepidoptera: Pieridae). *Funct. Ecol.* **10**: 457–464.

Bonneaud, C., Mazuc, J., Chastel, O., Westerdahl, H. & Sorci, G. 2004. Terminal investment induced by immune challenge and fitness traits associated with major histocompatibility complex in the house sparrow. *Evolution* **58**: 2823–2830.

Bowers, E. K., Bowden, R. M., Sakaluk, S. K. & Thompson, C. F. 2015. Immune activation generates corticosterone-mediated terminal reproductive investment in a wild bird. *Am. Nat.* **185**: 769–783.

Calatayud, P.-A., Polanía, M. A., Guillaud, J., Múnera, D. F., Hamon, J. C. & Bellotti, A. C. 2002. Role of single amino acids in phagostimulation, growth, and development of the cassava mealybug *Phenacoccus herreni*. *Entomol. Exp. Appl.* **104**: 363–367.

Calos, J. B. & Sakaluk, S. K. 1998. Paternity of offspring in multiply–mated, female crickets: the effect of nuptial food gifts and the advantage of mating first. *Proc. R. Soc. Lond. B Biol. Sci.* **265**: 2191–2195.

Candolin, U. 1999. The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? *Anim. Behav.* **58**: 1261–1267.

Clutton-Brock, T. H. 1984. Reproductive effort and terminal investment in iteroparous animals. *Am. Nat.* **123**: 212–229.

Comrey, A. D. 1973. *A first course in factor analysis*. Academic Press, New York.

Copeland, E. K. & Fedorka, K. M. 2012. The influence of male age and simulated pathogenic infection on producing a dishonest sexual signal. *Proc. R. Soc. B Biol. Sci.* **279**: 4740–4746.

Cote, J., Arnoux, E., Sorci, G., Gaillard, M. & Faivre, B. 2010. Age-dependent allocation of carotenoids to coloration *versus* antioxidant defenses. *J. Exp. Biol.* **213**: 271–277.

Cratsley, C., Rooney, J. & Lewis, S. 2003. Limits to Nuptial Gift Production by Male Fireflies, *Photinus ignitus*. *J. Insect Behav.* **16**: 361–370.

Creighton, J. C., Heflin, N. D. & Belk, M. C. 2009. Cost of reproduction, resource quality, and terminal investment in a burying beetle. *Am. Nat.* **174**: 673–684.

Eggert, A., Reinhardt, K. & Sakaluk, S. K. 2003. Linear models for assessing mechanisms of sperm competition: the trouble with transformations. *Evolution* **57**: 173–176.

Gershman, S. N., Barnett, C. A., Pettinger, A. M., Weddle, C. B., Hunt, J. & Sakaluk, S. K. 2010. Give 'til it hurts: trade-offs between immunity and male reproductive effort in the decorated cricket, *Gryllodes sigillatus*. *J. Evol. Biol.* **23**: 609–610.

Gershman, S. N., Mitchell, C., Sakaluk, S. K. & Hunt, J. 2012. Biting off more than you can chew: sexual selection on the free amino acid composition of the spermatophylax in decorated crickets. *Proc. R. Soc. B Biol. Sci.* **279**: 2531–2538.

Gershman, S. N., Hunt, J. & Sakaluk, S. K. 2013. Food fight: sexual conflict over free amino acids in the nuptial gifts of male decorated crickets. *J. Evol. Biol.* **26**: 693–704.

González-Tokman, D. M., González-Santoyo, I. & Córdoba-Aguilar, A. 2013. Mating success and energetic condition effects driven by terminal investment in territorial males of a short-lived invertebrate. *Funct. Ecol.* **27**: 739–747.

Gwynne, D. T. 2008. Sexual conflict over nuptial gifts in insects. *Annu. Rev. Entomol.* **53**: 83–101.

Hughes, L., Siew-Woon Chang, B., Wagner, D. & Pierce, N. E. 2000. Effects of mating history on ejaculate size, fecundity, longevity, and copulation duration in the ant-tended lycaenid butterfly, *Jalmenus evagoras*. *Behav. Ecol. Sociobiol.* **47**: 119–128.

Hunt, J. & Hosken, D. J., eds. 2014. *Genotype-by-Environment Interactions and Sexual Selection*. Wiley Blackwell, Chichester.

Ivy, T. M. & Sakaluk, S. K. 2005. Polyandry promotes enhanced offspring survival in decorated crickets. *Evolution* **59**: 152–159.

Ivy, T. M., Johnson, J. C. & Sakaluk, S. K. 1999. Hydration benefits to courtship feeding in crickets. *Proc. R. Soc. Lond. B Biol. Sci.* **266**: 1523–1527.

Ivy, T. M., Weddle, C. B. & Sakaluk, S. K. 2005. Females use self-referent cues to avoid mating with previous mates. *Proc. R. Soc. B Biol. Sci.* **272**: 2475–2478.

Jia, F.-Y. & Greenfield, M. D. 1997. When are good genes good? Variable outcomes of female choice in wax moths. *Proc. R. Soc. Lond. B Biol. Sci.* **264**: 1057–1063.

Kerr, A. M., Gershman, S. N. & Sakaluk, S. K. 2010. Experimentally induced spermatophore production and immune responses reveal a trade-off in crickets. *Behav. Ecol.* **21**: 647–654.

Kivleniece, I., Krams, I., Daukšte, J., Krama, T. & Rantala, M. J. 2010. Sexual attractiveness of immune-challenged male mealworm beetles suggests terminal investment in reproduction. *Anim. Behav.* **80**: 1015–1021.

Krams, I., Daukšte, J., Kivleniece, I., Krama, T., Rantala, M. J., Ramey, G. *et al.* 2011. Female choice reveals terminal investment in male mealworm beetles, *Tenebrio molitor*, after a repeated activation of the immune system. *J. Insect Sci.* **11**: 1–14.

Lehmann, G. U. C. & Lehmann, A. W. 2000. Spermatophore characteristics in bushcrickets vary with parasitism and remating interval. *Behav. Ecol. Sociobiol.* **47**: 393–399.

Leventhal, G. E., Dünner Robert, P. & Barribeau, S. M. 2014. Delayed virulence and limited costs promote fecundity compensation upon infection. *Am. Nat.* **183**: 480–493.

Lewis, S. & South, A. 2012. The evolution of animal nuptial gifts. *Adv. Study Behav.* **44**: 53–97.

Lewis, S. M., Vahed, K., Koene, J. M., Engqvist, L., Bussière, L. F., Perry, J. C. *et al.* 2014. Emerging issues in the evolution of animal nuptial gifts. *Bio. Lett.* **10**: 20140336.

Michod, R. E. 1979. Evolution of life histories in response to age-specific mortality factors. *Am. Nat.* **113**: 531–550.

Minchella, D. J. & Loverde, P. T. 1981. A cost of increased early reproductive effort in the snail *Biomphalaria glabrata*. *Am. Nat.* **118**: 876–881.

Nielsen, M. L. & Holman, L. 2012. Terminal investment in multiple sexual signals: immune-challenged males produce more attractive pheromones. *Funct. Ecol.* **26**: 20–28.

Parker, G. A. & Simmons, L. W. 1989. Nuptial feeding in insects: theoretical models of male and female interests. *Ethology* **82**: 3–26.

Pianka, E. R. & Parker, W. S. 1975. Age-specific reproductive tactics. *Am. Nat.* **109**: 453–464.

Quinn, J. S. & Sakaluk, S. K. 1986. Prezygotic male reproductive effort in insects: why do males provide more than sperm? *Florida Entomol.* **69**: 84–94.

Roff, D. A. 1992. *The Evolution of Life Histories: Theory and Analysis*. Chapman and Hall, London.

Sadd, B., Holman, L., Armitage, H., Lock, F., Marland, R. & Siva-Jothy, M. T. 2006. Modulation of sexual signalling by immune challenged male mealworm beetles (*Tenebrio molitor*, L.): evidence for terminal investment and dishonesty. *J. Evol. Biol.* **19**: 321–325.

Sakaluk, S. K. 1984. Male crickets feed females to ensure complete sperm transfer. *Science* **223**: 609–610.

Sakaluk, S. K. 1985. Spermatophore size and its role in the reproductive behaviour of the cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae). *Can. J. Zool.* **63**: 1652–1656.

Sakaluk, S. K. 1986. Is courtship feeding by male insects parental investment? *Ethology* **73**: 161–166.

Sakaluk, S. K. 1987. Reproductive behaviour of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae): calling schedules, spatial distribution, and mating. *Behaviour* **100**: 202–225.

Sakaluk, S. K. 1997. Cryptic female choice predicated on wing dimorphism in decorated crickets. *Behav. Ecol.* **8**: 326–331.

Sakaluk, S. K. 2000. Sensory exploitation as an evolutionary origin to nuptial food gifts in insects. *Proc. R. Soc. B Biol. Sci.* **267**: 339–343.

Sakaluk, S. K. & Eggert, A.-K. 1996. Female control of sperm transfer and intraspecific variation in sperm precedence: antecedents to the evolution of a courtship food gift. *Evolution* **50**: 694–703.

Sakaluk, S. K., Schaus, J. M., Eggert, A.-K., Snedden, W. A. & Brady, P. L. 2002. Polyandry and fitness of offspring reared under varying nutritional stress in decorated crickets. *Evolution* **56**: 1999–2007.

Sakaluk, S. K., Avery, R. L. & Weddle, C. B. 2006. Cryptic sexual conflict in gift-giving insects: chasing the chase-away. *Am. Nat.* **167**: 94–104.

Sakaluk, S. K., Weddle, C. & Hunt, J. 2014. Signal reliability, sex-specific genotype-by-environment interactions in cuticular hydrocarbon expression, and the maintenance of polyandry through chemosensory self-referencing in decorated crickets, *Gryllodes sigillatus*. In: *Genotype-by-environment interactions and sexual selection* (J. Hunt, D. J. Hosken, eds), pp. 312–330. Wiley Blackwell, Chichester.

Savalli, U. M. & Fox, C. W. 1999. The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. *Funct. Ecol.* **13**: 169–177.

Sheldon, B. & Verhulst, S. 1996. Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**: 317–321.

Stearns, S. C. 1992. *The evolution of life histories*. Oxford University Press, Oxford.

Svärd, L. & Wiklund, C. 1989. Mass and production rate of ejaculates in relation to monandry/polyandry in butterflies. *Behav. Ecol. Sociobiol.* **24**: 395–402.

Thanda Win, A., Kojima, W. & Ishikawa, Y. 2013. Age-related male reproductive investment in courtship display and nuptial gifts in a moth, *Ostrinia scapulalis*. *Ethology* **119**: 325–334.

Thornhill, R. 1976. Sexual selection and paternal investment in insects. *Am. Nat.* **110**: 153–163.

Trivers, R. 1972. Parental investment and sexual selection. In: *Sexual selection and the descent of man* (B. Campbell, ed), pp. 1871–1971. Aldine, Chicago.

Vahed, K. 1998. The function of nuptial feeding in insects: a review of empirical studies. *Biol. Rev.* **73**: 43–78.

Vahed, K. 2007. All that glisters is not gold: sensory bias, sexual conflict and nuptial feeding in insects and spiders. *Ethology* **113**: 105–127.

Velando, A., Drummond, H. & Torres, R. 2006. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. *Proc. Biol. Sci.* **273**: 1443–1448.

Warwick, S., Vahed, K., Raubenheimer, D. & Simpson, S. J. 2009. Free amino acids as phagostimulants in cricket nuptial

gifts: support for the "Candymaker" hypothesis. *Biol. Lett.* **5**: 194–196.
Weddle, C. B., Mitchell, C., Bay, S. K., Sakaluk, S. K. & Hunt, J. 2012. Sex-specific genotype-by-environment interactions for cuticular hydrocarbon expression in decorated crickets, *Gryllodes sigillatus*: implications for the evolution of signal reliability. *J. Evol. Biol.* **25**: 2112–2125.
Wedell, N. & Ritchie, M. G. 2004. Male age, mating status and nuptial gift quality in a bushcricket. *Anim. Behav.* **67**: 1059–1065.
Weil, Z. M., Martin, L. B., Workman, J. L. & Nelson, R. J. 2006. Immune challenge retards seasonal reproductive regression in rodents: evidence for terminal investment. *Biol. Lett.* **2**: 393–396.
Will, M. W. & Sakaluk, S. K. 1994. Courtship feeding in decorated crickets: is the spermatophylax a sham? *Anim. Behav.* **48**: 1309–1315.
Williams, G. C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's principle. *Am. Nat.* **100**: 687–690.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Identification of the 22 free amino acids and the internal standard (Norvaline) contained in the male spermatophylax of *Gryllodes sigillatus*, as well as the qualitative ion used to quantify each amino acid.
Figure S1 A typical chromatograph showing the amino acids contained in the spermatophylax of male *Gryllodes sigillatus*.
Table S2 The Principal Component Analysis (PCA) of the 19 amino acids examined in the spermatophylax of male *Gryllodes sigillatus* in the selection analysis of Gershman *et al.* (2012).
Table S3 The **M** matrix of eigenvectors from the canonical analysis of γ for the amino acid composition of the spermatophylaxes of male *Gryllodes sigillatus* taken from the selection analysis of Gershman *et al.* (2012).

Data deposited at Dryad: doi: 10.5061/dryad.tm706

Received 7 May 2015; revised 14 July 2015; accepted 18 July 2015