# Trait Integration as a Constraint on Phenotypic Evolution

Submitted by William Ronald Pitchers, to the University of Exeter as a thesis for the Degree of Doctor of Philosophy in Biological Sciences, June 2010.



I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signed: W. R. Pitchers

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## ABSTRACT

One of the most compelling features of biology is the apparent complexity of phenotypes. The morphology and behaviour of organisms are wonderfully varied, and as evolutionary biologists we attempt to understand the patterns and mechanisms that underlie this diversity. Though evolution leads to changes in gene frequency over time, it is upon the phenotype that selection acts. The integration that allows phenotypes to function as coherent systems, by exposing only certain trait combinations to selection, may therefore act to divert or constrain phenotypic evolution.

I begin this thesis with a quantitative review, where I uncover a pattern of stronger potential integrative constraint on sexual signals than morphology. I then present empirical work using the black field cricket, *Teleogryllus commodus,* as a model system. Specifically, I employ estimates of the phenotypic variance-covariance matrix (**P**) to summarise integration within a five-dimensional characterization of the structure of the males' sexual advertisement call. In Chapters 3 and 4, I show that despite changes in trait means, the structure of **P** for the advertisement call is stable among genetically divergent populations, over time and between diets. In Chapter 5, I reveal a novel link between the size and shape of the male forewing, which is used in the production of calls, and call structure. Finally, I use artificial calls to test for divergence in female call preference across populations and whether this varies with diet, and show that female choosiness is condition-dependent.

Collectively, my results highlight the utility of **P** as a tool for studying the integration of complex traits. The extreme stability of **P** in *T. commodus* suggests that it is likely to act as a constraint on the evolution of call structure in this species. This insight, together with the link between call structure and wing morphology, illustrates the value of treating evolution as a multivariate process.

## ACKNOWLEDGEMENTS

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Since joining the CEC I've always found someone willing to discuss ideas (whether over coffee or something stronger) or to critique my writing and analyses; all my colleagues in the postgrad office deserve thanks in this regard, but particularly Sahran Higgins, Damo Smith, Xav Harrison, MD Sharma, MattLab Witt, Emma Barrett and Princess Stott. Most of all, thanks to Fiona Ingleby for believing in me and being right all the time.

Before I could undertake any empirical work, I had to establish captive populations of crickets for study. John Hunt and I made a collection trip to Australia, and we thank all the landowners who allowed us to collect crickets from their properties. During this trip we were assisted by Rob Brooks and Erik Postma, and thanks are particularly due to Michael Jennions for his generosity with both time and resources at ANU, without which our collections might not have been possible.

My time in the Hunt Lab was made immeasurably more enjoyable by the friendship and teamwork of my colleagues: thanks to Ruth Archer, Chris Mitchell and Probie. Also, it should be said that the Lab itself would be a far less pleasant place to work without the indefatigable efforts of Corrina Lowry.

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## AUTHOR'S DECLARATIONS

During the research contributing to this thesis I was supported by a studentship from NERC.

All of the chapters presented in this thesis were written by W. R. Pitchers, with comments and editing from J. Hunt and T. Tregenza. Nevertheless, there are others whose contributions to some chapters are appreciated. These are detailed below;

**Chapter 2**: As a quantitative review, this chapter necessitated the use of matrices estimated by a large number of researchers at enormous cumulative effort. See the search strategy in Section 2.3 for details of what was included and Appendix I for a reference list for the dataset that was generated.

**Chapter 3**: The cricket calls analysed for this chapter were originally recorded by J. Hunt as part of a previous experiment, conducted at the University of New South Wales.

**Chapter 4**: I made the call recordings for this chapter using a semi-automated call recording setup that was developed by T. Tregenza and J. Hunt, with advice from M. Jennions, and built by J. Wood of Ruthern Instruments, J. Hunt and me.

**Chapter 5**: For this chapter I combined the call data from Chapter 3 with morphometrics data gathered from preserved crickets provided by J. Hunt, and analysed using software donated by C. P. Klingenberg (University of Manchester).

**Chapter 6**: M. Cupper and C. Mitchell helped set up this experiment, and the phonotaxis trials were carried out in an arena that J. Hunt and I constructed for the purpose.

# CHAPTER 1: An Introduction to Integration

## *1.1. INTEGRATION*

"*It is also necessary to bear in mind that, owing to the law of correlation, when one part varies, and the variations are accumulated through natural selection, other modifications, often of the most unexpected nature, will ensue*."

(Darwin, 1859)

Animals are a complex mosaic of traits that must necessarily function as a cohesive unit to achieve fitness. Biologists have long been aware that not all traits vary independently, but rather are frequently correlated with each other. This correlation can be extremely strong between some pairs of traits, and weak to non-existent between others.

The subject of phenotypic integration has been sporadically addressed by researchers from a number of different disciplines. Palaeontologists Olson & Miller (1958) wrote extensively about the phenomenon and developed analytical tools to quantify integration in the early years of the modern synthesis. Shortly after this, Berg (a botanist) proposed specific hypotheses concerning the strength of patterns of integration ('Correlation Pleiades') in relation to ecological pressures (Berg, 1960). Meanwhile, Clausen and collaborators (also botanists) were involved in a long-term research program concerned with what they refer to as 'character coherence' (e.g. Clausen & Hiesey, 1960), before studies of integration faded into the background somewhat, at the expense of the intense focus of research interest on the application of new molecular techniques. Pigliucci & Preston (2004) suggest that a second reason for this hiatus in the study of integration was the persistence of "formidable analytical challenges" with the multivariate statistics required, and the lack of a coherent conceptual framework within which to connect theory to

empiricism. Latterly, fresh empirical efforts were inspired by Schlichting's (1989) connection of phenotypic integration to the related subject of phenotypic plasticity (e.g. Bossdorf & Pigliucci, 2009; Kolodynska & Pigliucci, 2003; Pigliucci, 2002; Pigliucci & Byrd, 1998), whilst Wagner and colleagues have made much progress in placing the study of integration into context within evolutionary theory (Schwenk & Wagner, 2001; Wagner & Schwenk, 2000; Wagner & Altenberg, 1996). Recent progress has also been made on the methodological front, with the introduction of new analytical tools (e.g. Bookstein, 1997; Zelditch et al., 2004; Roff, 2002; Phillips & Arnold, 1999) that build on the traditional fields of quantitative genetics and morphometrics (e.g. Chapters 3 and 5).

Integration can be thought of as a pattern of correlations between traits; these correlations can arise via a number of different mechanisms, ranging from the physical or biochemical to the genetic or behavioural. At a most basic level, physical scaling laws can lead to a predictable relationship between disparate characters of an organism's phenotype. For example, Kleiber's law that body mass predicts metabolic rate is a consequence of the operation of physics and geometry on animal circulatory systems. Moreover, Galileo's cube-square law tells us that body mass must inevitably influence skeletal (or exoskeletal) morphology and thermal efficiency, such that large size, low metabolic rate, robust stature and high resting body temperature form a suite of traits that tend to be co-expressed. While such extrinsic causes of trait correlation are undoubtedly powerful as constraints on the limits of macro-evolution, they are unlikely to be of central importance in the differential reproductive success that drives phenotypic micro-evolution. More interesting for evolutionary geneticists are causes of trait correlation that result from the mechanisms of trait inheritance and expression; such as linkage disequilibrium or pleiotropy.

These mechanisms all provide for the co-expression of certain properties by an individual organism, but in order for this co-expression to become a pattern of correlation it must recur (i.e. be transmitted intact across generations), as segregation and random assortment should serve to break these correlations each generation. When traits co-occur due to pleiotropy or linkage disequilibrium, they will recur wherever the alleles involved are inherited together. If loci involved in

these interactions are polymorphic, and this variation is heritable, then selection can act upon the pattern of trait correlation and this genetic integration can evolve. In the case of co-expression due to physical constraint, the recurrence is trivial, since all organisms will share their experience of physics. This phenomenon, referred to as functional or developmental integration, has the potential to impose absolute constraints. Since evolutionary change occurs in small, incremental steps however, such constraints are more likely to be important at macro-evolutionary scales. However, other than in cases of absolute constraint, it is currently unclear whether integration should be expected to act as a constraint or a facilitator of phenotypic evolution (Pigliucci, 2003).

## *1.2. VIEWING INTEGRATION FROM A MATRIX PERSPECTIVE*

In a quantitative genetics framework, the relationships between traits are represented by trait covariance matrices. The most commonly encountered are additive genetic and phenotypic covariance matrices (by convention usually referred to as **G** and **P** matrices), although environmental and mutational (**E** and **M**) matrices are sometimes used. These are all symmetrical, square matrices; with *n* rows and columns to represent *n* traits. The diagonal values are the *n* trait variances, and the off-diagonal values are the $n^2$-$n$ bivariate covariances for the pairwise relationships between traits (Figure 1.1). These values are calculated as phenotypic variances and covariances for **P** or as additive genetic variances ($V_A$) and covariances ($Cov_A$) for **G**.

One of the fundamental equations in quantitative genetics is the breeder's equation; $\boldsymbol{R} = \boldsymbol{h^2 S}$ that predicts the response of a mean trait value to selection ($\boldsymbol{R}$) as the product of the heritability ($\boldsymbol{h^2}$) of the trait and the selection differential ($\boldsymbol{S}$) applied to it. The use of covariance matrices allows multiple traits to be evaluated within the same framework, using the multivariate extension of the breeder's equation, which can be written; $\Delta\bar{z} = \mathbf{GP^{-1}S}$ where $\Delta\bar{z}$ is a vector of changes to the means of $\boldsymbol{n}$ traits whose additive genetic variances and covariances make up the $\boldsymbol{n}$ dimensional **G** matrix, **P** is the **P** matrix for the same traits and **S** is a vector of selection gradients (Lande, 1979). From this equation it is easy to see why **G** and **P**

| | Trait 1 | Trait 2 | Trait 3 | Trait 4 | Trait 5 |
|---|---|---|---|---|---|
| Trait 1 | $VAR_{T1}$ | $COV_{(T1,T2)}$ | $COV_{(T1,T3)}$ | $COV_{(T1,T4)}$ | $COV_{(T1,T5)}$ |
| Trait 2 | $COV_{(T1,T2)}$ | $VAR_{T2}$ | $COV_{(T2,T3)}$ | $COV_{(T2,T4)}$ | $COV_{(T2,T5)}$ |
| Trait 3 | $COV_{(T1,T3)}$ | $COV_{(T2,T3)}$ | $VAR_{T3}$ | $COV_{(T3,T4)}$ | $COV_{(T3,T5)}$ |
| Trait 4 | $COV_{(T1,T4)}$ | $COV_{(T2,T4)}$ | $COV_{(T3,T4)}$ | $VAR_{T4}$ | $COV_{(T4,T5)}$ |
| Trait 5 | $COV_{(T1,T5)}$ | $COV_{(T2,T5)}$ | $COV_{(T3,T5)}$ | $COV_{(T4,T5)}$ | $VAR_{T5}$ |

**Figure 1.1**: The components of a 5-dimensional covariance matrix. In the case of **P** 'VAR' and 'COV' are variance and covariance values; in the case of **G** they are additive genetic variance ($V_A$) and covariance ($Cov_A$). Note that the upper off-diagonal terms are identical to their counterparts below the diagonal.

are important, since the change in the mean of any individual trait depends not only on the product of its variance and its selection differential, but also partially on the products of its covariances with all the other traits multiplied by their selection differentials. This means that selection on one trait applies correlational selection to other, correlated traits. If traits *x* and *y* are correlated therefore, trait *y* may evolve due to indirect selection on trait *x*.

The **G** matrix can therefore be thought of as a summary of the structure of genetic integration in a suite of traits. However, as discussed above (section 1.1), integration may also arise non-genetically by the action of the environment on the developing organism; the resulting pattern of covariances can also be measured and is referred to as the **E** matrix. Integration at the level of the phenotype can therefore be brought about via genetic or environmental influences, or the interaction of these factors during development. The **P** matrix summarises the pattern of integration among the component parts of a complex trait, irrespective of whether the root cause is genetic or environmental, and is thus invaluable as an integral part of the description of a complex trait (Dobzhansky, 1956). Whilst the degree to which the covariance structures of **G** and **P** matrices are likely to

correspond is uncertain (Cheverud, 1988; Willis et al., 1991; McGuigan & Blows, 2007), there is evidence to suggest that **G** and **P** may be more similar in some circumstances than others (Roff, 1995; Roff, 1996), with **G** and **P** expected to be similar if there is relatively little influence of the environment, or if environment and genotype influence traits through the same developmental pathways (Cheverud, 1984; Cheverud, 1988; Klingenberg & Leamy, 2001).

When only two component traits are involved, the covariance between them is relatively easy to visualise (Figure 1.3, and section 1.3 below) on a scatterplot of trait 1 values versus trait 2 values. When a **G** or **P** matrix is multidimensional, however, matters are more complex, and in these cases matrix diagonalization is a powerful tool for characterising the spread of variance. Diagonalization decomposes an ***n***-dimensional matrix into ***n*** orthogonal vectors; each of which can be thought of as a composite trait defined by a linear combination of partial trait values. Each vector has a magnitude (eigenvalue) associated with it that represents the length of that vector in ***n***-dimensional space; that is to say the amount of variance present for that composite trait. These vectors can be thought of as 'genetic degrees of freedom' (Kirkpatrick & Lofsvold, 1992), since they represent independent directions in which the population mean can evolve.

Crucially, diagonalization of a matrix extracts vectors in order of magnitude, meaning that the principle vector is the most variable composite trait, the second vector is the most variable composite trait that is orthogonal to the first, and so on (by convention, these vectors are referred to as $\mathbf{g}_{max}$, $\mathbf{g}_2$, $\mathbf{g}_3$… $\mathbf{g}_n$ respectively if calculated from a **G** matrix; $\mathbf{p}_{max}$, $\mathbf{p}_2$, $\mathbf{p}_3$… $\mathbf{p}_n$ if calculated from a **P** matrix). This means that a given movement of the multivariate mean (say +***x*** units) would entail changes of different magnitude and/or direction for the population means of different traits. A trait with a large and positive coefficient for the principle vector might experience a mean increase of 0.70***x***, whereas one with a smaller negative coefficient might see its mean decrease by 0.01***x***. Thus not all vectors are equivalent, and in some cases the latter vector(s) of a matrix may contain no variance at all. In these cases we would say that the matrix is singular (Strang, 2003), i.e.; there are fewer dimensions that contain variance than there are traits (Figure 1.2). Once it is understood that an ***n***-dimensional matrix may have <***n***

directions of independent variance, then it is relatively easy to envisage that asking questions about the evolution of a complex suite of traits without considering the pattern of integration between them may lead to erroneous findings.

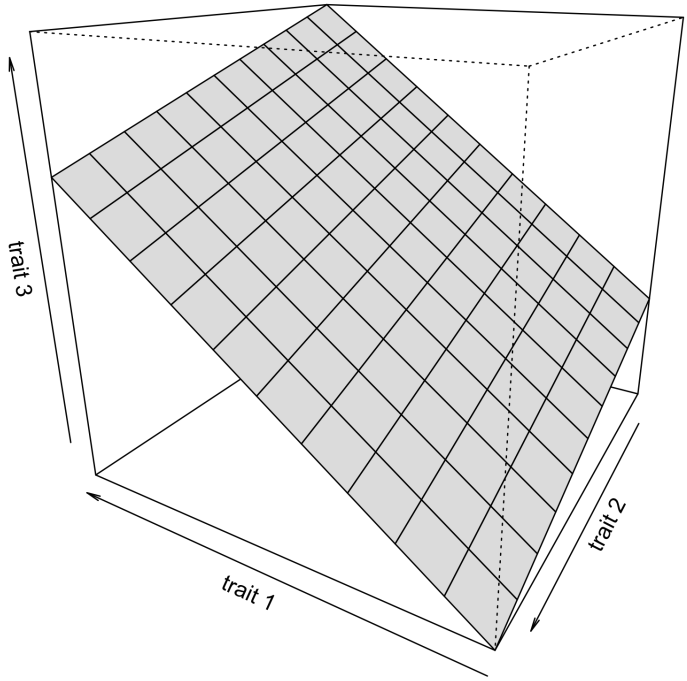


**Figure 1.2**: The plane described by a singular matrix. In this case, the plane describes the covariance between three hypothetical traits. There is variance in all three dimensions of trait-space, but the range of multivariate values defines a two-dimensional plane. There are, in fact, only two independent directions of variance present, but they are composite vectors; analogous to the two non-zero eigenvectors that would be extracted by diagonalization of this hypothetical covariance matrix. If plotted on axes aligned with these eigenvectors, the shape of this three-dimensional surface would be completely described in two dimensions.

## *1.3. THE EVOLUTION OF INTEGRATED PHENOTYPES*

From a quantitative genetic perspective, the starting point for evolutionary change is the quantity of standing genetic variance in phenotypic traits, since this trait variance is required for any phenotypic response to selection. As discussed above, however, this response can also be influenced by selection on other traits and the

covariance between them and the focal trait of interest. A trait without heritable variance cannot evolve. The multivariate corollary of this in the case of integrated traits is that a direction without variance is a direction in which the multivariate mean cannot evolve. For complex traits with a singular **G** or **P** matrix there may therefore be trait combinations that would, should they arise in the population, confer a high fitness value on the bearer, but that are prevented from evolving because they exist in a dimension that lacks heritable variance (Blows & Hoffmann, 2005).

The possibility of selection operating in a direction entirely devoid of variation, however, is unlikely to occur outside of quantitative genetic models (e.g. Brakefield, 2003). Moreover, the findings of the many single-trait heritability experiments and studies of artificial selection have, as Blows & Hoffmann (2005) point out, led to the widespread conclusion that genetic variance is likely to be found wherever it may be searched for (Brakefield, 2003; Lynch & Walsh, 1998; Barton & Partridge, 2000). However, it is entirely possible for a complex trait to possess heritable variance for all its ***n*** components, but for those components to be integrated in such a way that there are **<*n*** independent directions of variance (Figure 1.2). Thus, an absolute genetic constraint might be imposed on the evolution of a complex trait not by a lack of variance in any individual component, but by a lack of independent variance in some directions, since trait configurations that exist outside the distribution of variance present in multi-dimensional trait-space will not be exposed to selection. How often such constraints actually occur is currently unclear.

Whether or not a given complex trait experiences absolute genetic constraints, when traits covary and variance is distributed unequally among eigenvectors, there is potential for the 'shape' of this covariance to influence the direction and speed of phenotypic evolution. Although this is an inherently multivariate process, an intuitive way to visualise this effect is to imagine a scatterplot of breeding values for two traits that covary such that individuals with high values for trait 1 also tend to have high values for trait 2 (Figure 1.3). If selection were to act on trait 1, trait 2 also would change in value even without any direct selection on trait two. For example if large values of trait 1 carried a fitness benefit and the population

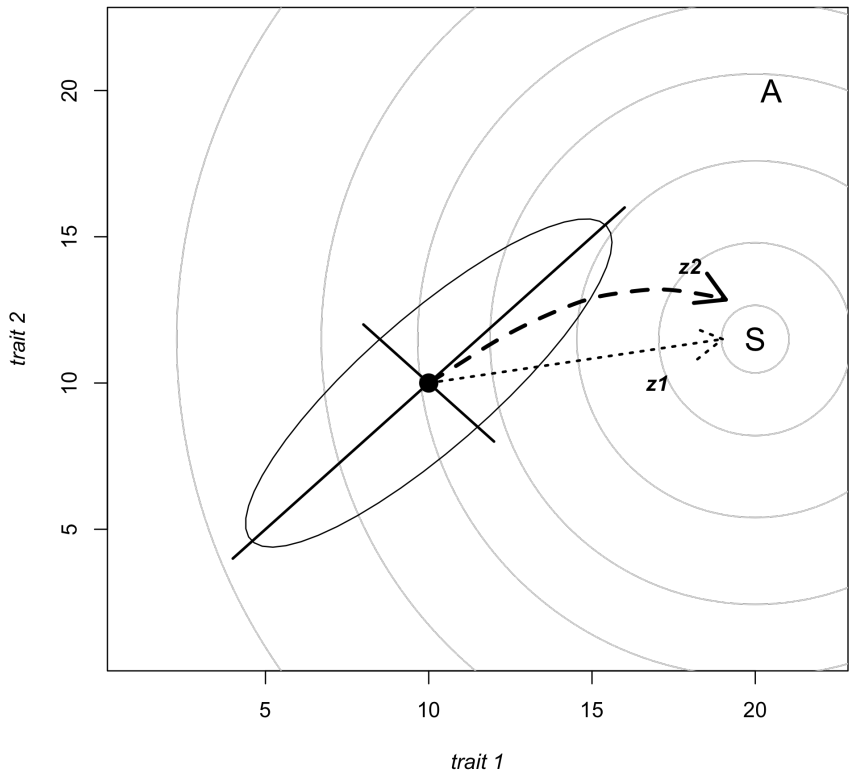


**Figure 1.3**: The biasing effect of $\mathbf{g}_{max}$ on response to selection when traits covary. The axes represent the breeding values for 2 hypothetical traits; the solid point representing the population mean for traits 1 and 2, and the surrounding ellipse; the 95% confidence region for the distribution of trait values about the mean. That these traits covary is evident as the ellipse is at an angle relative to the trait axes. The axes of the ellipse represent the 2 orthogonal directions (eigenvectors) of variance present; there is more standing genetic variance along the major axis ($\mathbf{g}_{max}$) than the minor axis. They grey lines are 'contours' on a fitness landscape, with an adaptive peak at 'S'. Rather than evolving directly toward the peak (response '*z1'*), the influence of $\mathbf{g}_{max}$ may cause the population to evolve along an indirect course (response '*z2'*). In some cases this may even, result in the population evolving toward an alternate fitness peak (e.g. at 'A') in line with $\mathbf{g}_{max}$, even though it is more distant from

evolved to increase them, then the value of trait 2 would increase also. The ellipse in Figure 1.3 represents a 95% confidence region around the population mean. Since it is elliptical, this visualisation indicates that there is more variance in one

dimension (the major axis of the ellipse) than in the other (minor axis). In cases where variance is unevenly distributed among dimensions, evolution can be expected to proceed most quickly along the longest axis of the ellipse (i.e. $\mathbf{g}_{max}$) than in other directions. It currently is unclear how frequently the trajectory of phenotypic evolution is likely to be influenced by the distribution of variance in this way (section 1.6 below). Where an evolutionary optimum exists in line with the direction of $\mathbf{g}_{max}$, the response will therefore be more rapid than where the local optimum lies elsewhere; these directions have been characterised as 'genetic degrees of freedom' (Kirkpatrick & Lofsvold, 1992) or 'lines of least evolutionary resistance' (Schluter, 1996). Where the optimum is not aligned with $\mathbf{g}_{max}$, the **G** matrix may constrain the evolutionary trajectory such that the population takes an indirect path to the optimum. This may lengthen the time needed reach the optimum, and if the optimum is moving, even slowly, the population may never reach it (Jones et al., 2004). Even more dramatically, on adaptive landscapes with multiple optima, the shape of **G** may bias the evolution of a population toward those that lie along lines of least evolutionary resistance, even if a closer, higher peak exists (Steppan et al., 2002).

## *1.4. ADVERTISEMENT CALLS AS A COMPLEX SEXUAL TRAIT*

The males of many acoustic insect species broadcast an advertisement call. It is thought to have evolved as a mechanism for both species recognition and mate assessment (Lewis, 1985). Orthopterans produce this advertisement call by stridulation, and in male field crickets (Gryllidae) the forewings develop as specialized sound-production structures (Zuk, 1987) and are not used in flight.

Singing is a cyclic process, with a silent phase as the forewings are opened and raised, and sound produced by a toothed 'file' moving against a 'plectrum' as the forewings are closed. When raised, a plectrum on the (typically) left forewing engages with a toothed file on the ventral surface of the right forewing; the movement of the plectrum over the file as the forewings are closed sets up a vibration in resonant 'harp' and 'mirror' structures of both wings (Bennet-Clark, 2003; Bennet-Clark & Bailey, 2002). As the file and plectrum are opposed they

function as an escapement (analogous to the devices that regulate the speed of clockwork mechanisms) that regulates the catch and release of the plectrum (Koch et al., 1988; Bennet-Clark & Bailey, 2002; Prestwich et al., 2000). The stridulatory mechanism employed by crickets constrains aspects of the calls that they are able to produce. Most obviously, the alternation of the silent 'upstroke' with the sound producing 'down stroke' of the forewings necessitates a call that consists of pulses separated by pauses. In some species there is little variation on this base and the call remains simple, but others have evolved more elaborate sequences of pulses grouped into chirp or trill 'syllables', and of syllables grouped into phrases (Otte 1992). The call thus has a temporal pattern consisting of the durations of pulses and inter-pulse intervals, numbers of pulses comprising a syllable, number of syllable repeats to name a few parameters. It has been suggested that species recognition in crickets is based mostly on this temporal pattern (Robinson & Hall, 2002). This idea is supported by the general finding that call temporal patterning is often highly variable between species but stereotyped within species, with generally low levels of inter-individual variation (Hoy, 1974; Hoy & Paul, 1973; Hoy et al., 1977). Indeed, advertisement calls are the principal character in determining species identity for a number of cryptic species (Robinson & Hall, 2002; Shaw, 1996). This role in species recognition has made cricket advertisement calls an attractive system for researchers studying speciation (e.g. Otte, 1992).

For those studying phenotypic integration cricket advertisement calls also represent an excellent model system. First integration is predicted to be an adaptive response to sustained stabilising selection on a complex trait (Lande, 1980; Cheverud, 1984) and species-specific stereotypy is strongly suggestive of such a regime. Second, as an acoustic trait, advertisement call is relatively tractable. It is straightforward to take measures of both temporal and spectral properties from a recording, and since it is composed of discreet sound pulses, a recording can be digitally manipulated without specialist equipment (e.g. Bentsen et al., 2006). Third, these synthesised calls can then be played back to females meaning that the sexual selection operating on the parameters of interest can be measured.

## *1.5. TELEOGRYLLUS COMMODUS AS A MODEL SYSTEM*

The model species I use in my empirical research is *Teleogryllus commodus;* a widely distributed field cricket that is found across much of the southern half of Australia. *T. commodus* is univoltine diapausing species, being active only through spring and summer in the wild, but is amenable to continuous breeding when reared under constant conditions in the lab.

Once adult, male *T. commodus* spend a substantial proportion of their time broadcasting their advertisement call (Hunt et al., 2004), which has been shown to be energetically costly (Kavanagh, 1987). The *T. commodus* advertisement call is relatively complex (Appendix III), consisting of a single 'chirp' syllable followed by a variable number of 'trill' syllables and then a pause; with a variable number of pulses in each syllable, gaps of variable duration between them, and variation in the length of the inter-call pause (Bentley & Hoy 1972; Hill et al. 1972). This offers many possible call structure traits that could be measured. Furthermore, Female *T. commodus* show preferences for both temporal (Pollack & Hoy, 1979) and spectral (Hennig & Weber, 1997) properties of this call, resulting in a regime of multivariate stabilising sexual selection (Brooks et al., 2005; Bentsen et al., 2006).

Crickets advertisement calls have been studied by a number of researchers interested in the role of sexual signals in population divergence and speciation due to their ability to act as pre-mating isolation barriers (e.g. Gray & Cade, 2000; Honda-Sumi, 2005; Izzo & Gray, 2004; Jang & Gerhardt, 2006). Though I am not, for the purposes of this thesis, concerned with population divergence *per se*, the wide distribution of *T. commodus* also suits it to its role as a model organism. Animals used in the empirical work that follows (Chapters 3-6) were taken from six laboratory stock populations established from wild-caught stock. In order to establish these stock populations, founder animals were collected at six widely dispersed sites; from Canberra in the Australian Capital Territory, from Kioloa and Smith's Lakes in New South Wales, from McLaren Vale in South Australia, from Richmond in Tasmania and from Walpole in Western Australia (See Appendix II for a map showing the locations of these populations). Since this model system was new to the UK, it was necessary for me to make fresh collections from these sites. These collections were made in February and March of 2007.

The distance between my source populations, and the dramatic environmental differences between their habitats means, it is safe to assume, that there has been ample opportunity for local adaptation. Furthermore, given the relatively short dispersal range of field crickets (Cade, 1979) and the unimpressive flight capabilities of *T. commodus* (personal observation), not to mention the formidable geographical barriers between them (Appendix II), it is also seems reasonable to assume that the rate of gene flow between these populations is extremely slow. Taken together, these observations suggest the comparison of patterns of integration between these populations is likely to be informative as to the robustness of such patterns during population divergence.

## *1.6. THESIS OUTLINE*

The primary focus of this thesis is to examine the potential for trait integration to influence phenotypic evolution. The chapters in this thesis are presented as self-contained papers, and therefore each contain summaries of relevant literature, descriptions of methodology and results, and discussions that attempt to place the findings in an appropriate theoretical context. While each chapter was motivated by the same question of the influence of integration on phenotypic evolution, the literature referred to and the theories discussed in each differ. Below, I shall outline how the chapters of this thesis are intended to fit together within the framework of existing theory on phenotypic integration.

Though the majority of the chapters report my empirical work on the *T. commodus* model system, the thesis begins with a quantitative review, using data from the quantitative genetic literature. As mentioned above, empirical progress on trait integration has, until recently, been handicapped by a dearth of suitable tools and approaches (Pigliucci & Preston, 2004). In particular, the generality of integration effects on evolution is not known. Though demonstrating absolute genetic constraint (i.e. a singular covariance matrix) is a formidable empirical undertaking (Hine & Blows, 2006), the approach used by Schluter (1996) enables data about trajectory biasing 'lines of least evolutionary resistance' to be extracted from published trait relationships. In Chapter 2, I collate published **G** and **P** matrices and

use a similar method to quantify the prevalence of lines of least evolutionary resistance (LLER's) in an attempt to estimate how frequently these LLER's may have the potential to influence evolutionary trajectories. Previous quantitative reviews have found evidence that traits of different types may differ in their quantitative genetics; morphological traits tend to be more heritable than life-history or behavioural traits (Mousseau & Roff, 1987), and whereas morphology most commonly experiences stabilising selection (Pomiankowski & Moller, 1995), selection on sexual signals is more likely to be directional (Andersson & Iwasa, 1996). I therefore examined my matrix dataset to determine if there were patterns in the distribution or strength of LLER's among the different types of complex trait represented.

Quantitative geneticists are able model the relationship between integration and selection using the multivariate extension of the breeders' equation (Lande, 1979), which can be stated as: $\Delta\bar{z} = \mathbf{GP^{-1}S}$ (where $\Delta\bar{z}$ is the vector of mean responses, **G** and **P** are the **G** and **P** matrices, and **S** is the vector of selection differentials). However, the predictions of the breeders' equation only hold as long as both **G** and **P** are constant, and there is a considerable body of evidence indicating that we ought to expect these matrices to evolve (Agrawal et al., 2001; Jones et al., 2004; Phillips & McGuigan, 2006; Phillips et al., 2001; Roff, 2000; Roff & Mousseau, 1999). In other words, the shape of **G** determines what trait combinations are exposed to selection each generation, but in the long term **G** is shaped by selection. In Chapter 3, I address the question of what 'long term' means in the previous sentence, since the timescale over which covariance matrices evolve is not currently clear. I use data from common-garden rearing to estimate the **P** matrix for advertisement calls for my six divergent study populations. By comparing those matrices I can then search for differences in **P** both over the timescale of their divergence, and that of their lab adaptation. Comparison of covariance matrices is one of those areas (mentioned in section 1.3) in which rapid progress has been made recently (Blows & Higgie, 2003; Houle et al., 2002; Mezey & Houle, 2003; Roff, 2002; Steppan et al., 2002), but consensus as to the most appropriate methodology is currently lacking. In this chapter I therefore make use of a number of available matrix analysis tools in order that my findings can be directly

compared between approaches, in the hope that this information may prove useful to future researchers.

As I mentioned in section 1.3, researchers are becoming increasingly aware of the link between the phenomena of trait integration and phenotypic plasticity. In Chapter 4 I present an experimental manipulation of diet, intended to impose developmental stress and expose what condition dependence might be found for call structure. Once again I compare **P** matrices in order to determine how stability/plasticity of phenotypic integration underlies changes measured in call structure traits.

Having quantified the extent of phenotypic integration of the advertisement call, in Chapter 5 I examine the relationship between call structure and forewing morphology. As discussed above (section 1.4) the call is produced by stridulation using the forewings, which are not used in flight. Since the structure of the call has presumably been shaped by sexual selection (Brooks et al., 2005; Bentsen et al., 2006), this experiment is intended to measure how, and to what extent, call structure is integrated with the morphology of the organs used in its production. For this purpose I utilise geometric morphometric techniques (Klingenberg & McIntyre, 1998; Klingenberg et al., 1998; Dryden & Mardia, 1998) to quantify the shape of the forewings from those males whose calls I analyse in Chapter 3.

In Chapter 3 I find evidence for genetic differentiation for advertisement call between my study populations. I also find evidence that these populations respond differently to environmental variation; between field and lab conditions in Chapter 3, and between diet treatments in Chapter 4. These findings are suggestive of a genotype by environment interaction (GxE). Theory predicts that in cases of GxE interaction for a sexual signal, coevolution of the signal and the preference function for that signal ought to be favoured in order to maintain signal coherence (Kokko & Heubel, 2008; Higginson & Reader, 2009). Since I had uncovered a signal divergence and a potential GxE, I aimed to test the coherence of the advertisement call in Chapter 6. I used artificial advertisement calls to present a wide range of variation for inter-call duration; an element of call structure that is both divergent and under sexual selection (Brooks et al., 2005). I then presented these calls to

females in acoustic choice trials in order to calculate preference functions. By using females raised on the same diets used in Chapter 4, I was also able to test for condition dependence of in female preference. This is of interest since the analysis of effects associated with both environmental (diet) and genetic (population) differences would allow me to uncover (tentative) evidence for the presence or absence of a GxE interaction in female preference, similar to that suggested for male call structure.

In Chapter 7, I conclude by discussing my findings from these studies in the wider context of theory on the evolution of integrated traits.

# CHAPTER 2: The Prevalence of 'Evolutionary Lines of Least Resistance' in Morphology, Life-History and Sexual Signals.

## *2.1 ABSTRACT*

The **G** matrix summarises the structure of covariance among a suite of traits. Under the quantitative genetic framework, the **G** matrix is of primary importance with respect to evolvability, since those directions in trait space whose variance is independent may be extracted from it. The spread of variance among these vectors is of interest because vectors with most variance represent 'evolutionary lines of least resistance', along which a population's immediate response to selection is predicted to be greater than in other directions. Systematic surveys of the level of standing genetic variance (diagonal elements of the **G** matrix), and of the nature and strength of selection in populations can be found in the literature, but to our knowledge, no such survey has been published for evolutionary lines of least resistance. Here we present a survey of **G** matrices and the related **P** matrices from the literature, with analyses using a diversity index to measure the unevenness of the spread of variance among their orthogonal vectors. Comparison of diversity indices between **G** and **P** matrices found no difference, nor did comparison among taxa. Between trait types however, variance in complex traits classified as life-history or sexually selected was found to be distributed more unevenly than in those classified as morphological. This difference in the distribution of variance among vectors suggests that evolutionary lines of least resistance may have more influence on the direction of evolution for life-history and sexually selected traits than for morphology.

**Keywords**; **G** matrix; **P** matrix; Genetic architecture; Genetic integration; Additive genetic variance; Lines of least evolutionary resistance; Evolvability; Quantitative genetics

## *2.2 INTRODUCTION*

The concept of evolvability, in various forms, has received considerable attention in the literature for the last two decades (Cheverud, 1996a; Hansen, 2006; Hansen & Houle, 2004; Jones et al., 2007; Pigliucci, 2008; Schluter, 1996; Wagner & Altenberg, 1996). In a quantitative genetic framework, evolvability is closely related to the level of standing genetic variance in phenotypic traits, and to the covariances between them; trait variance is required for any phenotypic response to selection, and inter-trait covariances can magnify or constrain this response (Lande, 1979; Lynch & Walsh, 1998). Genetic variance and covariance is described by the **G** matrix (additive genetic variance-covariance matrix); a symmetrical matrix with values for additive genetic variances on its diagonal, with values for genetic covariances – arising from pleiotropy and linkage disequilibrium – above and below (Lande, 1979). The **G** matrix is an integral component of the theoretical framework of quantitative genetics, particularly due to its role in Lande's multivariate extension of the breeders' equation (Lande, 1979), which is routinely used to predict the short-term response to selection. The related **P** matrix (phenotypic variance-covariance matrix) also summarizes variance-covariance structure, but includes environmental and non-additive genetic effects in addition to the additive genetic values of **G**.

Hypothesis testing and biological interpretation of results based on individual elements of the **G** or **P** matrix (i.e. on single-trait variances or bivariate covariances) are common in the literature, but the value of such tightly focused investigations is limited (Blows & Hoffmann, 2005; Hunt et al., 2007; Roff, 2006). Moreover, in most cases the genetic and developmental architecture of the traits of interest is not well understood, and data from a suite of *n* phenotypic traits does necessarily comprise *n* independent units, due to trait integration (Dobzhansky, 1956; Phillips & McGuigan, 2006; Schluter, 2000). This covariance between traits that are functionally or developmentally linked means that selection experienced by each trait is affected by selection applied to the others. A useful concept here is that of 'genetic degrees of freedom' (Kirkpatrick & Lofsvold, 1992), each of which is a composite trait; a vector defined by a linear combination of partial trait values. Diagonalization of the **G** matrix reveals these vectors, and also quantifies the

amount of variance in each direction as an eigenvalue (Blows, 2007). By convention eigenvectors are listed by decreasing size of their eigenvalue, with eigenvector 1 (the multivariate direction of maximum variance) usually referred to as $\mathbf{g}_{max}$. If variance is not distributed evenly among the eigenvectors of **G**, then some evolutionary trajectories are more accessible than others; these are evolutionary 'lines of least resistance' that may deflect the course of evolution away from the direct route toward the selective optimum, at least in the short term (Schluter, 1996). Needless to say, the utility of this approach is limited by the choice of measured traits: diagonalization will reveal the relationships among a suit of traits, but it must be kept in mind that each trait measured may covary with one or more traits that were not measured in a particular study.

The quantitative genetic enterprise has benefited from reviews that have established the prevalence of heritable genetic variation (Barton & Turelli, 1989) and the strength of selection (Kingsolver et al., 2001). Both Schluter (2000) and Kirkpatrick and Lofsvold (1992) have taken samples of **G** matrices from the literature and found that much of the available variance in each case was concentrated in the first few directions, but we know of no systematic review that reveals how generally this is the case. While estimating **P** is a non-trivial exercise, the empirical challenge in estimating **G** can be considerable to say the least. From a standpoint of efficient experimental design, it has long been noted that it would therefore be useful to know how closely **P** predicts **G** (Cheverud, 1988). In terms of lines of least evolutionary resistance (LLER's), one could predict that the inclusion of environmental variance in **P** could obscure a pattern present in the underlying **G** if that variance were differently allocated among vectors, and to emphasise that pattern if **G** and **P** are similar in structure (Arnold et al., 2008).

Kingsolver et al. (2001) found that selection tended to be stronger on morphological traits than on life-history traits, and Mousseau & Roff (1987) found that morphological traits had, on average, higher heritabilities than life-history traits, but whether LLER's are more prevalent in some trait types than others is currently unknown. There is evidence that some types of traits – such as morphology (Pomiankowski & Moller, 1995) – tend to be under stabilising selection, whereas selection on other types – such as sexual signals (Andersson &

Iwasa, 1996) – tends to be directional. For this reason we may expect LLER's to be more prevalent in sexually selected traits than in morphological ones, and for the relative weakness of selection on life-history traits to correlate with a relatively lower prevalence of LLER's. When making comparisons between trait types, we must acknowledge that these classifications may not be directly equivalent among taxa: in particular, sexual selection could conceivably have different consequences for plants and animals. We shall therefore group our matrices by taxon and test for differences between plants and animals.

In this paper, we describe a systematic survey of **G** and **P** matrices from the literature. A diversity index was used to quantify unevenness in the distribution of variance among vectors for each matrix. This gave us a dataset with which to explore how this unevenness varies; between matrix types (**G** or **P** matrices), across taxa and between types of traits. Comparing the spread of variance among eigenvectors allows us assess the relative strength of LLER's, and therefore the extent to which they are likely to influence the direction of evolution.

## *2.3 METHODS*

### Search Strategy

Between November 2006 and March 2010, we queried the ISI Web of Science citation database for the search terms; "G matrix" (or "G-matrix"), "covariance matrix" (or "co-variance matrix" or "(co)variance matrix") and "quantitative genetics", restricting ourselves to hits from the four most common biology-related categories: 'Genetics & Heredity', Evolutionary Biology', 'Zoology' and 'Environmental Sciences & Ecology'. This gave us a preliminary list of 2,675 papers. The assignment of categories is non-exclusive on the Web of Science database – all papers on our list were tagged with more than one category – and so we feel confident that our search, even if not exhaustive, will have missed very few appropriate references.

We next refined the preliminary list of references on the basis of their title, abstract and keywords; and attempted to obtain the full text for all papers not excluded in this way. In addition to recording **G** and **P** matrices, we also noted matrices constructed with genetic correlations and narrow sense heritabilities. In those cases where values for phenotypic variance had been presented alongside genetic correlations and heritabilities, we were able to back-calculate the variances and covariances of **G** as: $V_A = h^2 V_P$ and $Cov_{(x,y)} = rG\sqrt{V_A{}^x V_A{}^y}$ where $V_A$ and $V_P$ are the additive genetic and phenotypic variances, $h^2$ is the narrow sense heritability and *r*G is the genetic correlation between traits *x* and *y*. In some cases not all of the above statistics were presented in the paper and it was not possible for us to calculate **G** in this way. In these cases we contacted the corresponding author(s) wherever possible to request these unpublished statistics. A few authors were no longer working in the academic arena, and many had moved from one institution to another, but most were both contactable and helpful. S. F. McDaniel and W. U. Blanckenhorn were kind enough to give us access to datasets that are not yet in press, and we have also included a set of unpublished **P** matrices of our own (Chapter 3).

We compiled a list of 156 **G** and 87 **P** matrices, which we then classified by taxon; vertebrate, invertebrate or plant (following Kingsolver et al., 2001); and by trait type; either morphological, life-history or sexually selected (Table 2.1). All classifications were exclusive. For animals, male secondary sexual characters or signals that have been shown to be the focus of female choice were classified as sexually selected (Table 2.1). In the case of matrices calculated from plant measurements, traits were classified as sexually selected only if they were measures of flower morphology.

Those matrices whose traits did not fit into any of these categories were not included in our analysis. Matrices containing traits from more than one category – or traits that fitted none of our three categories – were split to produce smaller sub-matrices that could be included in the appropriate category. In cases where we found more than one matrix for the same traits, measured in the same population, the first matrix to be published was used and others were excluded. The final list of sources providing matrices to the dataset can be found in Appendix I.

**Table 2.1**: Summary table for all animal traits assigned as sexually selected; specifying the study from which the matrices were drawn, and a supporting reference to demonstrate that the traits are an object of female choice.

| Study | Species | Matrices | Traits | Reference |
|---|---|---|---|---|
| Blows et al 2004 | *Drosophila serrata* | G | cuticular hydrocarbons | Howard et al. (2003) J. Chem. Ecol. |
| Brandt & Greenfield 2004 | *Achroia grisella* | G | advertisement song | Cremer & Greenfield (1998) Ethology |
| Brooks & Endler 2001 | *Poecilia reticulata* | G (2) | male colour patches | Sheridan & Pomiankowski (1997) Anim. Behav. |
| Hine & Blows 2006 | *Drosophila serrata* | G (2) | cuticular hydrocarbons | Howard et al. (2003) J. Chem. Ecol. |
| Hunt et al 2007 | *Teleogryllus commodus* | G | calling song | Bentsen et al. (2005) Am. Nat. |
| McGuigan et al 2008 | *Drosophila bunnanda* | G | cuticular hydrocarbons | McGuigan et al (2008) Am. Nat. |
| Pitchers & Hunt (unpub.) | *Teleogryllus commodus* | P (12) | calling song | Bentsen et al. (2005) Am. Nat. |
| Simmons 2004 | *Teleogryllus oceanicus* | G (2) | calling song | Simmons et al. (2001) Evolution |
| Svensson et al 2005 | *Calopteryx splendens* | P (2) | male wing colour | Svensson et al. (2004) Heredity |

## Analysis

The matrices in this final list were then diagonalized – we used the eigenanalysis function of the Poptools add-in program for Microsoft Excel (Hood, 2009). When covariance matrices are estimated, small errors can cause them to be non-positive-definitive: i.e. to return negative eigenvalues when diagonalized (Jorjani et al., 2003). These values, like a negative variance, are mathematical artifacts and cannot be interpreted. We therefore 'bent' such matrices to ensure that their eigenvalues were non-negative before analysis, using the bending method described by Jorjani *et al* (2003). Following Schluter (2000), we initially calculated Levin's diversity index (LDI) to describe the unevenness of the distribution of variance among the eigenvectors of each matrix (calculated as: $LDI = 1 / \sum p_i^2$ where $p_i$ is the magnitude of the eigenvalue for eigenvector $i$ as a proportion of the sum of the eigenvalues for all vectors). However, since LDI is free to vary between zero and the number of traits/vectors in the matrix, using this index means that smaller matrices are constrained to a narrower range of potential values than larger ones. This means that the diversity values are effectively unstandardised for matrix rank. When we analysed the resulting LDI values we found that this led to multiple higher-order interactions when matrices of different size were compared. In order to better control for the effect of matrix size, we calculated Shannon's Equitability index ($E_H$), which is defined as:

$$E_H = \sum_{i=1}^{S} p_i \ln p_i \Big/ \ln S$$

where $S$ is the number of traits/vectors in the matrix. The value of $E_H$ may vary only between one and zero for matrices of any size. An $E_H$ value of one indicates an even spread of variance between all vectors; a value of zero indicates that all the variance is to be found on the principal eigenvector of **G**.

We analysed the dataset of $E_H$ values using randomisation tests based on general linear models. Each model was tested by comparing its $F$ value to a sample distribution of 10,000 $F$ values from the same model with the response variable ($E_H$) randomised relative to the explanatory variables. In this approach the proportion of pseudo-$F$ values more extreme than the true $F$ value gives a 1-tailed $p$-value; the 2-tailed $p$ is then calculated as either $2p$ if $p<0.05$, or as $2(1-p)$ if

$p$>0.05. This approach was chosen because our data broke parametric assumptions; randomisation allowed us to use GLM model reduction despite our data being non-parametric. A full model, containing all factors, was not helpful in addressing our hypotheses as a number of 2 and 3-way interactions were significant. In order to interpret the data in a meaningful way, we therefore analysed models specific to each hypothesis of interest. All analyses were performed using R (R Development Team, 2009).

## *2.4 RESULTS*

A full factorial general linear model with matrix type (**G** or **P**), taxon, trait type and trait number as main effects and $E_H$ as the response variable showed significant effects of both trait type and trait number ($p$<0.0001 and $p$=0.0006 respectively). However, the interactions of matrix type by trait type ($p$<0.0001), matrix type by trait number ($p$=0.03), taxon by trait number ($p$<0.0001) and trait type by trait number ($p$=0.0003) were also significant. (See Table 2.2 for full model summary) More problematically, three of the four 3-way interactions were significant (matrix type by taxon by trait number: $p$=0.03, matrix type by trait type by trait number: $p$=0.0002, taxon by trait type by trait number: $p$<0.0001). These higher-order interactions are not informative with respect to our hypotheses, but they make the interpretation of lower-order relationships difficult. Hence we used reduced models to test our individual hypotheses. Trait number was included as a covariate in all models in order to control for an effect of matrix size.

We used a two factor GLM to test for difference between **G** and **P** matrices, whilst controlling for an effect of size. We found no effect of matrix type ($p$=0.8) and no interaction between matrix type and trait number ($p$=0.2), but there was a significant effect of trait number ($p$=0.001).

We used similar GLM to test for an effect of taxon, also with trait number as a covariate. Since we had found no difference between **G** and **P**, all matrices were included in this analysis. We found no effect of taxon ($p$=0.9) and no taxon by trait number interaction ($p$=0.6), but the effect of trait number was again significant ($p$=0.002).

**Table 2.2**: Results of a randomised full-factorial general linear model. The model was run for 10,000 permutations. 2-tailed *p* values are in bold where significant.

| Effect | True F value | Proportion of permuted F values < true F value (1-tailed p value) | 2-tailed *p* value |
|---|---|---|---|
| matrix type (**G** or **P**) | 0.62 | 0.57 | 0.851 |
| taxon (Animal or Plant) | 1.46 | 0.77 | 0.461 |
| trait type (M, S or L) | 50.82 | 1.00 | **0.000** |
| trait no. (matrix rank) | 11.98 | 1.00 | **0.001** |
| matrix.type x taxon | 4.01 | 0.98 | **0.042** |
| matrix.type x trait.type | 28.75 | 1.00 | **0.000** |
| taxon x trait.type | 2.04 | 0.91 | 0.180 |
| matrix.type x trait.no | 5.87 | 0.98 | **0.033** |
| taxon x trait.no | 10.83 | 1.00 | **0.000** |
| trait.type x trait.no | 6.85 | 1.00 | **0.003** |
| matrix.type x taxon x trait.type | 1.78 | 0.81 | 0.387 |
| matrix.type x taxon x trait.no | 4.17 | 0.98 | **0.034** |
| matrix.type x trait.type x trait.no | 17.27 | 1.00 | **0.000** |
| taxon x trait.type x trait.no | 13.99 | 1.00 | **0.000** |

Having found no effects of either matrix type or taxon, we included both **G** and **P** matrices from all taxa in a GLM to test for an effect of trait type, once again with trait number included as a covariate. We found a significant difference between trait types ($p$<0.0001), but no effect of trait number ($p$=0.2) or trait number by trait type interaction ($p$=0.5). As trait type was a 3-level factor we re-ran this analysis three times, excluding one trait type in each case, to look for pairwise differences. There was no significant difference between life-history and sexually selected traits ($p$=0.7), but both sexually selected and life-history traits were found to have significantly lower $E_H$ values than morphological traits ($p$<0.0001 in both cases, Figure 2.1). Trait number and the interaction of trait number by trait type remained non-significant in all three of these pairwise models.

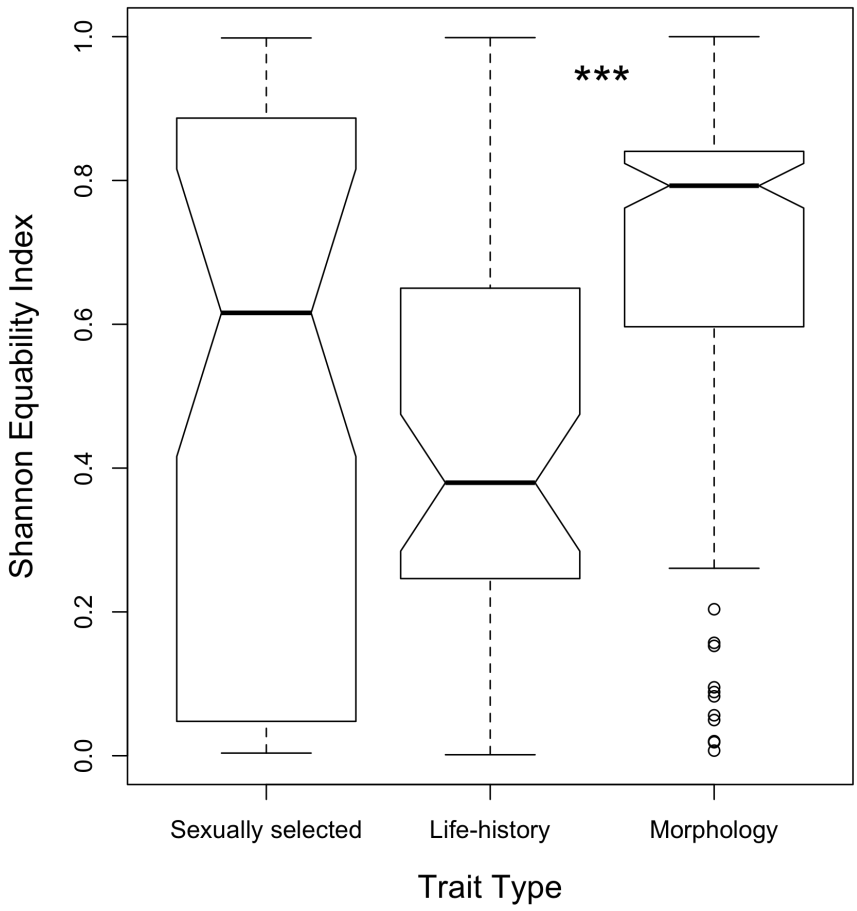


**Figure 2.1**: Boxplot to show differences in $E_H$ between trait types for both **G** and **P** matrices. *** Both sexually selected traits and life-history differ from morphological traits at the *p*=0.0001 level.

## *2.5 DISCUSSION*

Comparison of diversity indices for the spread of variance among eigenvectors is an approach for assessing the relative strength of lines of least evolutionary resistance (LLER's). A lower diversity index indicates a less even distribution of variance among vectors which means (as vectors are extracted hierarchically) a greater concentration of variance in the dominant vectors; the LLER's. It should not be forgotten that, though variance may be assigned to all dimensions, some dimensions may lack statistical support (McGuigan & Blows, 2007). With this caveat in place, our results do suggest the existence of a pattern relating trait type to the strength of LLER's. We found that life-history and sexually selected matrices had lower diversity index values (combined median 0.43, mean 0.45 ±SE 0.04)

than morphological matrices (median 0.80, mean 0.71 ±SE 0.02), and that no difference could be detected between life-history and sexual selected traits (Figure 2.1). The higher values for morphological traits suggest that the influence of LLER's on the direction of evolutionary change may be weaker for these traits than for life-history or sexually selected traits. In other words; morphology may be more able to evolve in the direction of minor dimensions than are secondary sexual characters or life-history traits.

Life-history traits have been shown to have significantly more additive genetic variance, on average, than morphological traits (Houle, 1992). If this variance represents genetic variance for fitness, then we would expect this high level of additive variance to be shared by sexually selected traits (Rowe & Houle, 1996), and there is some evidence that this is the case (Pomiankowski & Moller, 1995). Life-history traits tend to be more polygenic than other traits (Price & Schluter, 1991). Condition is also likely to be polygenic, and sexually selected traits likely to be condition dependent; and so sexually selected/life-history traits can together be characterised as traits with high levels of additive genetic variance spread among many genes of small effect. With many genes contributing to these trait types, we might also expect higher levels of non-additive genetic variance than in other traits, even if non-additivity is distributed at random throughout the genome. This would fit with our finding that these traits display more pronounced unevenness in the distribution of variance among dimensions, since pleiotropy and epistasis could contribute to trait covariance.

Since both sexually selected and life-history traits display this pattern, it begs the question of whether similar selection regimes are responsible in each case. Selection on sexual traits is typically directional in nature (Andersson, 1994; Andersson & Iwasa, 1996), while selection measured on morphology tends to be stabilising (Pomiankowski & Moller, 1995), though this distinction is by no means absolute (Brooks et al., 2005). As for the comparative strength of selection, a review by Kingsolver et al (2001) found that selection was in general stronger on morphology than on life-history, but unfortunately the data were not available to compare either with sexual selection.

We found no evidence for a difference between **G** and **P** matrices in the strength of LLER's; even in a pairwise analysis using only those studies where both matrices were measured. **G** and **P** matrices are expected to differ markedly in the absolute amount of variance they contain (in our sample of matrices, for example; the mean eigenvalue for **P** matrices was more than an order of magnitude larger than the mean **G** eigenvalue). The structure of the extra variance in **P**; from the environment, and non-additive genetic effects; may thus determine whether the non-additive-genetic variance in **P** acts to preserve or mask the smaller signal of **G**, depending on the degree to which the structures of **G** and **P** are aligned.

There are a number of reasons why this might be so, and the first is a straightforward one: in any study that estimates both matrices from the same system, the sample sizes from which **G** and **P** are calculated will inevitably differ. The sample size for **P** ($n_{\mathbf{P}}$) will be the number of individuals measured, whereas $n_{\mathbf{G}}$ will be the number of *families* in the study – estimates for **P** are therefore likely to be more precise than those for the corresponding **G**. Secondly, the difficulty of running a quantitative genetic breeding design in order to estimate **G** means that the majority of such work is done in a laboratory setting, with external sources of variance explicitly controlled. If environmental variance were to be spread differently among dimensions from genetic variance (i.e. the **E** matrix has a different structure to **G**) then this signal might not be detected when good experimental controls will, by definition, reduce the magnitude of **E**. However, though the influence of **G** may be misaligned to that of selection in the short to medium term (McGuigan et al., 2005; Schluter, 1996), in the long term we expect selection to influence the shape of **G**. Simulation work suggests that, over a large number of generations, **G** will come to be aligned with the dimensions of the adaptive landscape (Arnold et al., 2008). In this situation, with **G** and **E** aligned, we would be surprised to find that **G** and **P** did not share LLER's – though they may differ greatly in magnitude.

The lack of detectable difference between taxa may seem unsurprising, but the reader should note that the classification criteria for trait type were necessarily slightly different between animals and plants. Though there is nothing controversial about applying the 'morphology' and 'life-history' categories to plant

matrices, the assumption that flower morphology could be grouped with the sexually selected traits of animals was more uncertain.

We note that diversity index values are generally low (grand mean 0.62), particularly for life-history (mean 0.41) and sexually selected traits (mean 0.49), as opposed to morphology (mean 0.71). Where LLER's have been studied empirically, the evidence suggests that they can influence the direction of phenotypic evolution as predicted (McGuigan et al., 2005; Schluter, 1996), and our results suggest the potential for such bias may be present in many systems. This underlines Schluter's (2000) conclusion that we should be wary of using $V_A$ and $h^2$ as measures of evolvability, since even heritable traits with additive genetic variance present may not be able to respond to selection due to the alignment of that trait's covariance with other traits. The generality of our finding that LLER's are common and particularly strong in life-history and sexually selected traits is another reason to heed the advice of Blows (2007) and Houle (2007) and acknowledge the inherently multivariate nature of adaptation.

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# CHAPTER 3: Stability of the Phenotypic Variance-Covariance Matrix (P) over Evolutionary Time and Geographic Space

## *3.1 ABSTRACT*

One of the assumptions of classical quantitative genetic theory is the stability of phenotypic (co)variance matrices (**P**). More recent work strongly suggests that these matrices are temporally plastic, spatially variable and evolvable, but more empirical data on **P** matrix evolution and plasticity is needed. We calculated **P** matrices for the structure of the complex advertisement call of six divergent allopatric populations of the Australian black field cricket – *Teleogryllus commodus*. We then calculated a second set of matrices for the same populations after three generations of common-garden rearing. Inter-population variation of **P** was found, but the variation was largely in terms of means and variances, with the covariance structure of **P** changing little. Inter-generation variation in **P** showed a similar pattern of difference, but with a conserved eigenstructure. We used multiple statistical tools for matrix comparisons and where therefore able to make a direct evaluation of their relative merits; information that has previously been scarce elsewhere.

**Keywords**; *Teleogryllus commodus*; Advertisement call; **P** matrix; Phenotypic plasticity; Phenotypic integration; Common garden; Matrix comparison; Common principal components

## *3.2 INTRODUCTION*

The phenotypic variance/covariance matrix (**P**) summarises the variances of, and covariances among, a suite of traits. The covariances in the **P** matrix arise both from developmental, environmental and genetic effects (pleiotropy/linkage disequilibrium), or the interaction of these factors during development. Phenotypic integration as represented by **P** therefore comprises the overall structure of genetic, environmental and interaction effects that connect the various components parts of a complex trait. The **P** matrix is thus an integral part of the description of that complex trait (Roff & Mousseau, 2005).

The level of integration in complex traits has implications for the understanding of phenotypic evolution, both interpretive and predictive. In line with quantitative genetic theory, stabilising selection is predicted to lead complex traits to evolve genetic correlations that are consistent with the structure of developmental/functional relationships between component traits (Lande, 1980; Lande, 1979; Lande, 1984). A **P** matrix showing strong integration, with high covariances that show little environmental plasticity, can therefore be interpreted as evidence for a history of stabilising selection on the complex trait in question (Armbruster & Schwaegerle, 1996; Schluter, 2000). In addition, an understanding of the covariance structure of a complex trait enables predictions about the magnitude and direction of that trait's response to future selection (Pigliucci, 2003; Pigliucci & Preston, 2004; Schluter, 1996). It is currently unclear, however, whether integration should be expected to act as a constraint or a facilitator of phenotypic evolution (Pigliucci, 2003).

The relationship between integration and selection is modelled by the multivariate breeders' equation $\Delta\bar{z} = \mathbf{GP^{-1}S}$, where $\Delta\bar{z}$ is the vector of mean responses, **G** is the genetic variance/covariance matrix (**G** matrix), **P** is the **P** matrix, and **S** is the vector of selection differentials (Lande, 1979). This allows either the estimation of the future response to known selection, or the back-calculation of past selection if **P** and **G** are known. In both cases, the predictive power of the multivariate breeders equation relies on the assumption that **G** and **P** matrices are stable over the timescale in question. As there is now a considerable body of evidence that these matrices evolve (Agrawal et al., 2001; Jones et al., 2004; Phillips & McGuigan,

f2006; Phillips et al., 2001; Roff, 2000; Roff & Mousseau, 1999), it is important for empiricists to quantify the scale over which the **P** matrix varies – either by measuring **P** at time intervals or, more tractably, measuring **P** between divergent allopatric populations.

Most organisms encounter some level of heterogeneity in their environment, and a frequent solution to the problems this poses is phenotypic plasticity – a plastic phenotype allows the organism to express each trait in the way best suited to the current environmental conditions (reviewed in DeWitt & Scheiner, 2004; Stearns, 1989). The components of complex traits may also exhibit differential expression in alternate environments. In cases where the complex is weakly integrated, the component traits will be free to vary more independently of each other, and we would expect the structure of trait covariance to change. In cases where integration is strong, though the component traits may still vary in terms of means and variances in response to environmental change, we would expect that the covariance structure between those traits would be maintained. Thus plasticity and integration may co-occur, but when integration is strong we expect that the differences between alternate phenotypes would be less likely to perturb the covariance struture of the complex (Pigliucci & Preston, 2004).

Changes in the covariance structure of trait complexes are predicted to have evolutionary consequences (Lande & S. Arnold, 1983), and the role of phenotypic plasticity in evolution has received much attention of late (West-Eberhard, 2003). However, most studies that have addressed patterns of phenotypic integration have done so in a single environment (Pigliucci & Preston, 2004). Of those studies that have tackled the issue of the plasticity of phenotypic integration, most have focussed on plants, and have found very different levels of integration. In some cases authors have found traits to be highly plastic (Nicotra et al., 2007; Pigliucci et al., 1999; Tomkins et al., 2005) with correspondingly low integration levels. Elsewhere, studies have found suites of traits that display much tighter integration in the face of presumed local adaptation pressure (Game & Caley, 2006) or, intriguingly, that show a plastic response to some stimuli, but remain integrated in spite of others (Pigliucci & Hayden, 2001).

The complex trait we shall focus on is the advertisement call of *Teleogryllus commodus*; a field cricket with a wide distribution across the southern half of Australia. Field cricket advertisement calls are a species-specific signal and are know to be important in species recognition in *Teleogryllus* (Honda-Sumi, 2005a; Simmons, 2004). Females also use advertisement call cues in mate choice, and the call traits of males are known to experience sexual selection (Bentsen et al., 2006; Brooks et al., 2005). As long as there is additive genetic variation available to selection, these conditions may be expected to promote signal-receiver coevolution. This prediction is supported by a number of studies that have found signal-receiver coupling maintained through environmental change across a range of cricket genera (Grace & Shaw, 2004; Olvido & Mousseau, 1995; Pires & Hoy, 1992; Walker, 2000).

As advertisement call must provide both a species recognition signal, and information on male quality, we have reason to expect that the various traits of the advertisement call should display phenotypic covariance, i.e.; that call structure should be functionally integrated. The call components included in the **P** matrix were chosen because they are known to be under sexual selection in this species (Bentsen et al., 2006; Brooks et al., 2005) and are relatively simple to quantify.

In this study I compare **P** both among populations, and within populations between field and common garden. As there are currently a number of matrix-comparison tools to be found in the literature, we shall make multiple comparisons using all the most commonly used tools (discussed in section 3.3). Since **P** is a product of **G** and **E**, a finding of variation in **P** among populations that remains in common garden (i.e. when the contribution of **E** is controlled) would indicate that these populations have diverged genetically (i.e. divergence in **G**). Variation in call parameters within populations between wild-caught and lab-reared generations would indicate environmental plasticity of the call phenotype. Such plasticity may or may not extend to the covariance structure of **P**.or within populations between field and common garden would suggest that **E** has little effect on **P**. In other words, such a result would be evidence for limited plasticity in the structure of the advertisement call, implying strong integration.

By contrast, if there were substantial variation in **P**, this would imply that **P** is able to respond plastically to change in the environment. Furthermore, the extent of variation in **P** could be informative about the relative contributions of **G** and **E** to **P**. If **P** differs markedly between generations, but not among populations in common garden, then a strong contribution of **E** is suggested. If **P** were to differ among populations in common garden, however, then this would suggest that the difference is due to the contribution of **G**.

## *3.3 METHODS*

### Collection and Common-Garden rearing of cricket populations

Approximately 400 adult *Teleogryllus commodus* were collected from each of six populations spanning the southern distribution of this species in Australia (Appendix II). Crickets were collected between January and April in 2004 and air-freighted back to the laboratory at the University of New South Wales. In the laboratory, each population was established in a separate 80L culture container and provided with food (Friskies Go-Cat Senior) and water *ad libitum*, an abundance of egg cartons for shelter and maintained in a constant temperature room set to 28° ± 1°C with a 14L:10D light regime. A hazard sample sample of males from each population was recorded within 3 nights of being established in the laboratory. As this sample of males had completed their development and reached sexual maturity in the field, we refer to these as *field* males.

We maintained our populations under common environmental conditions for 3 generations prior to measuring calls from each population for a second time. In each generation, populations were maintained by haphazardly pairing 100 males each with a virgin female. Each pair was placed in an individual plastic container (5 cm x 5 cm x 5 cm) for 3 days to mate. Males were then removed and females provided with food, water and a small Petri-dish of moist cotton wool for a week to lay eggs. At hatching, approximately 25 nymphs were haphazardly selected from each mating pair and reared in a small plastic container (15 x 10 x 15cm) until they reached 4$^{th}$ instar. Nymphs were then sexed and 5 crickets of each sex per container were haphazardly selected to contribute to the next generation. These crickets were reared to eclosion in a single, large plastic container (80L) for each

population. Containers were checked daily and adults removed and maintained in sex-specific containers (80L) until sexually mature. When sexually mature, these crickets were used to propagate the next generation following the protocol outlined above. As this sample of males has been maintained in the laboratory for 3 generations, we refer to them as *laboratory* males.

### Call Recordings

After 3 generations of common garden rearing, 50 males from each population were isolated at eclosion, established in individual plastic containers (5 cm x 5cm x 5 cm) and provided with food, water and a piece of egg carton for shelter. At 10 days of age, we recorded the advertisement call of all males that were calling. Each male was housed in an individual recording chamber (5 cm x 5 cm x 5 cm) with a condenser microphone (C1163, Dick Smith) mounted in the lid. To reduce disturbance, each microphone was connected to a 3m length of shielded acoustic cable with its terminal end located outside of the recording room. This end was then connected to a 9-volt amplifier to power the microphone and a 2 minute sample of each call was recorded with a Sony Professional Walkman (WM-D6C) on high quality chrome tapes (BASF CEII, Germany). Calls were recorded opportunistically between 21:00 and 03:00 each night by scanning recording chambers hourly. The calls of all males were recorded at 28 ± 1°C during the dark phase of a 14L:10D light regime. The morning after recording, the pronotum width and body weight of each cricket was measured using an eyepiece graticule fitted on a binocular dissecting microscope (Leica, MZ5) and an analytic balance (Mettler-Toledo, 345G), respectively.

### Call Analysis

We digitized calls from tape and used Canary software (version 1.2.4, Bioacoustics Research Program, Cornell University) to measure the following properties of six haphazardly selected calls per male: the number of pulses in the chirp (CPN, chirp pulse number), the interval between pulses in the chirp (CIPD, chirp inter-pulse duration), the number of trills in the call (TN, trill number), the interval between the last trill pulse of one call and the first chirp pulse of the next call (ICD, inter-call duration) and the dominant frequency of the call (DF, dominant frequency: see

Appendix III for an illustration of call traits.) We used the mean of these call properties for each male in all subsequent analyses. In total, we measured the calls of 142 males (WA = 20, SA = 25, TAS = 17, ACT = 25, KL= 25, SL = 30) collected from the field and 173 males (WA = 25, SA = 35, TAS = 32, ACT = 26, KL = 28, SL = 27) after 3 generations of common garden rearing in the laboratory.

### Statistical Analysis

To determine if the structure of the advertisement call had genetically diverged across our populations, we analysed our call data using a multivariate analysis of covariance (MANCOVA)**.** We employed a full model including population (i.e. WA, SA, TAS, ACT, KL and SL), time (i.e. field versus laboratory males) and their interaction as fixed effects and male pronotum width as a covariate. Male size (measured as pronotum width) was included as a covariate in our model because it is known to influence the structure of the advertisement call in other field cricket species (Simmons, 1995; Simmons & Zuk, 1992) and because male size differed significantly across populations ($F_{5,315} = 4.543$, $P = 0.001$), with time ($F_{1,315} = 197.708$, $P = 0.0001$) and there was a significant interaction between population and time ($F_{5,315} = 14.517$, $P = 0.0001$). As our aim was to remove the effect of male size from the analysis of call structure, rather than to examine the specifics of how male size interacts with population and time in determining call structure, we did not examine all possible interactions between population, time and male size. All call structure parameters, as well as male size, were log transformed prior to analysis.

We calculated phenotypic variances and covariances for each of our 5 call parameters (using the 'VAR' and 'COVAR' functions of Microsoft Excel) and used these values to construct two **P** matrices for each population: one for field males and the other for laboratory males. There is currently no single best method of comparing matrices, with each available technique testing slightly different aspects of the matrices being compared (Blows & Higgie, 2003; Houle et al., 2002; Mezey & Houle, 2003; Roff, 2002; Steppan et al., 2002). We therefore used a number of complementary methods to test whether **P** differed across populations and with time: the Mantel test, the Jackknife-MANOVA approach (Roff, 2002), Common Principal Component (CPC) analysis using the Flury hierarchy (Phillips &

Arnold, 1999) and Geometric subspace analysis (Krzanowski, 1979). By using multiple methods of comparing matrices, our aim is to reach the most harmonious interpretation on the stability of **P**, as well as to directly facilitate comparison with other studies in the literature (Game and Caley, 2006).

The Mantel test is a randomisation test used to examine the correlation between two matrices. The elements of one matrix are iteratively randomised and the Pearson product-moment correlation calculated (Sokal & Rohlf, 1995). Pairwise Mantel tests were made within populations between generations, and within generations between populations, using the 'PopTools' add-in for Microsoft Excel (Hood 2009). In all comparisons, we used 10,000 iterations.

Roff's (2002) Jackknife-MANOVA approach uses resampling to generate a column of pseudovalues for each matrix element of each matrix. These columns of pseudovalues can then be analysed using a standard multivariate analysis of variance. Unlike the Mantel test, the Jackknife-MANOVA approach allows any differences between matrices to be partitioned with respect to population, generation and their interaction. We used PopTools to calculate our Jackknife pseudo-values and SPSS (version 15) to conduct the MANOVA analysis.

Whereas the Mantel test merely quantifies the similarity of two matrices, and the Jackknife-MANOVA approach allows us to partition the variance between them by variable effect, the Common Principal Components analysis sequentially tests for differences (or similarity) between matrices in a hierarchical manner (Phillips & Arnold, 1999). Matrices can have completely unrelated structures (unrelated model), they may share one or more (but not all) principal components (partial common principal components (PCPC) model), they may share all principal components and differ only in their eigenvalues (full common principal components (CPC) model), they may have identical principal components and eigenvalues which differ by a proportional constant (proportional model), or they may be identical (equal model). Phillips & Arnold (1999) discuss three methods for determining the position of a relationship on this hierarchy the 'step-up', 'jump-up' and 'model-building' approaches. The step-up approach tests each model against its next lowest neighbour, and a move up the hierarchy is made only if the test is

non-significant. The jump-up approach tests each level against an unrelated structure until a significant result is achieved and the model building approach determines the position on the hierarchy by the model that gives the best fit to the data, evaluated by the Akaike Information Criterion (AIC). We used the 'CPC' program (Phillips, 1997) to perform analyses using all three approaches.

A potential limitation of the CPC program is that it includes all eigenvectors of the matrices being compared, irrespective of the relative size of their eigenvalues. This means that any similarity detected could be due to principal components which have a small eigenvalue in one matrix and a large eigenvalue in the other; potentially making the results of CPC analysis overly conservative (Blows et al., 2004). We therefore compared the eigenstructure of the **P** matrices geometrically (Krzanowski, 1979). Krzanowski's method compares *k*-dimensional subspaces, where *k* is the number of eigenvectors being compared in each sample, by calculating the angles between the most similar pairs of orthogonal components. To be meaningful, not all of the *n* vectors of *n* x *n* matrices can be included in the analysis. This is because including more than half of the *n* vectors will constrain the analysis to recover common dimensions (i.e. angles of 0°) and if all *n* vectors are included in the analysis, the two subspaces will be identical (Blows et al., 2004). In our analyses we include the first 2 eigenvectors in the subsets as together they explain greater than 99% of the variation in the structure of the advertisement call. If the *k*-dimensional subsets of matrices 1 and 2 are represented by **A** and **B** respectively, we can calculate a new matrix as $\mathbf{T} = \mathbf{A}^{\mathsf{T}}\mathbf{B}\mathbf{B}^{\mathsf{T}}\mathbf{A}$ (Krzanowski designates this as **S**, but I shall use **T** to avoid confusion with Lande's **S**). The minimum angle between any pair of orthogonal axes in matrices **A** and **B** can then be calculated as $\cos^{-1}\sqrt{\lambda_1}$ where $\lambda_1$ is the largest eigenvalue of **T**. Furthermore, the sum of the eigenvalues of **T** is equal to the sum of squares of the cosines of the angles between the two sets of orthogonal axes. This sum will lie in the range of 0 to *k*, as each of the eigenvalues of **T** have values between 0 and 1, and this equates to an angle between 0° and 90°. A sum close to 0 indicates that the two subspaces are dissimilar and are approaching orthogonal (90°), while a sum close to *k* indicates that the two matrices being compared share the same orientation.

## *3.4 RESULTS*

A MANOVA of the call trait data showed significant differences both between the populations (Wilks' $\lambda = 0.256$, $F_{25,1112} = 174$, $P < 0.001$), and between generations (Wilks' $\lambda = 0.479$, $F_{5,299} = 9.76$, $P < 0.001$). There was also a significant interaction effect of population x generation (Wilks' $\lambda = 0.64$, $F_{25,1112} = 5.62$, $P < 0.001$, Table 3.1).

### Matrix Comparison

The results of the pairwise Mantel tests were largely non-significant; only the SA and WA populations showed significant differences between the field and lab **P** matrices ($P = 0.016$ and 0.033 respectively). Between populations in the field, only the KL-SL, KL-WA, SA-TAS and SL-WA comparisons showed significant differences ($P = 0.012$, 0.034, 0.034 and 0.036 respectively). Between populations in the lab the only comparison to show a significant differences was ACT-TAS ($P = 0.020$, Table 3.2).

Matrix comparison by MANOVA of Jackknife pseudovalues for the matrix elements showed significant difference between populations (Wilks' $\lambda < 0.001$, $F_{75,1388} = 765$, $P < 0.001$) and between generations (Wilks' $\lambda = 0.007$, $F_{15,289} = 2672$, $P < 0.001$). There was also a significant interaction of population x generation (Wilks' $\lambda < 0.001$, $F_{75,1388} = 937$, $P < 0.001$, Table 3.3). Tests of between subject effects show significant differences in the pseudovalues of all fifteen matrix elements (five variances and ten covariances) taken individually; due to population ($P < 0.001$ in all cases), due to generation ($P \leq 0.01$ in all cases) and due to the interaction of population and generation ($P < 0.001$ in all cases).

**Table 3.1:** Results of a MANOVA on the structure of the advertisement call of *T. commodus* among populations and between generations. Significant *p*-values are in italics.

| MANOVA | | Wilks' Lambda | F | Degrees of freedom | | *P* -value |
|---|---|---|---|---|---|---|
| | | | | Hypothesis | Error | |
| | Population | 0.256 | 174 | 25 | 1112 | *<0.001* |
| | Generation | 0.479 | 9.76 | 5 | 299 | *<0.001* |
| | Population x Generation | 0.643 | 5.62 | 25 | 1112 | *<0.001* |

post-hoc ANOVAs

| | Population Means (± SE) | | | | | | Degrees of freedom | | F | *P* -value |
|---|---|---|---|---|---|---|---|---|---|---|
| Traits | ACT | KL | SA | SL | TAS | WA | Hypothesis | Error | | |
| field-caught generation | | | | | | | | | | |
| CPN | 6.43 (0.30) | 6.73 ( 0.25) | 6.73 (0.26) | 6.02 (0.20) | 6.95 (0.21) | 8.15 (0.48) | 5 | 136 | 5.92 | *<0.001* |
| TN | 2.59 (0.21) | 2.84 (0.20) | 2.43 (0.20) | 3.34 (0.22) | 2.70 (0.18) | 2.63 (0.20) | 5 | 136 | 2.00 | 0.08 |
| ICD | 209 (18.3) | 246 (18.4) | 305 (23.2) | 215 (33.1) | 263 (16.2) | 298 (22.5) | 5 | 136 | 6.55 | *<0.001* |
| CIPD | 26.7 (0.94) | 30.3 (1.19) | 29.3 (0.71) | 25.3 (0.83) | 32.3 (1.43) | 37.4 (0.87) | 5 | 136 | 17.6 | *<0.001* |
| DF | 3.87 (0.03) | 3.78 (0.03) | 3.66 (0.05) | 3.40 (0.04) | 3.49 (0.04) | 3.89 (0.08) | 5 | 136 | 16.0 | *<0.001* |
| lab-reared generation | | | | | | | | | | |
| CPN | 5.59 (0.14) | 5.60 (0.14) | 5.79 (0.15) | 5.77 (0.12) | 5.79 (0.12) | 5.56 (0.14) | 5 | 167 | 1.57 | 0.17 |
| TN | 2.96 (0.18) | 2.93 (0.18) | 2.06 (0.23) | 2.41 (0.17) | 2.42 (0.16) | 2.64 (0.24) | 5 | 167 | 2.52 | *0.03* |
| ICD | 157 (5.13) | 157 (5.13) | 136 (4.34) | 152 (4.19) | 140 (4.00) | 128 (5.32) | 5 | 167 | 9.73 | *<0.001* |
| CIPD | 19.0 (0.75) | 18.9 (0.75) | 18.1 (0.61) | 18.1 (0.38) | 18.1 (0.49) | 18.6 (0.57) | 5 | 167 | 4.14 | *0.001* |
| DF | 4.06 (0.02) | 4.06 (0.02) | 4.15 (0.03) | 3.99 (0.03) | 4.08 (0.03) | 4.15 (0.03) | 5 | 167 | 6.85 | *<0.001* |

Units: CPN and TN are counts, ICD and CIPD are measurements in milliseconds and DF is measured in kilohertz. See text for population abbreviations.

Comparing our **P** matrices using CPC analysis yielded results from close to the top of Flury's hierarchy using all three approaches. When comparing matrices with 5 eigenvectors the hierarchy has 9 steps from 'unrelated' to 'equality', and all the results of all comparisons were of the top three ranks – the rank assigned differed in a number of cases depending on which of the three approaches was used. When the 6 populations' matrices for the field generation were compared, both the step-up and model building approaches returned a result of common principal components (CPC), but the jump-up result was proportionality (Table 3.4). Comparing the 6 matrices for the lab generation also gave a common principal components result using the step-up approach, but indicated proportionality using the model building and jump-up approaches (Table 3.4). Within-population CPC comparisons of field vs. lab matrices were also somewhat inconsistent between approaches. The step-up and model building approaches both returned common principal components results for all populations except KL, where the step-up approach returned a result of equality. The jump-up approach returned results of proportionality for KL, SA, TAS and WA populations, common principal components for the ACT population and a single common principal component for the SL population (Table 3.4).

**Table 3.2:** Pairwise comparisons between populations using Mantel test for correlation (10,000 iterations). 2-tailed *P* values: field matrices above and lab matrices below the diagonal (**bold** if significant).

| | Pairwise population comparisons | | | | | | Field-Lab comparison within pop. |
|---|---|---|---|---|---|---|---|
| | **ACT** | **KL** | **SA** | **SL** | **TAS** | **WA** | |
| **ACT** | | 0.084 | 0.162 | 0.082 | 0.506 | 0.233 | 0.116 |
| **KL** | 0.308 | | 0.636 | **0.012** | 0.167 | **0.034** | 0.777 |
| **SA** | 0.592 | 0.611 | | 0.382 | **0.034** | 0.164 | **0.016** |
| **SL** | 0.267 | 0.236 | 0.084 | | 0.160 | **0.036** | 0.313 |
| **TAS** | **0.020** | 0.337 | 0.660 | 0.099 | | 0.198 | 0.136 |
| **WA** | 0.547 | 0.193 | 0.219 | 0.133 | 0.359 | | **0.033** |

**Table 3.3:** Matrix comparison by MANOVA of Jackknife pseudovalues calculated for all fifteen matrix elements (five variances and ten covariances).

| | Wilks' Lambda | Approx. F | Degrees of freedom | | *P* -value |
|---|---|---|---|---|---|
| | | | Hypothesis | Error | |
| Population | 1.59E-08 | 767.9 | 75 | 1388 | <0.001 |
| Generation | 2492.7 | 2493 | 15 | 289 | <0.001 |
| Population x Generation | 5.35E-09 | 698.7 | 75 | 1388 | <0.001 |

Although CPC analysis is currently the most popular method of matrix comparison (McGuigan, 2006), it is wise to keep in mind that it was not developed specifically for biological applications. Houle et al. (2002) systematically tested the performance of CPC analysis and found that any similarity of structure that is detected may not be due to the same underlying causal factors. That said, we are confident in accepting the general finding of common principal components from our CPC analyses because the pattern of eigenvector loadings is similar across all matrices, and particularly so for the first two eigenvectors; which account for >99% of variation. One situation in which biological interpretation of CPC results does appear tenable is in the case of finding common PC's: Mezey & Houle's (2003) modelling study suggested that (co)variance matrices should be expected to show common PC's only in the case of there being a shared modular organisation.

Geometric matrix comparisons between generations within population showed that the eigenvectors of the field and lab matrices lie in similar subspaces (Table 3.5) The angle between the closest vectors was < 0.6° for all populations, and the sum of **T** eigenvalues was > 1.9 for all populations ($k$ = 2). Pairwise comparisons between populations for the field generation gave an angle of < 0.3° in all cases, and the sum of **T** eigenvalues was > 1.9 in all cases ($k$ = 2). Similarly, pairwise comparisons for the lab generation gave an angle of < 0.4° in all cases, and the sum of **T** eigenvalues was > 1.9 in all cases ($k$ = 2).

**Table 3.4:** The results of matrix comparison by CPC analysis using Flury's hierarchy. The upper part of the table shows the results for among-population comparisons within generations, the lower part for within-population comparisons between generations.

| | *Approach used* | **Step-up** | **Model building** | **Jump-up** |
|---|---|---|---|---|
| *Pop. x Pop.* | **Field Generation** | CPC | CPC | Proportional |
| | **Lab Generation** | CPC | Proportional | Proportional |
| *Field x Lab* | **ACT** | CPC | CPC | CPC |
| | **KL** | Equality | CPC | Proportional |
| | **SA** | CPC | CPC | Proportional |
| | **SL** | CPC | CPC | CPC(1)* |
| | **TAS** | CPC | CPC | Proportional |
| | **WA** | CPC | CPC | Proportional |

* CPC(*n* ) indicates a result of *n* common principal components

**Table 3.5:** The results of geometric matrix comparisons. Values in *italics* are angles in degrees (between 0° and 90°), and values in **bold** represent the sum of **T** matrix eigenvalues (between a minimum of 0 and a maximum of 2).

| Field x Lab within population | | **ACT** | **KL** | **SA** | **SL** | **TAS** | **WA** |
|---|---|---|---|---|---|---|---|
| Sum of **T** eigenvalues | | **1.992** | **1.993** | **1.999** | **1.999** | **1.998** | **1.975** |
| *Closest eigenvector angle* | | *0.106* | *0.056* | *0.340* | *0.574* | *0.379* | *0.385* |
| Pairwise by population | | | | | | | |
| Sum of **T** eigenvalues above the diagonal, closest eigenvector angles below. | | | | | | | |
| | **ACT** | | **1.995** | **1.990** | **1.999** | **1.997** | **1.986** |
| | **KL** | *0.081* | | **1.999** | **1.998** | **2.000** | **1.965** |
| Field | **SA** | *0.158* | *0.058* | | **1.994** | **1.998** | **1.955** |
| | **SL** | *0.059* | *0.103* | *0.044* | | **1.999** | **1.978** |
| | **TAS** | *0.218* | *0.106* | *0.066* | *0.019* | | **1.971** |
| | **WA** | *0.226* | *0.147* | *0.071* | *0.096* | *0.026* | |
| | **ACT** | | **1.999** | **1.996** | **1.996** | **1.997** | **1.997** |
| Lab | **KL** | *0.426* | | **1.993** | **1.995** | **1.998** | **1.996** |
| | **SA** | *0.209* | *0.064* | | **1.999** | **1.996** | **1.999** |
| | **SL** | *0.185* | *0.255* | *0.334* | | **1.998** | **2.000** |
| | **TAS** | *0.173* | *0.295* | *0.265* | *0.105* | | **1.999** |
| | **WA** | *0.194* | *0.140* | *0.106* | *0.372* | *0.069* | |

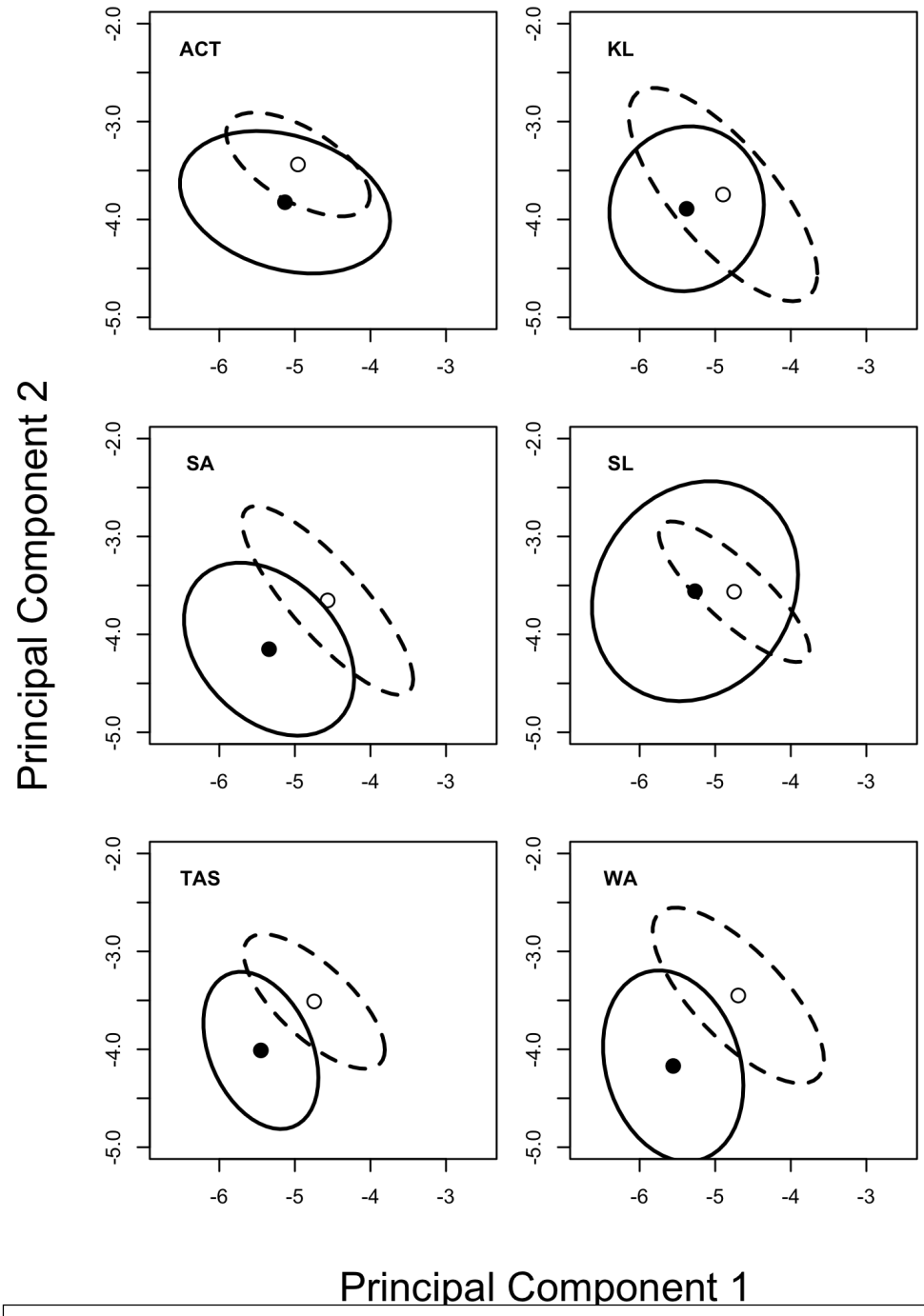


**Figure 3.1**: Means and 95% confidence ellipses for each population and each generation for the first 2 principal components of call structure. The filled circles and solid lines represent the wild-caught generation, and the open circles and dashed lines represent the lab-reared generation. Since these populations appear to share principal components, the axes are the same in each case (Table 4). Note the difference in means, but the conserved shape of the probability distributions.

## *3.5 DISCUSSION*

From our initial analysis by MANOVA, the five call traits are shown to differ between our study populations, and also between generations, in terms of means and variances (Table 3.1). The finding that significant differences remained after common-garden breeding indicates that the divergence of our populations' call traits has a genetic basis. This interpretation is supported by the significant interaction between population and generation effects, which shows that our six populations responded differently to the common-garden conditions of the lab. This is not altogether surprising as geographic variation in the call structure of Gryllids has been demonstrated before (Simmons et al., 2001) and these structural components of *T. commodus* call are known to have a genetic basis (Hunt et al., 2007).

The relationship between our covariance matrices is somewhat more complicated to interpret. Most of our **P** matrices are quite highly correlated with each other – the Mantel test works by comparing correlation pseudovalues, and of the 36 pairwise tests performed, only 7 found significant differences (Table 3.2). However, the Jackknife-MANOVA comparison shows that there are significant levels of variation both between and among populations. The results of our common principal components analysis, though they are somewhat inconsistent between approaches, show remarkable similarity of **P** between populations and between generations (Table 3.4). The most inconsistent result was for the between generations comparison for the SL population, where the three approaches assigned different ranks that were 4 steps apart on the hierarchy. The between generations comparison for the KL population was inconsistent by 2 ranks, but no other disagreement between the approaches involved ranks more than 1 step apart on the hierarchy. We can then be reasonably confident that, though the matrices differ, their principal components largely do not. Our interpretation of these results is that among-population differences and between-generation differences are of a similar scale, and are mean/variance differences, with little or difference in the pattern of covariance (Figure 3.1).

This interpretation is supported by the geometric subspace comparisons. In all cases the angles between closest vectors are <0.07°, and the sums of **T** eigenvalues

are >1.97 (of a possible *k*=2); indicating that the subspaces spanned by the eigenvectors of all our **P** matrices are very closely aligned. We thus have a clear indication of variance difference from our Mantel and MANOVA comparisons, and evidence for eigenstructure similarity from geometric and common principal component comparisons; demonstrating the value of employing multiple tools when comparing variance-covariance matrices (Game & Caley, 2006).

From the low level of inter-population divergence in the field, and the similarly low level of inter-generation change between field-caught and common garden reared males, it appears that there is strong integration among the component traits of the advertisement call. The component traits themselves are plastic when considered singly, but their integration is strong enough to be conserved, at least in the face of environmental differences on the scale of those between field and lab environments. Plasticity in the face of a heterogeneous environment is an extremely common adaptation (DeWitt & Scheiner, 2004; West-Eberhard, 2003), and yet one of the most noticeable properties of most metazoans is the cohesion and integration of their organs and appendages. Selection can be expected to favour integration insomuch as it contributes to the development of robust and functionally resilient body-plans (Pigliucci, 2003), but this does not mean that the processes of plasticity and integration need be thought of as mutually exclusive (Phillips & McGuigan, 2006).

In those cases where empiricists have assessed the stability of integration by comparing covariance matrices between populations they have often found intermediate levels of difference (Brodie, 1993; Carr & Fenster, 1994; Cowley & Atchley, 1990; Jernigan et al., 1994; Platenkamp & Shaw, 1992; Shaw & Billington, 1991) or more recently, detected conserved eigenstructure (Game & Caley, 2006; Roff et al., 2004) as we find here for *T. commodus* calls (but see also Badyaev & Hill, 2000). Our finding of phenotypic integration in these populations fits well with theoretical predictions; it is known that the call parameters we measured as component traits are under multivariate stabilising selection (Brooks et al., 2005), and this type of selection has been predicted to promote the evolution of integration (Cheverud, 1984). Our widely spaced sample populations seem to be diverging by local adaptation – at least in terms of means and variances – and

there is evidence from modelling work (Wolf et al., 2001) that some conditions can enable trait variances to evolve independently of covariances.

As mentioned previously, knowledge of **P** and **G** is central to quantitative genetic predictions, but these predictions are based on the assumption that the matrices are conserved over the temporal or spatial distance to which those predictions relate (Steppan et al., 2002). Complex traits that are integrated between environments, such as the advertisement call addressed here, have been thought of as sources of evolutionary constraint (Arnold, 1992; Wagner et al., 1997). However, the relationship between integration and constraint is more subtle, as elucidated by Schluter (1996; 2000); integration can be thought of as an uneven distribution of variance available to selection among the eigenvectors that describe the covariance matrix. When integration is strong, what variance there is will lie disproportionately along the first (or first few) eigenvectors. The maximum response to selection will result when selection is aligned with one of these vectors. If selection acts in a different direction in multivariate space, then the eigenvector(s) where the majority of the variance lies can be expected to constrain the rate, and potentially bias the direction of the response to that selection (Schluter, 1996). Thus, to understand the influence of integration on phenotypic evolution, we must take multivariate selection into account, in addition to **P** and **G** (Lande & Arnold, 1983).

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# CHAPTER 4: The Stability of the Phenotypic Variance-Covariance Matrix under Nutritional Stress

## *4.1 ABSTRACT*

One of the assumptions of classical quantitative genetic theory is the stability of phenotypic (co)variance matrices (**P** matrices). Recent empirical work, however, strongly suggests that these matrices are temporally plastic, spatially variable and evolvable, but more empirical data are needed. This study used a diet treatment to induce a plastic response in the **P** matrix. We reared 6 populations of black field crickets (*Teleogryllus commodus*) on 2 diets; one nutrient-rich and one nutrient-poor. When adult males eclosed, we recorded their advertisement calls. We then calculated **P** matrices for 5 call traits known to be subject to sexual selection. Inter-population variation of **P** was found, but the variation was largely in terms of means and variances, with the covariance structure of **P** varying little. Variation between levels of nutritional stress was associated with a similar pattern of small differences in **P**, but with a conserved covariance structure. Given the lack of a single sufficient tool for matrix comparisons, we used multiple statistical approaches with broadly consistent results.

**Keywords**; **P** matrix; Advertisement call; Condition dependence; Phenotypic plasticity; Phenotypic integration; *Teleogryllus commodus*

## *4.2 INTRODUCTION*

The **P** matrix (or simply **P**) is a variance-covariance matrix calculated from phenotypic measurements of a suite of traits. As such, the values of **P** represent both additive and non-additive genetic (co)variance, in addition to environmental (co)variance. **P** therefore summarises the structure of integration between traits, by whatever mechanism that integration may arise. Readers with a background in quantitative genetics will be familiar with the **P** matrix from the multivariate extension of the breeders equation: $\Delta\bar{z} = \mathbf{G}\mathbf{P}^{-1}\mathbf{S}$, where $\Delta\bar{z}$ is the vector of change in trait means, **G** is the genetic variance-covariance matrix, **P** is the **P** matrix and **S** is the vector of selection differentials (Lande, 1979). This multivariate version of one of the fundamental equations of quantitative genetics is used both for predicting the response of traits to a known selective regime and for estimating selection vectors from the recent past after measuring **P** and **G**.

Use of the breeders' equation in this form is based on the assumption that **P** and **G** matrices will remain stable for the duration of the selective bout of interest. However, there is now a substantial body of work – both theoretical and empirical – to show that these matrices evolve, both in response to selection and by drift (Agrawal et al., 2001; de Oliveira et al., 2009; Eroukhmanoff et al., 2009; Jones et al., 2004; Phillips & McGuigan, 2006; Phillips et al., 2001; Roff, 2000; Roff, Mousseau, et al., 1999; Roff, Tucker, et al., 1999). There is, therefore a need to quantify the scale over which **P** may vary, either temporally or geographically.

Variation in the structure of **P** represents variation in the degree to which component traits form an integrated unit. The robustness of this relationship among individuals and during development has implications for the course of evolution due to the interaction of integration with selection. Consistent stabilising selection is predicted to favour the evolution of genetic correlations between traits that reflect their developmental or functional relationships (Cheverud, 1984; Cheverud, 1996a; Jones et al., 2004; Lande, 1979; Lande, 1980), and disruptive selection to disfavour the accumulation of such correlations. **P** is not only a product of selection however, as the more stable the structure of integration, the fewer trait combinations that are exposed to selection. Hence patterns of trait integration are built and modified by selection, but the functional, developmental

and genetic relationships underlying those patterns of integration may constrain or channel the traits' evolutionary trajectory (Pigliucci, 2003; Jernigan et al., 1994). This means that an understanding of the plasticity of **P** is required for prediction of the capacity for complex phenotypes to adapt to environmental changes (Olson & Miller, 1958; Pigliucci & Preston, 2004; Lande & Arnold, 1983; Schluter, 2000).

Some aspects of phenotype tend to be more plastic and responsive to change than others, with animal behaviour being at the extreme end of this scale (West-Eberhard, 2003). Sexually selected ornaments and signals are likewise expected to be expressed plastically, in order that they should represent the quality/health/condition of each male, but males are also expected to express their signal in the most exaggerated form possible, in order to appear as attractive as possible to females (Zahavi, 1975; Andersson, 1994). In terms of integration, there are conflicting predictions regarding sexually selected signals (Badyaev, 2004). In the first instance, in order that sexual signals may function as an honest summary of male quality and health, we might predict strong integration between signals and the traits that are advertised by those signals (Wedekind, 1992; Bischoff et al., 2009; Johnstone, 1995). In the second instance, when the expression of signals is selected to be exaggerated, we might predict that this should minimise the integration between sexual signals and other elements of the phenotype; allowing the signal to evolve with the minimum of correlational selection pressure on traits that are not sexually selected (Emlen & Nijhout, 2000). The strength and persistence of patterns of trait integration are predicted to bias the direction of evolution (Schluter, 1996; Schluter, 2000), and are therefore of interest to those who seek to predict or explain evolutionary trajectories. Given the conflicting pressures upon them, however, the integration of sexual signals is a topic that warrants more attention than it has so far received.

Adult male Australian field crickets (*Teleogryllus commodus*) spend much time (Hunt et al., 2004) and expend much effort (Kavanagh, 1987) stridulating to attract females. Males' calling effort (in terms of time spent calling) is known to be under positive directional sexual selection (Bentsen et al., 2006). The advertisement call itself is comparatively complex; beginning with a single chirp sequence, which is followed by a variable number of trill sequences (Bentley & Hoy 1972; Hill et al.

1972: see also Appendix II). Female preference for this call structure has also been studied, and females have been shown to be sensitive to both temporal (Pollack & Hoy, 1979) and spectral (Hennig & Weber, 1997) call properties. The selection imposed by these female preferences has been measured both in the lab (Brooks et al., 2005) and in the field (Bentsen et al., 2006), and in contrast to sexual selection measured on calling effort, the dominant form of selection on call structure was found to be multivariate stabilising. If persistent, this is a selection regime that would facilitate trait integration (Lande, 1980; Cheverud, 1984; McGlothlin et al., 2005).

Call structure traits are known to vary between disparate populations of *T. commodus*, and there is evidence from a previous common-garden study (Chapter 3 of this thesis) that these differences have a genetic basis. This study also showed significant intra-population differences in call traits between wild-caught and lab-reared generations, indicating a plastic response to rearing conditions. Moreover, despite these robust differences in the means and variances of structure traits, the covariances between traits are conserved between populations; suggesting that the pattern of call trait integration has remained stable despite population divergence (Chapter 3). Local adaptation appears to have been associated with changes in the genetics of call structure that are elaborative as opposed to innovative; *sensu* Endler et al. (2005).

A lack of matrix divergence is a result that has been found before, for example; by Arnold & Phillips (1999) in divergent grass snake populations, by Game & Caley (2006) in species of coral reef fishes and by de Oliveira et al. (2009) in New World monkeys. These studies have concentrated on divergence between populations/taxa. The relationship between integration and plasticity has also been investigated, most prominently in the morphology of *Arabidopsis* (Pigliucci, 2002; Pigliucci & Kolodynska, 2002b; Bossdorf & Pigliucci, 2009; Kolodynska & Pigliucci, 2003). In addition to differences between populations, these studies also examine patterns of phenotypic correlation under experimentally induced stress. Despite finding plastic responses associated with changes in soil moisture content (Pigliucci & Kolodynska, 2002), in light intensity (Pigliucci & Kolodynska, 2002b) and in wind speed (Bossdorf & Pigliucci, 2009), these studies found the patterns of

phenotypic correlation to be remarkably stable. This work has all been based on morphological traits however, and given the particular relevance of these issues to sexual signals (Badyaev, 2004: see above), there is a need for empirical evidence on the plasticity of sexual trait integration.

In light of the conservation of call trait covariance structure despite changes in individual call traits, the current study attempts to provoke a plastic response in the pattern of call trait integration using a controlled manipulation of rearing environment in the lab, and animals from laboratory stock populations maintained in common garden. This experiment owes its methodology to earlier work by Hunt et al. (2005; 2004), who manipulated condition in the Australian black field cricket, *Teleogryllus commodus*, by supplementing diets with protein. Here, we examine the effects of a simplified diet manipulation on the call structure of six divergent populations under common garden conditions.

## *4.3 METHODS*

### Establishment of Stock Populations

The populations used in this study are derived from collections made in February and March of 2007. Approximately 200 field-mated females were taken from each of six sites spanning the southern distribution of *T. commodus*. Populations from Western Australia, South Australia, Tasmania, Smith's Lakes and Kioloa (both in New South Wales), and the Australian Capital Territory are referred to subsequently as WA, SA, TAS, SL, KL and ACT respectively (See Appendix II for details). Stocks were maintained in a constant temperature room at 28° ± 1°C, with a 16:8 hours light:dark regime. Stock animals are kept in 100L ventilated plastic containers with ad lib water and food – 'Go-Cat senior' cat food pellets – and cardboard egg boxes to provide shelter. Our captive populations had been lab reared for 5 generations under common-garden conditions before the start of this experiment, with at least 100 haphazardly assigned breeding pairs per generation. Even in the absence of genetic differences among populations, maternal effects are known to induce adaptive plastic responses that can resemble local adaptation

(Agrawal et al. 1999), but after 5 generations of rearing under lab-standard conditions any differences in maternal effects resulting from differences in the habitat of collection ought to have been reduced to a negligible level.

### Experimental Design

Hatchling nymphs were collected from 6 captive populations and reared on 2 experimental diets. Experimental animals were collected on the day of hatching, and housed individually in small plastic containers (7 x 7 x 5.5cm), each being provided with a water source and a small cardboard shelter. Thereafter these animals were kept in the same constant temperature room as the stock populations; at 28° ± 1°C, with 16:8 hours light:dark. All the animals within each population were collected at the same time and assigned haphazardly to treatment in order that related hatchlings (sibs or half-sibs) were not grouped within treatment. Each animal's food and water were changed at weekly intervals and then, once the first experimental animals reached their final instar, all the boxes were checked daily for eclosion to record development time.

Within each population, 300 individuals were reared; 150 in each of the 2 treatment groups ($n$ = 1800 nymphs). The 'high-nutrient' diet group were fed the same cat food as the stock animals, whereas the 'low-nutrient' diet group were fed a 50:50 mixture (by weight) of cat food and ground oats. Both diets were fed as pellets in order to control for compensatory feeding. Cat food and oats were ground to a fine powder and sieved to remove lumps. Each diet (100% cat food or 50:50 cat food and oats by weight) was then mixed with a small quantity of water to make a paste, which was then spread across a ~1cm thick rigid polymer sheet perforated with holes. After drying for 24hrs at 30°C, the identically sized pellets thus produced could be pushed out from the holes. This protocol was simplified from that used by Hunt et al. (2004; 2005) to manipulate resource acquisition. These pellets were stored in sealed containers at room temperature and discarded if any sign of moisture or mould became apparent. Both diets were presented as powder (using a lid of an 1.5ml centrifuge tube as a feeding bowl) for the first 4 weeks because young nymphs can have difficulty breaking the surface of food pellets.

Food and water were replenished, the container cleaned and nymph survival recorded on a weekly basis. On reaching the fifth instar, nymphs were checked daily for eclosion. Imagos were weighed on the day of eclosion, and their pronotum widths were measured using a binocular microscope and graticule (measurements made to the nearest 0.1mm).

### Call Recording

Males were moved to a recording chamber to record their advertisement call between 8 and 10 days post-eclosion. The call recording chamber was maintained at the same environmental settings as the rearing chamber (28°C and 16:8 hours light:dark). In the recording chamber males were placed in individual sonically insulated boxes, each with a microphone built into the lid. All call recordings were made using an automated multi-channel digital recorder connected by a National Instruments data acquisition ('NI-DAQ') interface to a PC, allowing it to be controlled by software written in LabView. This system automatically monitors up to 256 channels and makes recordings only when the sound amplitude on a channel exceeds a user-defined threshold at two points in time (for instance 10 seconds apart to ensure that the cricket is singing consistently). The system then makes a recording from this channel and ignores subsequent inputs from the same channel until a 1 minute recording has been obtained.

The recordings were then measured using 'Raven' software version 1.1 (Bioacoustics Research Group: Cornell Lab of Ornithology). We measured 5 call traits, 1 spectral and 4 temporal; dominant frequency (DF), chirp pulse number (CPN), chirp inter-pulse interval (CIPD), trill number (TN) and inter-call interval (ICD). These traits (Appendix III) have been previously investigated and are known to be under multivariate sexual selection (Bentsen et al., 2006; Brooks et al., 2005).

### Analysis

I used MANOVA to test for population, treatment and population x treatment effects on development time (no. days from hatching to eclosion), pronotum width (mm) and weight at eclosion (g) and the 5 measured call traits. I then calculated **P**

matrices for each population under each treatment using the "cov" function in the 'R' statistical package (R Development Core Team, 2009). I used four approaches to compare these matrices; firstly the Mantel test of matrix correlation, computed using Poptools (Hood, 2009). Secondly we applied Roff's (2002) Jackknife MANOVA method to test for differences in the variance of matrix elements, using the resampling function of Poptools and running the MANOVA in SPSS (SPSS, 2007). We also performed a common principal components analysis on Flury's hierarchy (Phillips & Arnold, 1999) using the 'CPC' program (Phillips, 1997), and finally, a geometric comparison of eigenvector angles after translation into a shared subspace (Krzanowski, 1979), also using Poptools. Each of these methods is described in more detail in Chapter 3.3. It is prudent to use multiple tools in this way, given the current lack of a consensus as to the relative value of metrics for matrix comparison, and the results are complementary in any case; providing more information than any method alone.

## *4.4 RESULTS*

From a total of 1374 animals reared I was able to obtain recordings for 388 males, with no less than 56 recordings per population, and at least 190 recordings per diet treatment (actual numbers; ACT 66, KL 59, SA 56, SL 72, TAS 63, WA 71, high-nutrient diet 190, low-nutrient diet 197).

### Life-history

A MANOVA of the life-history parameters we measured (weight and pronotum width at eclosion, and development time) revealed significant differences between populations (Wilks' $\lambda$ =0.57, $F_{15,1737}$ =26.55, $P < 0.001$), which after 5 generations in common-garden, indicate underlying genetic differences (Table 4.1). Population mean development times ranged from 54.2 days (WA) to 66.5 days (SL), mean weights from 0.41g (WA) to 0.53g (SL) and pronotum widths from 8.73mm (TAS) to 9.57mm (SL). There was a significant effect of diet (Wilks' $\lambda$ =0.95, $F_{3,629}$ =12, $P < 0.001$); with development time increasing from a mean of 59.4 days (high-nutrient diet) to 61.9 days (low-nutrient diet), weight and pronotum width increasing from

**Table 4.1**: Results of a MANOVA showing the effects of population and diet treatment on male life-history. Significant *p*-values are in italics.

| MANOVA | | Wilks' Lambda | F | Degrees of freedom Hypothesis | Error | *P* -value |
|---|---|---|---|---|---|---|
| Population | | 0.58 | 54.6 | 15 | 3755 | *<0.001* |
| Diet | | 0.93 | 34.4 | 3 | 1360 | *<0.001* |
| Population x Diet | | 0.96 | 3.99 | 15 | 3755 | *<0.001* |

post-hoc ANOVAs

| Traits | Population Means (± SE) ACT | KL | SA | SL | TAS | WA | Degrees of freedom Hypothesis | Error | F | *P* -value |
|---|---|---|---|---|---|---|---|---|---|---|
| high-nutrient diet | | | | | | | | | | |
| pronotum width | 9.03 (0.04) | 9.00 (0.04) | 8.82 (0.04) | 9.26 (0.05) | 8.71 (0.05) | 8.65 (0.04) | 5 | 659 | 22.3 | *<0.001* |
| development time | 61.1 (0.58) | 59.6 (0.52) | 56.8 (0.49) | 64.3 (0.49) | 54.3 (0.53) | 52.8 (0.35) | 5 | 659 | 78.3 | *<0.001* |
| weight | 0.44 (0.01) | 0.42 (0.01) | 0.39 (0.01) | 0.46 (0.01) | 0.39 (0.01) | 0.39 (0.01) | 5 | 659 | 21.8 | *<0.001* |
| low-nutrient diet | | | | | | | | | | |
| pronotum width | 9.24 (0.05) | 9.59 (0.04) | 9.11 (0.04) | 9.54 (0.04) | 8.78 (0.08) | 8.73 (0.03) | 5 | 703 | 50.9 | *<0.001* |
| development time | 64.5 (0.69) | 62.5 (0.60) | 57.3 (0.58) | 66.1 (0.54) | 58.5 (1.09) | 53.3 (0.44) | 5 | 703 | 62.2 | *<0.001* |
| weight | 0.46 (0.01) | 0.50 (0.01) | 0.43 (0.01) | 0.52 (0.01) | 0.40 (0.01) | 0.39 (0.01) | 5 | 703 | 56.2 | *<0.001* |

Units: Pronotum width is measured in millimeters, weight is measured in grams and development time is a count of no. days.

0.44g and 8.96mm (high-nutrient) to 0.48g and 9.29mm (low-nutrient). A significant population-by-diet interaction effect was also found (Wilks' $\lambda$ =0.91, $F_{15,1737}$ =4.02, $P < 0.001$), with the difference in development time between diets varying from +0.09 days (WA) to +3.92 days (ACT). For weight and pronotum width the interaction was more pronounced, with differences varying from –0.02g and –0.05mm (TAS) to +0.08g and +0.59mm (KL). (Figure 4.1)

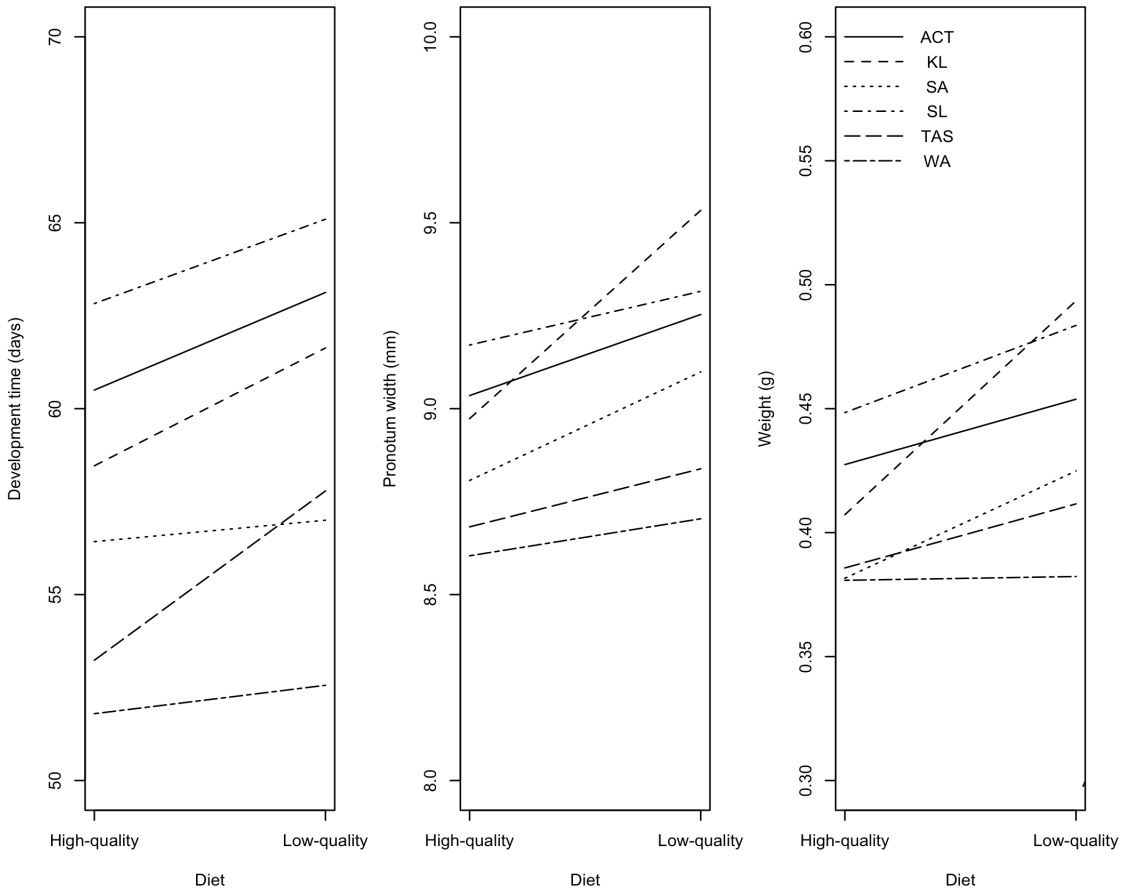


**Figure 4.1:** Reaction norms for the three life-history traits measured; from left to right development time (days), pronotum width (mm) and weight at eclosion (g). Populations are abbreviated as in the main text.

## Call Traits

We next analysed our five call traits. Since these data were not distributed normally we used using a randomisation test based on the MANOVA. The model was tested by comparing its *F* value to a sample distribution of 10,000 *F* values from

the same model with the call trait values randomised relative to the explanatory variables between iterations. In this approach the proportion of pseudo-*F* values more extreme than the true F value gives a 1-tailed *p*-value; the 2-tailed *P* is then calculated as either 2*p* if $p<0.05$, or as 2(1-*p*) if $p>0.05$. This analysis revealed significant differences among populations ($P<0.001$), but no effect of diet (Table 4.2). There was, however, a marginally significant population-by-diet interaction effect ($P=0.046$, Figure 4.2). Post-hoc analyses revealed that these inter-population differences were driven mostly by differences in inter-call duration (ICD).

### Matrix Comparison

Pairwise mantel tests were performed between diet treatments within populations, and between populations within diet treatments. None of our populations displayed a significant difference between call **P** matrices calculated for males on the high-nutrient diet and those on the low-nutrient diet (Table 4.3). Within the high-nutrient diet there were significant differences only between ACT and WA populations (65/10,000 iterations, $p=0.01$), between KL and TAS populations (9999/10,000 iterations, $p<0.001$) and between SA and TAS populations (155/10,000 iterations, $p=0.03$). Within the low-nutrient diet treatment there was a significant difference only between TAS and WA populations (9832/10,000 iterations, $p=0.03$).

Matrix comparison by MANOVA of Jackknife pseudovalues for matrix elements showed significant difference between populations (Wilks' $\lambda<0.001$, $F_{75,1733}=1241$, $p<0.001$) and between diet treatments (Wilks' $\lambda=0.02$, $F_{15,361}=1150$, $p<0.001$). There was also a significant interaction of population x diet treatment (Wilks' $\lambda<0.001$, $F_{75,1733}=612.4$, $p<0.001$, Table 4.4). Tests of between subject effects show significant differences in the pseudovalues of all fifteen matrix elements (five variances and ten covariances) taken individually; due to population ($p<0.001$ in all cases), between diet treatments ($p<0.001$ in all cases) and due to the interaction of population and diet ($p<0.001$ in all cases).

Between diets comparisons of **P** matrices using common principal components analysis yielded results that were qualitatively in agreement between step-up,

model building and jump-up approaches (Table 4.5). The results of all bar one test were of the top 3 ranks in the hierarchy – matrices classed as either equal, proportionally different, or sharing common principal components.

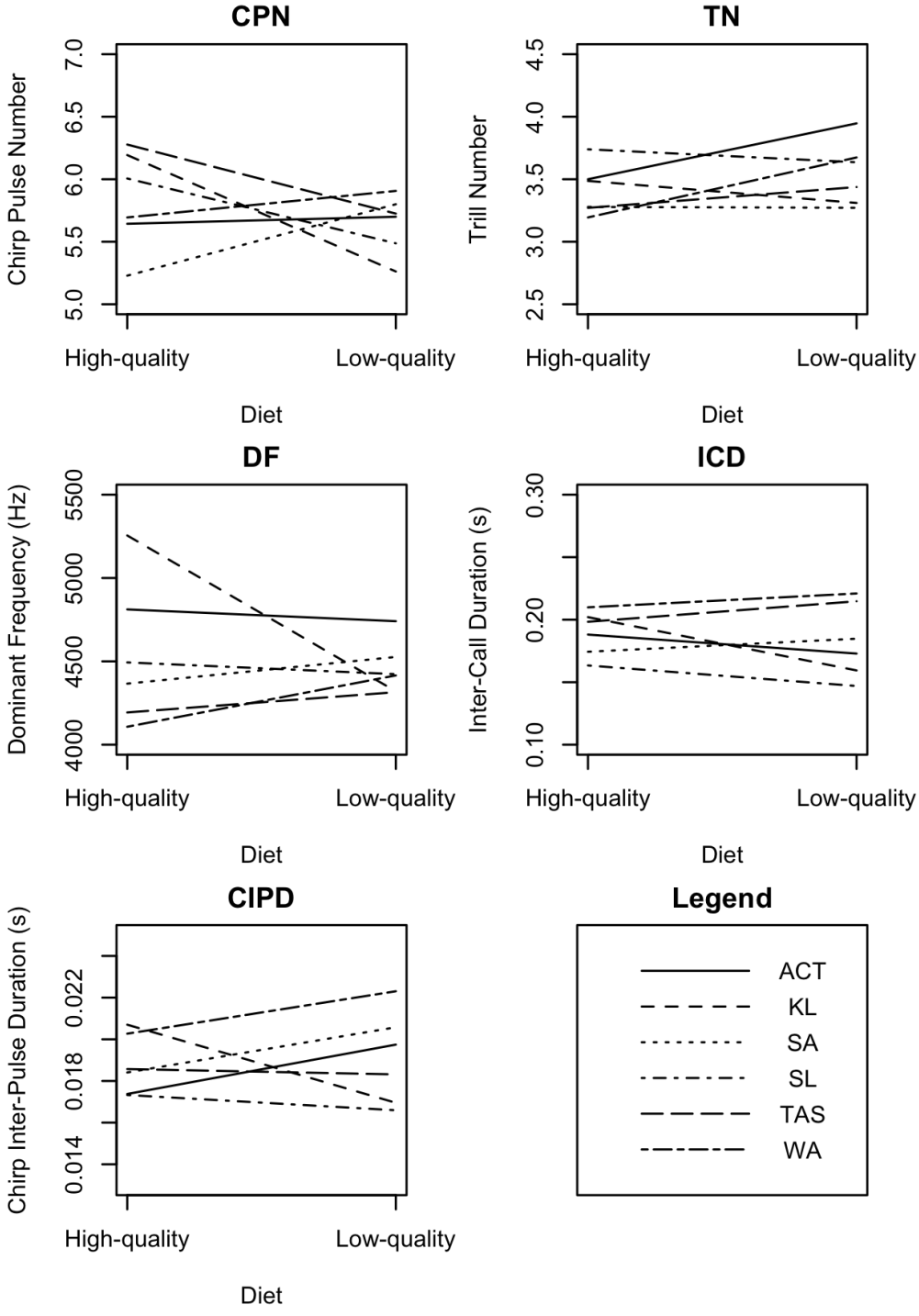


**Figure 4.2:** Reaction norms for the five call traits measured. Populations and traits are abbreviated as in the main text. The y-axes of the uppermost row have no units, as these traits are counts.

**Table 4.2**: Results of a MANOVA showing the effects of population and diet treatment on advertisement call structure. Significant *p*-values are in italics.

| randomised MANOVA | | no. Iterations | no. Pseudo-F vals > real F value | 2-tailed P value |
|---|---|---|---|---|
| | Population | 10,000 | 10,000 | *<0.001* |
| | Diet | 10,000 | 5178 | 0.964 |
| | Population x Diet | 10,000 | 9769 | *0.046* |

post-hoc ANOVAs

| | Population Means (± SE) | | | | | | no. Iterations | no. Pseudo-F vals > real F value | 2-tailed P value |
|---|---|---|---|---|---|---|---|---|---|
| Traits | ACT | KL | SA | SL | TAS | WA | | | |
| high-nutrient diet | | | | | | | | | |
| CPN | 5.64 (0.38) | 6.19 (0.50) | 5.22 (0.28) | 6.00 (0.39) | 6.27 (0.30) | 5.69 (0.23) | 10,000 | 3364 | 0.67 |
| TN | 3.5 (0.25) | 3.48 (0.32) | 3.27 (0.18) | 3.73 (0.32) | 3.27 (0.22) | 3.19 (0.22) | 10,000 | 7482 | 0.50 |
| ICD | 188 (19.3) | 202 (25.8) | 174 (15.2) | 163 (15.1) | 198 (28.0) | 209 (23.3) | 10,000 | 75 | *0.02* |
| CIPD | 17.3 (1.22) | 20.7 (1.40) | 18.4 (1.87) | 17.3 (1.21) | 18.5 (1502) | 20.2 (0.87) | 10,000 | 3760 | 0.75 |
| DF | 4.81 (0.30) | 5.25 (0.35) | 4.36 (0.18) | 4.49 (0.22) | 4.19 (0.17) | 4.10 (0.11) | 10,000 | 6561 | 0.69 |
| low-nutrient diet | | | | | | | | | |
| CPN | 5.7 (0.35) | 5.26 (0.27) | 5.8 (0.33) | 5.48 (0.19) | 5.72 (0.34) | 5.90 (0.31) | 10,000 | 7205 | 0.56 |
| TN | 3.94 (0.29) | 3.31 (0.28) | 3.27 (0.19) | 3.63 (0.24) | 3.43 (0.20) | 3.67 (0.30) | 10,000 | 4769 | 0.95 |
| ICD | 172 (12.3) | 159 (11.5) | 184 (10.4) | 146 (5.92) | 214 (25.5) | 220 (17.3) | 10,000 | 4 | *<0.001* |
| CIPD | 19.7 (1.43) | 16.9 (0.89) | 20.5 (2.04) | 16.5 (0.96) | 18.3 (1.34) | 22.3 (1.33) | 10,000 | 211 | *0.04* |
| DF | 4.74 (0.27) | 4.32 (0.19) | 4.52 (0.25) | 4.42 (0.18) | 4.31 (0.21) | 4.41 (0.22) | 10,000 | 7770 | 0.45 |

Units: CPN and TN are counts, ICD and CIPD are measurements in milliseconds and DF is measured in kilohertz. See text for population abbreviations.

**P** matrices were also compared using common principal components analysis using all three approaches. All three CPC approaches; 'Step-up', 'Model building' and 'Jump-up', yielded results from near the top of the Flury's hierarchy. When comparing 5 element matrices, the Flury's hierarchy has 7 steps from 'unrelated' to 'equality'. 2-way between-diet comparisons within populations yielded results from the top 3 ranks of the hierarchy ('equality', 'proportionality' or 'common principal components') in all cases except for the WA population using the Model building approach; which returned a result of 2 common principal components. 6-way among-population comparisons within diet treatments returned 'proportional' ranks for both treatments using Step-up and Model building approaches, but 'unrelated' rank using the Jump-up approach. Despite its current popularity as a method for comparing **P** or **G** matrices (McGuigan, 2006), the CPC method was not developed with biological matrices in mind, and the detection of similar structure may not indicate the conservation of causal factors (Houle et al., 2002). In the case of our data, however, we are reassured that there is a shared pattern, since the pattern of eigenvector loadings is similar across all matrices.

**Table 4.3**: 2-tailed *p*-values from matrix comparisons by Mantel test. Population names are abbreviated as in text. Between-population comparisons in the high-nutrient diet group are above the diagonal. Within-population comparisons between diet treatments are on the diagonal in **bold**. Between-population comparisons in the low-nutrient diet group are below the diagonal in *italics*. Significant p-values are underlined.

| | ACT | KL | SA | SL | TAS | WA |
|---|---|---|---|---|---|---|
| **ACT** | **0.16** | 0.07 | 0.53 | 0.12 | 0.33 | 0.01 |
| **KL** | *0.33* | **0.38** | 0.30 | 0.36 | 0.00 | 0.17 |
| **SA** | *0.15* | *0.97* | **0.12** | 0.68 | 0.03 | 0.83 |
| **SL** | *0.30* | *0.67* | *0.74* | **0.62** | 0.13 | 0.17 |
| **TAS** | *0.51* | *0.18* | *0.24* | *0.52* | **0.18** | 0.41 |
| **WA** | *0.60* | *0.17* | *0.53* | *0.58* | *0.03* | **0.14** |

Geometric matrix comparisons within populations between treatments found that the eigenvectors for the high- and low-nutrient diets lie in different, but similar subspaces (Table 4.6). The sum of **T** matrix eigenvalues was >1.5 for all populations except WA, whose score was 1.02 respectively (of a possible maximum k=2). The angle between the closest eigenvectors (θ) was also small in most cases: <6.6° in all cases except for the TAS population, where the closest eigenvectors were divergent at an angle of 12.5° (of a possible maximum of 90°). For all within-population comparisons, the mean sum of **T** eigenvalues was 1.66 and the mean θ was 5.8°. Geometric comparisons between populations within treatments showed similar levels of difference to within populations between treatments comparisons, with a mean sum of **T** eigenvalues of 1.6 and a mean θ of 6.7°. In summary, the shape of the covariance structure was changed very little by the diet treatment (Figure 4.3).

**Table 4.4**: Results of a MANOVA on Jackknife pseudovalues for call trait **P** matrix elements.

| | Wilks' Lambda | Approx. F | Degrees of freedom | | *P* -value |
|---|---|---|---|---|---|
| | | | Hypothesis | Error | |
| Population | 4.74E-09 | 1241 | 75 | 1733 | <0.001 |
| Diet | 2.00E-02 | 1157 | 15 | 361 | <0.001 |
| Population x Diet | 1.28E-07 | 612.4 | 75 | 1733 | <0.001 |

**Table 4.5**: Results of common principal components analysis comparing **P** matrices using the Flury's hierarchy. The upper part of the table shows results from within-population between-diet comparisons; the lower part shows results from among-population within-diet comparisons. All comparisons were carried out using all three CPC approaches. The Flury's hierarchy in this case has 7 ranks; equal, proportional, CPC, CPC(3), CPC(2), CPC(1), unrelated.

| | *CPC approach* | **Step-up** | **Model building** | **Jump-up** |
|---|---|---|---|---|
| between diets | **ACT** | Proportional | Proportional | Equal |
| | **KL** | CPC | CPC | CPC |
| | **SA** | Equal | Equal | Equal |
| | **SL** | Proportional | Proportional | Equal |
| | **TAS** | Equal | Equal | Equal |
| | **WA** | CPC | CPC(2) | Equal |
| | **high-nutrient diet** | Proportional | Proportional | Unrelated |
| | **low-nutrient diet** | Proportional | Proportional | Unrelated |

**Table 4.6**: Results of a geometric comparison of **P** matrix eigenvectors. Comparisons between diet treatments within populations are in the upper part of the table; the lower part contains comparisons between populations within diet treatments. The sum of **T** matrix eigenvalues (presented in **bold** and above the diagonal) is a measure of similarity; in this case between a minimum of 0 and a maximum of 2. 'Angle' is the angle between closest vectors (presented in *italics* and below the diagonal), in this case between 0° and 90°.

Between treatment comparisons within populations

| | **ACT** | **KL** | **SA** | **SL** | **TAS** | **WA** |
|---|---|---|---|---|---|---|
| **T** | **1.81** | **1.89** | **1.79** | **1.87** | **1.55** | **1.02** |
| *angle* | *4.73* | *5.02* | *6.6* | *2.12* | *12.5* | *3.84* |

Between population comparisons within treatment

| | | **ACT** | **KL** | **SA** | **SL** | **TAS** | **WA** |
|---|---|---|---|---|---|---|---|
| Control Diet | **ACT** | | **1.69** | **1.67** | **1.74** | **1.25** | **1.31** |
| | **KL** | *5.44* | | **1.59** | **1.88** | **1.47** | **1.6** |
| | **SA** | *8.85* | *7.07* | | **1.89** | **1.78** | **1.5** |
| | **SL** | *3* | *1.56* | *5.33* | | **1.74** | **1.68** |
| | **TAS** | *11.79* | *14.78* | *3.2* | *9.45* | | **1.6** |
| | **WA** | *2.93* | *7.39* | *15.26* | *3.54* | *17.11* | |
| Test Diet | **ACT** | | **1.9** | **1.85** | **1.94** | **1.09** | **1.54** |
| | **KL** | *7.03* | | **1.62** | **1.87** | **1.16** | **1.4** |
| | **SA** | *4.63* | *5.45* | | **1.82** | **0.96** | **1.77** |
| | **SL** | *4.48* | *9.05* | *2.64* | | **1.08** | **1.55** |
| | **TAS** | *4.79* | *1.73* | *11.20* | *11.86* | | **1.11** |
| | **WA** | *4.55* | *4.17* | *0.47* | *6.62* | *5.2* | |

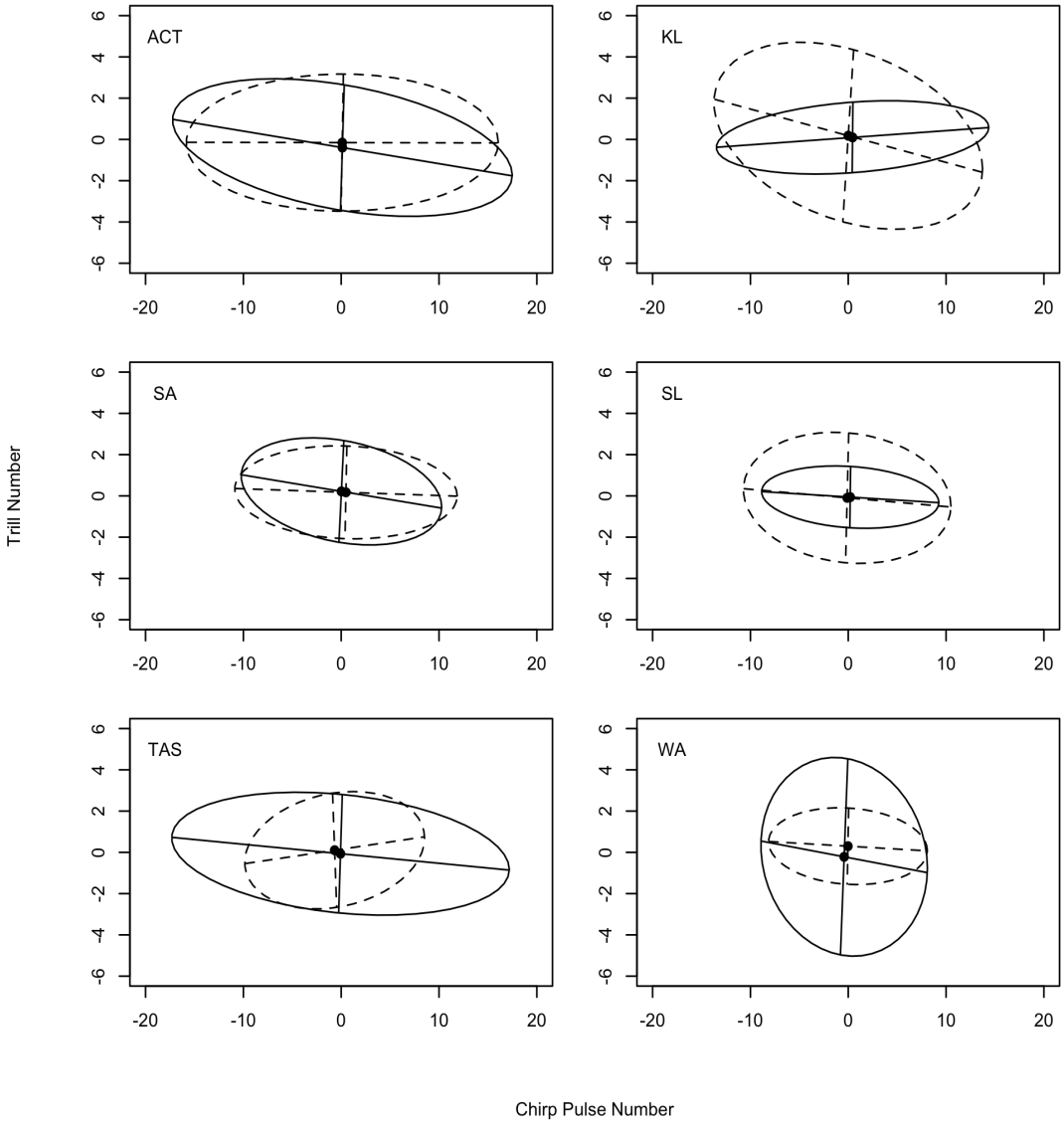


**Figure 4.3**: Ellipses representing the first two eigenvectors and associated eigenvalues of **P** for all 6 populations (abbreviations as above) and for both diets; broken lines represent **P** for high-nutrient diet, solid lines for low-nutrient diet; projected onto axes for chirp pulse number and trill number. These traits were chosen for the axes because they were consistently the most influential traits on the 1st and 2nd eigenvectors respectively. Eigenvector pairs do not appear orthogonal in all cases – this distortion is due to the matrices having been reflected into a common space.

## *4.5 DISCUSSION*

There are paradoxical predictions relating to the integration and plasticity of complex traits that function as sexual signals (Badyaev, 2004). *T. commodus* males produce a complex advertisement call, the multivariate structure of which is amenable to measurement and manipulation (Brooks et al., 2005: Figure 4.1). We reared crickets to adulthood on either their standard diet (henceforth 'high-nutrient') or an experimental diet (henceforth 'low-nutrient') in order to provoke a plastic response. Animals were drawn from common-garden reared stocks derived from divergent populations (Appendix II) to test for inter-population differences in integration and plasticity.There were four noteworthy results to emerge from this study. Firstly, we provide robust support for the findings that these populations of *T. commodus* are genetically divergent (Chapter 3). Secondly, our diet manipulation was associated with differences in life-history, though not with individual call parameters. Thirdly, the **P** matrices calculated from our call recordings were not identical, neither among populations nor between diets; a result in which we may have some confidence given the reasonable level of concordance between our analytical methods. Finally, although there were detectable differences to be found between **P** matrices, those differences are subtle and the covariance structures were largely concordant.

There are population-level differences in all the traits measured, both life-history parameters and call traits. Finding such differences in animals from established stock populations that have been reared under common garden conditions is indicative of a genetic divergence between populations. This is in line with my findings in Chapter 3.

### Developmental response to Diet

Our manipulation of diet was effective in perturbing the developmental trajectory of our test populations, as indicated by the differences seen in all life-history parameters I measured (Table 4.1). Animals reared on the low-nutrient diet took longer to reach adulthood, and were larger and heavier on average (similarly to the results reported by Hunt et al 2004), though these effects were not constant among populations; resulting in significant interaction effects (Figures 4.1 and

4.2). Given this evidence for genetic divergence between these populations, the population x diet interaction effects found for both life-history and call traits are, though not demonstrative, at least suggestive of genotype x environment interactions occurring in this system.

### Matrix stability

In this study, I compared the call **P** matrices using a suite of analytical methods. This was necessitated by the currently incomplete understanding of the behaviour of all of the currently available methods. Indeed, none of these methods in isolation have been shown to be sufficient for all examinations of **P** divergence (Pigliucci & Kolodynska, 2002a). It may well be the case that some methods are more sensitive to matrix differences than others, but it is also true that each methodology tests a subtly different hypothesis about the structure of the matrix. That being said, there is a pleasing complementarity in the results found from multiple tests here. Briefly; that CPC analysis (Phillips & Arnold, 1999) indicates that our **P** matrices share (some or all) principal components accords well with the small angles found between analogous eigenvectors using Krzanowski's geometric approach (Krzanowski, 1979). Given that Mantel tests reveal very few significant pairwise matrix differences, the overwhelming significant differences found using Roff's Jackknife MANOVA method (Roff, 2002) might seem counterintuitive, but the Jackknife MANOVA method has been submitted to rigorous testing (Begin et al., 2004), and so it seems likely that the differences between **P** matrices found – though real – are subtle enough to evade detection by the Mantel test.

How can we account for the concordance of covariance structure, despite the differences in traits? Similar results have appeared before (Arnold & Phillips, 1999; Game & Caley, 2006; de Oliveira et al., 2009; Garant et al., 2008; see also Chapter 3), though greater levels of **P** matrix divergence have also been observed (Eroukhmanoff et al., 2009). **P** is a product of the environmental variance-covariance matrix (**E**) and the additive genetic variance-covariance matrix (**G**) and we know that there has been genetic divergence between our populations, but a conserved structure of **E** might account for the stability of the **P** matrix, at least between wild populations. However, differences in **P** of a similar magnitude are found between these populations when wild-caught. Given that wild-caught males

can be safely assumed to have experienced differing contributions of **E**, the stability of **E** seems unlikely to be the full explanation.

Gene flow between populations could theoretically contribute to the maintenance of shared covariance structure, although this seems unlikely given the large geographical distances between source populations (particularly the separation of the TAS population from the Australian mainland by the Bass Strait and the large desert regions between Western and Eastern populations), and the measured divergence in individual call parameters between populations. **P** is shaped by selection operating on multivariate phenotypes, so shared covariance structure might be expected to result from a history of similar patterns of multivariate selection (Lande & Arnold, 1983; Badyaev & Hill, 2000). The sexual selection regime operating on call **P** matrices in this species has been characterised as multivariate stabilising (Bentsen et al., 2006; Brooks et al., 2005), but the shape of the fitness surface for the call traits measured here is known to differ between source populations (Hunt: unpublished data). It would appear, therefore, that the orientation of **P** in these populations has not responded to changes in the shape/position of the adaptive peak. This would be in line with the outcome of modelling (Jones et al., 2004) that showed that the orientation of **G** can remain stable in the face of significant shifts in the position of adaptive peaks. This result was found to be most likely in those instances where the direction of selection was orthogonal to $\mathbf{g}_{max}$ – the axis of greatest variance (Jones et al., 2004).

Mutation, drift and population size also have roles to play in determining the stability of **G** (Steppan et al., 2002), with possible knock-on influence on **P**. Population size influences how drift affects trait (co)variances (Falconer & Mackay, 1996; Steppan et al., 2002), and there is some evidence for a major role of population size in determining matrix stability (Jones et al., 2003). More recently, in simulations of **G** matrices evolving in response to moving optima, Jones et al. (2004) found large population size to be pivotal in encouraging the conservation of matrix orientation.

## Plasticity and Integration of P

We currently lack the data necessary to properly describe the genetics underlying the covariance patterns described here, but results from computer modelling studies suggest that stability of the **G** matrix can be promoted by certain genetic mechanisms, particularly by mutations with pleiotropic effects. Since the calls studied here are secondary sexual characteristics, they may be expected to be indicative of the condition and/or genetic quality of the whole organism (Andersson, 1994; Zahavi, 1975). Moreover, the advertisement call is a behaviour involving the finely timed coordination of wing muscles in order to produce the correct pattern of sound pulses. The development of wing musculature, and the nervous system required for its control, may be assumed to involve many developmental steps. Thus it seems likely that pleiotropic effects could be important and may be a contributory factor in maintaining the observed stability of **P**.

Modularity is a concept that has repeatedly come to the attention of those studying multivariate evolution (Badyaev, 2004; Bossdorf & Pigliucci, 2009; de Oliveira et al., 2009; Hallgrimsson et al., 2002; Hansen et al., 2003; Kraft et al., 2006; Mezey & Houle, 2003; Wagner & Altenberg, 1996), a phenotypic module being a suite of characters that display enhanced integration with each other and reduced integration with the rest of the organism. In light of the stability of **P**, despite the plasticity of its component traits and of the animals' life-history, it seems reasonable to suggest that the structure of the advertisement call in *T. commodus* meets this criterion. Of those mentioned above, Badyaev (2004) in particular has addressed the issue of modularity and integration in sexual signals, and suggests three predictions; that secondary sexual traits should be expected to show (1) weakened developmental integration with other traits, (2) strengthened functional integration and modularity, and (3) weaker genetic integration with the rest of the organism. The quasi-independence of call traits revealed here does indeed indicate weaker developmental integration between call traits and life-history traits than between call traits, in line with (1). The stability of **P** in the face of population divergence would seem to indicate tight functional integration (2), but as this

study deals with phenotypic measurements only, we can unfortunately not address prediction (3).

In summary, the *T. commodus* populations studied here appear to differ genetically with respect to all traits measured, yet have a high degree of shared covariance structure. Despite leading to life-history changes, a manipulation of diet did not lead to significant changes in the expression of call traits. Whilst the populations tested here displayed plasticity in their developmental trajectories and call parameters, and that plasticity appears to be genetically variable, the call **P** matrix remained stable throughout.

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# CHAPTER 5: Secondary Sexual Morphology and the Evolution of an Acoustic Signal


## *5.1 ABSTRACT*

The evolution of behaviour and morphology are often tightly linked. Lack of variation in the morphology of signalling traits therefore has the potential to constrain the evolution of the signal. This relationship is particularly likely to be important in field crickets, where males produce acoustic advertisement signals to attract females by stridulating using morphological structures on their forewings. In this study, we characterise the geometric size and shape of the forewings of males from populations of the black field cricket *Teleogryllus commodus* which are known to have divergent advertisement call behaviour. We sample from each of six populations over two generations, allowing us to test for relationships between wing morphology and call structure. In addition to correlations between wing size and a number of call structure traits, we find complex multivariate relationships between call structure and wing shape. The majority of variance associated with the primary axis of call - shape covariance is of opposite sign for the temporal aspects of call structure as compared to dominant frequency. Since sexual selection in this species favours a reduction of the values for all these parameters, any phenotypic evolution along this axis would therefore face a trade-off that may have the potential to influence the response of advertisement calls to selection.



**Keywords**; **P** matrix; Advertisement call; Geometric morphometrics; Acoustics; Phenotypic integration; *Teleogryllus commodus*

## *5.2 INTRODUCTION*

Behavioural traits, particularly social behaviours, are thought to evolve more rapidly than morphological traits (Puniamoorthy et al., 2009; Moore et al., 1998; Moore et al., 1997) for a number of reasons. Behaviour is more labile than morphology in metazoans; an animal may express a number of behaviours without the need to alter its morphology; for example flight and courtship 'song' in *Drosophila* (Bennet-Clark & Ewing, 1967). Additionally, whilst the expression of both morphology and behaviour can be 'switched on' by an appropriate environmental stimulus, the expression of a behaviour can be induced near-immediately, meaning that there are a great many more cues available for regulating behaviour, and thus the opportunity for more behavioural diversity (West-Eberhard, 1989).

The idea that the evolution of behaviour and morphology are connected has long been recognised (Baldwin, 1896). However, more recently researchers have described how behaviour may influence the evolvability of morphology (West-Eberhard, 2003; Wcislo, 1989) because selection on a behaviour will indirectly select on the morphology utilised in its performance. In some cases behaviours and the specialised morphologies that support them are intimately linked; for example phase polyphenisms in migratory locusts (Pener & Yerushalmi, 1998), male horn dimorphism and reproductive tactics in scarab beetles (Moczek & Emlen, 2000) and caste polyphenisms in eusocial insects (Nijhout, 1999). In these cases the alternative morphologies are discrete and intermediates are rare in the population, but variation in most traits is quantitative; making the relationship less easy to elucidate. A further complication is that most morphological traits will be utilised in many (if not all) behaviours expressed by the organism, meaning that conflicting indirect selection from different behaviours has the potential to influence the evolution of morphology. Secondary sexual traits that are not used for other behaviours may therefore be more tractable as model traits for the study of the relationship between morphological and behavioural evolution.

Male field crickets (Orthoptera: Gryllidae) display to potential mates acoustically (Zuk, 1987), and these signals are comparatively well-studied (e.g. Crnokrak & Roff, 1998; Ferreira & Ferguson, 2002; Gray & Cade, 1999; Gray & Cade, 1999;

Hoback & Wagner, 1997; Honda-Sumi, 2005; Jang & Gerhardt, 2006). These advertisement calls are produced using specialised stridulatory structures located on the forewings (tegmina). The forewings are not used in flight and so presumably their morphology is shaped principally by sexual selection. Numerous studies have shown that the advertisement calls of crickets are subject to strong sexual selection imposed by female mate choice. These advertisement calls also convey information about species identity and play an important role in minimizing hybridization (e.g. Scheuber et al., 2003a; Simmons, 1995; Simmons & Zuk, 1992; Ritchie et al., 1995).

When raised, a plectrum on the (typically) left forewing engages with a toothed file on the ventral surface of the right forewing; the movement of the plectrum over the file as the forewings are closed sets up a vibration in resonant 'harp' and 'mirror' structures of both wings (Bennet-Clark, 2003; Bennet-Clark & Bailey, 2002). As the plectrum is moved along the file the two opposed structures function as an escapement (analogous to the devices that regulate the speed of clockwork mechanisms), linking the catch/release of the plectrum to the resonant frequency of the forewings (Koch et al., 1988; Bennet-Clark & Bailey, 2002; Prestwich et al., 2000). The 'clockwork cricket' model developed by these authors predicts a negative relationship between the area of the resonant structures and the frequency of the call produced. Simmons & Ritchie (1996) found this relationship between the area of the harp and call dominant frequency in *Gryllus campestris*. Webb & Roff (1992) also found this relationship in *Gryllus firmus*; in addition to a negative relationship between body size and the number of pulses per chirp. Furthermore, a comparative study of katydids (Tettigonidae) found a phylogenetic association between changes in acoustic signals and the morphology of stridulatory structures (Montealegre-Z, 2009), including positive relationships between pulse duration and both body size and wing length, and negative relationships between frequency and both body size and wing length.

Male Australian field crickets (*Teleogryllus commodus*), spend a substantial proportion of their time calling in this way (Hunt, Brooks, et al., 2004). The structure of the advertisement call itself is relatively complex; beginning with a single 'chirp' sequence, which is followed by a variable number of 'trill' sequences

(Bentley & Hoy 1972; Hill et al. 1972). Female *T. commodus* show preferences for both temporal (Pollack & Hoy, 1979) and spectral (Hennig & Weber, 1997) properties of this call, resulting in a regime of multivariate stabilising sexual selection (Brooks et al., 2005; Bentsen et al., 2006). These same measures of call structure have been shown to vary between geographically distinct *T. commodus* populations, and there is a genetic component to this divergence (Chapter 3). Taken together, these populations represent a pool of standing genetic variance for call structure and are therefore an excellent system in which to evaluate the relationship between wing morphology and call structure.

In light of research showing a link between the morphology of stridulatory organs and the nature of the calls produced (Simmons & Ritchie, 1996; Montealegre-Z, 2009), it seems reasonable to predict that the size and/or shape of the cricket forewing has been shaped by sexual selection on acoustic performance, though this potential relationship is poorly understood. The best supported relationship between morphology and acoustic structure in crickets is the negative correlation between the dominant frequency of the call and body size (Scheuber et al., 2003a; Scheuber et al., 2003b; Gerhardt & Huber, 2002). This correlation (also seen in anurans e.g. Wagner & Sullivan, 1995) is assumed to be informative to females where large males are preferred; a mechanistic relationship whereby larger males bear larger resonant structures, which necessarily produce lower frequencies. However a review of the literature found no clear relationship between frequency and body size in crickets (Verburgt & Ferguson, 2010), and indeed the same study presented experimental evidence that female *Gryllus bimaculatus* cannot reliably predict male body size via the acoustic signal. Additionally, in *Gryllus campestris*, dominant frequency has been shown to change with male age; with older males calling with lower (i.e. more attractive) frequencies (Jacot et al., 2007). In fact, despite the assumption in the literature that body size predicts call frequency (e.g. Jacot et al., 2005; Gerhardt & Huber, 2002), a surprising number of studies report no association between body size and frequency. Clearly the relationship between morphology and acoustic performance is not yet fully understood.

Most of the studies described above have tested for a relationship between male wing morphology and acoustic performance focus on the length and/or area of

wing structures, and how they correlate with one or a few call parameters. The key point is that neither linear measurements, nor measures of area calculated from them, are particularly useful for describing geometric shape, which may also be an important determinant of call structure. The variety of multivariate techniques now available allow for the description of complex shapes in a more rigorous fashion, and permits statistical comparison of different morphological forms (Zelditch et al., 2004). Geometric morphometrics defines shape as the geometric properties of an object that are invariant to changes of location and scale (Bookstein, 1997), in contrast with traditional metrics such as straight-line measurements (e.g. Simmons & Ritchie, 1996), or area calculated from outlines (e.g. Moradian & Walker, 2008) that confound size with shape. Geometric morphometric techniques enable us to separate these two important aspects of morphology and examine the relationship between them (Dryden & Mardia, 1998), and such analyses may reveal patterns that would not be detectable by pairwise bivariate methods.

Here, we present an analysis of forewing shape and call structure in two experimental cohorts of male *T. commodus*; one wild caught and one after three generations of rearing under common-garden conditions. Six populations that are known to be genetically divergent for call parameters are represented in both cohorts. In addition to a multivariate characterisation of advertisement call, we employ the techniques of geometric morphometrics (Klingenberg & McIntyre, 1998; Klingenberg et al., 1998; Dryden & Mardia, 1998) to quantify the geometric size and shape of the forewing that is used to produce the advertisement call in this species. The use of divergent populations replicated with both wild-caught and common-garden-reared cohorts should enable us to include as wide a spread of variation as possible in both call parameters and wing morphology. This ought to maximise the power available for partial least squares analyses to characterise the relationships between these two multivariate datasets. Since the primary function of the tegmina is to produce an acoustic signal, characterising the relationship between morphology and acoustic structure should reveal the extent to which the evolution of calls may be constrained by integration between shape, size and acoustic performance.

## *5.3 METHODS*

### Collection of Animals and Common-Garden Rearing

Approximately 200 field-mated female *Teleogryllus commodus* were collected from each of six geographically isolated populations; Western Australia (WA), South Australia (SA), Tasmania (TAS), Smiths Lake (SL), Kioloa (KL) and Australian Capital Territory (ACT) (see Appendix II for locations of populations). Females were provided with egg-pads upon which to lay, and the resulting offspring were used to establish lab colonies representing each population. Lab reared animals were kept at a constant temperature of 28°C, with a 16:8 hours light:dark cycle. They were kept in large (100 L) plastic storage containers, supplied with water and fed *ad libitum* on 'Go-Cat senior' cat food pellets (Nestlé Purina PetCare). Stocks were replenished by rearing the offspring of 100 haphazardly paired adults per generation. In the field generation and after three generations of common-garden rearing, adult males' calls were recorded and their wings removed and mounted for geometric morphometric analysis (see below).

### Call recording and analysis

Males were moved to a recording chamber to record their advertisement call between 8 and 10 days post-eclosion. The call recording chamber was maintained at the same environmental settings as the rearing chamber (28°C and 16:8 hours light:dark). In the recording chamber males were placed in individual sonically insulated boxes, each with a microphone built into the lid. These microphones were sampled throughout the night by connecting them in turn to a Sony Walkman (WM DC6), which was manually activated if the male in question was calling. These recordings were then digitised from cassette and measured using the Raven application (Cornell Bioacoustics Group). We took measurements of five call traits; dominant frequency (DF), chirp pulse number (CPN), chirp inter-pulse interval (CIPD), trill number (TN) and inter-call interval (ICD) (Appendix III), which we know from previous work vary significantly between these populations (Chapter 3) and are known to be subject to strong sexual selection (Brooks et al., 2005; Bentsen et al., 2006). Each call parameter was measured five times for each call, and the means used for each male in our subsequent analyses. Since these traits

have different units of measurement, individual means were standardised (converted to z-scores) before analysis. We then used a MANOVA to test for differences among populations and between generations.

### Morphometric Analyses

We used landmark-based geometric morphometrics to quantify size and shape variation of the male forewing. We selected a suite of 19 points on the male forewing to serve as landmarks for morphometric analysis; these points were selected so as to define the margins of the known call-related structures and also to capture the outline shape of the wing (Figure 5.1). After successful call recording, each male's pronotum width was measured using a binocular microscope with an eye-piece graticule, before being killed by freezing. Forewings were raised and held by lightweight forceps, then removed by cutting through the articular sclerites at the attachment point to the thorax using iris dissecting scissors (just above landmark 2 on Figure 5.1). Cricket forewings have a flexible zone anterior to the Cubitus 1 vein (Figure 5.1 or Figure 1 in Bennet-Clark, 2003). When held at rest, this zone flexes almost to a right angle such that the two parts of the wing lie along the dorsal and lateral surfaces of the animal's body, respectively. We found that in *T. commodus* this zone was flexible enough that we could mount the wings whole; using transparent tape to secure them to a standard microscope slide. After mounting, each slide was photographed using a microscope-mounted digital camera. Before we recorded data, each photograph was reflected so that each wing appeared to be a right wing; this was done to control for error resulting from any perceptual or mechanical difference in digitising the same landmark from different parts of the image (Klingenberg & McIntyre, 1998). Coordinates for the landmark points on each wing were then digitised from these photographs using the ImageJ application (Rasband, 1997) and a macro written by C. P. Klingenberg. We used the generalised Procrustes superimposition, to separate variance in shape from variance in the orientation, alignment and size of the images. The resulting Procrustes coordinates contain information about shape only. This transformation of necessity involves the calculation of centroid size; calculated as the square root of the sum of squared distances from the centroid to the landmarks (Dryden & Mardia, 1998); a geometric measure of size that is independent of shape.

We initially used a full factorial MANOVA (type III sums of squares and cross-products) of Procrustes coordinates to test for differences between generations and among populations, and a two-way ANOVA (type III sums of squares) to test for differences in centroid size. Next we used partial least squares analysis (PLS) to test for covariance between morphometric parameters and call measures. In order to maximise power for our partial least squares analysis we pooled all populations and both generations in order to include the broadest spread of variance possible in the analysis. Lastly, in order to facilitate comparison with previous work and between metric and geometric methods, we regressed our 5 call measures against pronotum width (as an index of body size). Analyses of variance were performed using SPSS (version 16), and multiple regressions were performed in R (R Development Core Team, 2009). Other analyses were conducted in MorphoJ (Klingenberg, 2008).

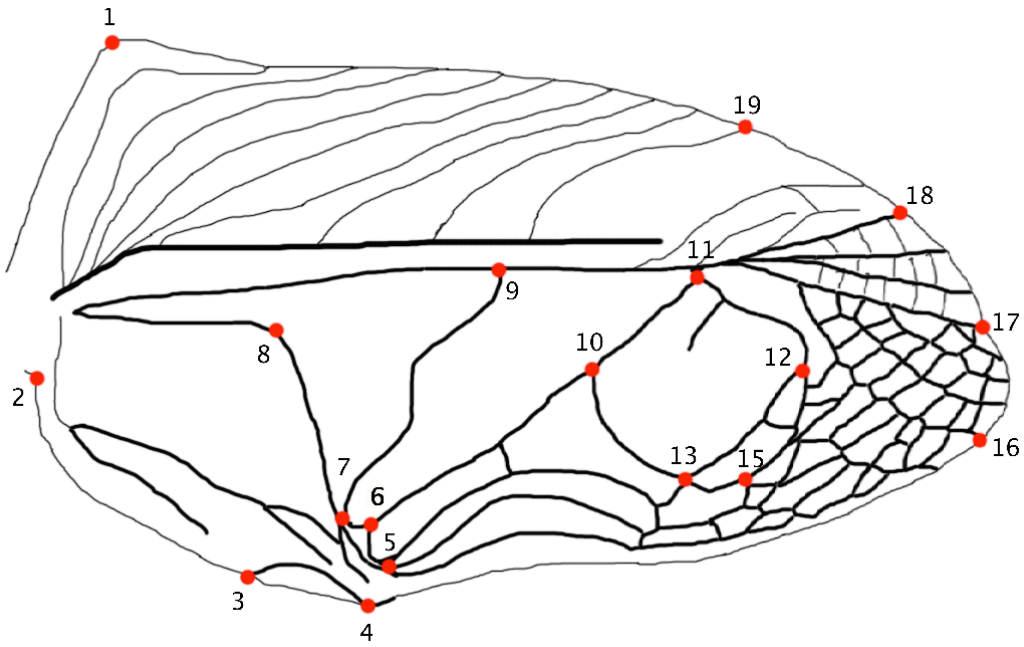


**Figure 5.1**: A simplified outline drawing of the venation of male forewings. Landmark points are indicated in red. The Cubitus 1 vein connects landmarks 17, 11 and 9 before terminating at the proximal wing boundary. The harp is the structure subtended by landmarks 5, 6, 7, 8, 9, 10 and 11. The file lies along the vein between 7 and 8 and the plectrum between 3 and 4. Landmarks 10, 11, 12 and 13 subtend the mirror.

## *5.4 RESULTS*

A total of 530 crickets were successfully recorded and had wings intact enough for morphometric analysis. The resulting dataset comprised 230 males from the field and 300 from the lab generation, with a minimum of 59 males per population.

### Population Divergence in Call Structure and Wing Morphology

A MANOVA (type III sums of squares and cross-products) of standardised call trait values showed significant differences among populations (Wilks' $\lambda$ =0.63, $F_{25,1911}$=10.24, $P$ <0.001) indicative of population divergence, and between generations (Wilks' $\lambda$ =0.27, $F_{5,514}$=28.00, $P$ <0.001) indicative of lab adaptation. There was also a significant interaction between population and generation effects (Wilks' $\lambda$ =0.55, $F_{25,1911}$=13.56, $P$ <0.001) indicating among-population differences in the lab adaptation response (Table 5.1).

We performed a MANOVA of Procrustes coordinates (type III sums of squares and cross-products) using a model that included population, generation and side (left or right wing) as main effects and centroid size as a covariate. This revealed that allometry is an important contributor to wing shape, as evidenced by a significant effect of centroid size (Wilks' $\lambda$ =0.52, $F_{34,482}$=13.2, $P$ <0.001), and that there is directional (left-right) asymmetry, indicated by the significant effect of side (Wilks' $\lambda$ =0.18, $F_{34,482}$=66.9, $P$ <0.001).

**Table 5.2**: Results of a MANOVA of Procrustes Coordinates after model reduction. The full model included generation, population, and side as main effects, and centroid size as a covariate.

| Effect | Wilk's Lambda | F value | Degrees of Freedom | | *p* -value |
|---|---|---|---|---|---|
| | | | Hypothesis | Error | |
| **Centroid Size** | 0.52 | 13.0 | 34 | 482 | <0.001 |
| **Population** | 0.16 | 6.41 | 170 | 2394 | <0.001 |
| **Generation** | 0.35 | 26.5 | 34 | 482 | <0.001 |
| **Side (L or R)** | 0.18 | 66.9 | 34 | 482 | <0.001 |
| **Gen. X Pop.** | 0.3 | 3.91 | 170 | 2394 | <0.001 |
| **Gen. X Side** | 0.88 | 1.98 | 34 | 482 | <0.001 |

**Table 5.1**: Results of a MANOVA of standardised call parameters, using a full-factorial model including generation and population, with post-hoc ANOVAs and population means for each trait. (Significant *p*-values are in *italics*.

| MANOVA | | Wilks' Lambda | F | Degrees of freedom Hypothesis | Error | *P* -value |
|---|---|---|---|---|---|---|
| | Population | 0.63 | 10.24 | 25 | 1911 | *<0.001* |
| | Generation | 0.27 | 280.5 | 5 | 514 | *<0.001* |
| | Population x Generation | 0.55 | 13.58 | 25 | 1911 | *<0.001* |

| post-hoc ANOVAs | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Population Means (± SE) | | | | | | Degrees of freedom | | F | *P* -value |
| Traits | ACT | KL | SA | SL | TAS | WA | Hypothesis | Error | | |
| field-caught generation | | | | | | | | | | |
| CPN | 6.43 (0.30) | 6.73 ( 0.25) | 6.73 (0.26) | 6.02 (0.20) | 6.95 (0.21) | 8.15 (0.48) | 5 | 224 | 5.26 | *<0.001* |
| TN | 2.59 (0.21) | 2.84 (0.20) | 2.43 (0.20) | 3.34 (0.22) | 2.70 (0.18) | 2.63 (0.20) | 5 | 224 | 5.37 | *<0.001* |
| ICD | 209 (18.3) | 246 (18.4) | 305 (23.2) | 215 (33.1) | 263 (16.2) | 298 (22.5) | 5 | 224 | 3.92 | *0.002* |
| CIPD | 26.7 (0.94) | 30.3 (1.19) | 29.3 (0.71) | 25.3 (0.83) | 32.3 (1.43) | 37.4 (0.87) | 5 | 224 | 20.3 | *<0.001* |
| DF | 3.87 (0.03) | 3.78 (0.03) | 3.66 (0.05) | 3.40 (0.04) | 3.49 (0.04) | 3.89 (0.08) | 5 | 224 | 33.5 | *<0.001* |
| lab-reared generation | | | | | | | | | | |
| CPN | 5.59 (0.14) | 5.60 (0.14) | 5.79 (0.15) | 5.77 (0.12) | 5.79 (0.12) | 5.56 (0.14) | 5 | 294 | 1.71 | 0.13 |
| TN | 2.96 (0.18) | 2.93 (0.18) | 2.06 (0.23) | 2.41 (0.17) | 2.42 (0.16) | 2.64 (0.24) | 5 | 294 | 2.47 | *0.04* |
| ICD | 157 (5.13) | 157 (5.13) | 136 (4.34) | 152 (4.19) | 140 (4.00) | 128 (5.32) | 5 | 294 | 15.7 | *<0.001* |
| CIPD | 19.0 (0.75) | 18.9 (0.75) | 18.1 (0.61) | 18.1 (0.38) | 18.1 (0.49) | 18.6 (0.57) | 5 | 294 | 10.8 | *<0.001* |
| DF | 4.06 (0.02) | 4.06 (0.02) | 4.15 (0.03) | 3.99 (0.03) | 4.08 (0.03) | 4.15 (0.03) | 5 | 294 | 16.8 | *<0.001* |

Units: CPN and TN are counts, ICD and CIPD are measurements in milliseconds and DF is measured in kilohertz. See text for population abbreviations.

We also found significant differences among populations (Wilks' λ =0.16, $F_{170,2394}$=6.41, $P$ <0.001) and a significant difference between generations (Wilks' λ =0.35, $F_{34,482}$=26.6, $P$ <0.001); indicating that wing shape had diverged among populations and that there was also a shape change associated with adaptation to common garden lab conditions (Figure 5.2). In addition, we found significant interactions between population and generation effects (Wilks' λ =0.30, $F_{170,2394}$=3.91, $P$ <0.001) and between generation and side effects (Wilks' λ =0.88, $F_{34,482}$=1.98, $P$ =0.001). The significant population by generation interaction indicates differences among populations in their response to lab conditions; since conditions in the lab were homogenous this is evidence for a genetic divergence between populations. The significant interaction between generation and side effects reflects a difference in the degree of directional asymmetry between generations (Table 5.2).

An ANOVA (type III sums of squares) was used to test for differences in centroid size between populations and with generation. Population and generation were included as main effects in the model and side was included as a covariate (Table 5.3). There was a significant effect of population ($F_{5,517}$=19.4, $P$ < 0.001) with males from the KL population having the largest wings, on average, (mean centroid size 21.7mm ± 0.12 S.E.) and males from the WA population having the smallest (mean centroid size 20.2mm ± 0.20 S.E.). The wings of the lab reared generation were significantly smaller than those of their field-caught ancestors effect ($F_{1,517}$=216.3, $P$ < 0.001), with mean centroid sizes of 21.5mm (± 0.08 S.E.) for the field generation, and 20.1mm (± 0.07 S.E.) for the lab generation. We also found a significant interaction between population and generation ($F_{5,517}$=12.81, $P$ < 0.001), indicating among-population differences in the response to the lab environment. The overall mean centroid size was 1.4mm smaller for the lab generation than for the field, though the change in mean values by population varied from 0.4mm (SA population) to 3.2mm (TAS population).

### Call Structure and Wing Morphology

We did not include shape (Procrustes coordinates) in the same analyses as wing centroid size since a regression of Procrustes coordinates against centroid size

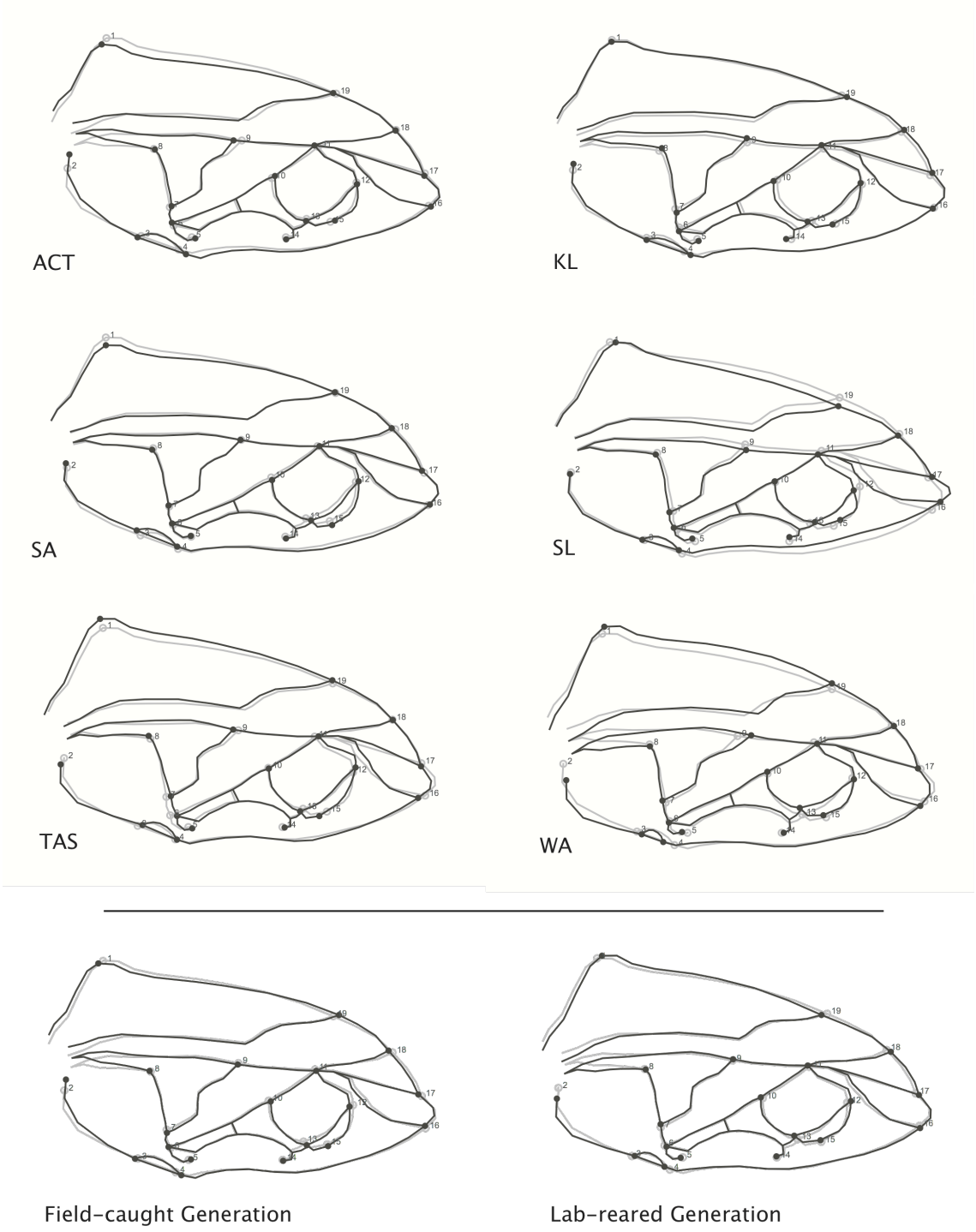


**Figure 5.2**: Mean wing shapes for our six populations (see text for abbreviations) and two generations. Figures show the mean for each population/generation are illustrated as dark grey outlines overlying the grand mean shape (light grey).

showed a significant difference from independence (permutation test; 0/10,000 iterations, $p<0.0001$). In order to remove this allometric shape variance, we conducted our shape analyses using the residuals from this regression in place of the Procrustes coordinates, and tested the relationship between call structure and centroid size separately.

**Table 5.3**: Results of a full factorial ANOVA of centroid size values after model reduction; the model included generation, population and side as main effects.

| Effect | Type III Sum of Squares | Degrees of Freedom | Mean Square | F value | *p* -value |
|---|---|---|---|---|---|
| **Population** | 99.40 | 5 | 19.88 | 19.36 | <0.001 |
| **Generation** | 222.0 | 1 | 222.0 | 216.3 | <0.001 |
| **Pop. X Gen.** | 129.8 | 5 | 25.29 | 25.29 | <0.001 |

We tested for covariance between size and call structure using a 2-block PLS analysis (including all males), with centroid size as block 1 and all five standardised call structure traits in block 2. This analysis can be thought of as a regression where have both predictor and response variables (analogous to the 2 blocks) may be matrices. PLS analysis works by extracting the multidimensional vector in the block 1 space that explains the maximum multidimensional variance in the block 2 space, followed by the largest vector orthogonal to the first and so on. This analysis produced 1 PLS axis (by definition encompassing 100% of the covariance, since centroid size (block 1) is a univariate measure) with a strongly significant RV correlation coefficient (conceptually similar to an $R^2$) of 0.160 (permutation test; 0/10,000 iterations, $p<0.0001$). The largest PLS coefficients were for DF and CIPD (0.559 and –0.557, respectively), indicating that the direction of this axis is determined mostly by the covariance of these two traits with body size. It is interesting to note that whilst the coefficient for DF was positive, those for all four temporal parameters were negative (Table 5.4), indicating that a change of size would predict correlated changes of opposite sign for the four temporal parameters than for DF.

**Table 5.4**: Coefficients for call traits (see text for abbreviations) from analyses of the covariance of wing centroid size and call traits. Test 1 is a 2-block partial least squares analysis. Test 2 is a pooled within-group 2-block PLS analysis, individuals grouped by generation.

| Call Traits | PLS1 coefficients | |
|---|---|---|
| | test 1 | test 2 |
| CPN | -0.340 | -0.377 |
| TN | -0.191 | -0.393 |
| ICD | -0.474 | -0.460 |
| CIPD | -0.557 | -0.125 |
| DF | 0.559 | 0.690 |
| RV coefficient | 0.160 | 0.009 |

We also ran a pooled within-group 2-block PLS analysis using the same blocks, and pooling by generation. This analysis extracts vectors for the same relationships as the unpooled analysis, but here the inter-generational variation is excluded from the analysis. This is achieved by focusing on the covariance arising from the deviance from the group means within each block of variables. The resulting PLS axis had a much smaller RV coefficient of 0.009, indicating a weaker relationship between size and call structure when considered only within generations. This was nonetheless a statistically significant relationship (permutation test; 0/10,000 iterations, $p<0.0001$). The pattern of coefficient magnitudes was largely the same as for the unpooled analysis; with DF having the largest, and the only positive, PLS coefficient (0.690; Table 5.4).

We also used a 2-block PLS analysis to test for covariance between wing shape and call structure, with the regression residuals for shape (from regression against centroid size; see above) as block 1 and our five standardised call traits as block 2. Since both blocks are now matrices, this analysis extracted five PLS axes (since the smaller matrix of call traits has a rank of 5), with a small but significant overall RV coefficient of 0.087 (permutation test; 0/10,000 iterations, $p<0.0001$). PLS1 was the only statistically significant axis (permutation test, 0/10,000 iterations, $p<0.0001$), accounting for 87.8% of the total covariance. The largest coefficients on

PLS1 were for CIPD and DF (0.652 and –0.489 respectively). See Figure 5.4 for an illustration of the shape variance associated with PLS1. The PLS1 coefficients for all for temporal call traits were positive in this case, with only DF having a negative coefficient (Table 5).

**Table 5.5**: Coefficients for call traits (see text for abbreviations) from analyses of the covariance of wing shape (regression residuals for Procrustes coordinates) and regression residuals for standardised call traits. Test 1 is a 2-block partial least squares analysis. Test 2 is a pooled within-group 2-block PLS analysis, individuals grouped by generation.

| call traits | PLS1 coeffients | |
|---|---|---|
| | test1 | test2 |
| CPN | 0.35 | 0.42 |
| TN | 0.06 | 0.12 |
| ICD | 0.43 | 0.70 |
| CIPD | 0.65 | 0.39 |
| DF | -0.49 | -0.30 |
| *P* -value | <0.0001 | <0.0001 |
| % total covariance | 87.6 | 59.8 |

Once again we also ran a pooled version of this analysis using the same blocks as before, and with data pooled by generation to exclude inter-generational variance. In this case we found a significant overall RV correlation coefficient of 0.034 (permutation test; 0/10,000 iterations, $p<0.0001$). PLS1 accounted for 53.4% of the total covariance when the analysis was run in this way, and was the only axis that was significant (permutation test, 0/10,000 iterations, $p<0.0001$). See Figure 5.5 for an illustration of the shape variance associated with PLS1. The PLS1 coefficients displayed a similar pattern to those from the un-pooled analysis, with the coefficient for DF being the negative and those for temporal parameters being positive (Table 5.5).

A multiple regression analysis found our five call structure measures to be highly predictive of pronotum width (Adjusted $R^2=0.22$, S.E.=0.08, $F_{5,524}=31.6$ , $P<0.001$), and revealed significant positive associations with ICD ($\beta=0.38$, S.E.=0.004, $F_{1,528}=89.5$, $P<0.001$) and with CIPD ($\beta=0.43$, S.E.=0.004, $F_{1,528}=117$, $P<0.001$).

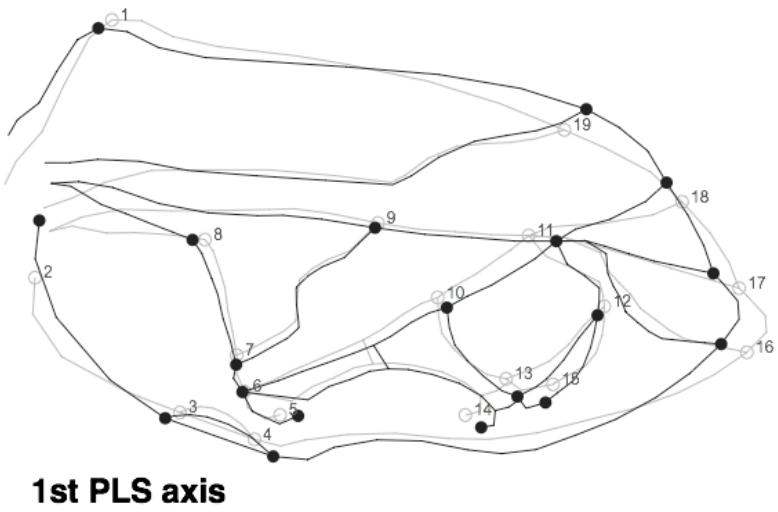


**Figure 5.4**: Shape variance associated with the 1st PLS axis from a partial least squares regression with Procrustes coordinates as block 1 and standardised call traits as block 2. The light grey outline represents the mean shape; the dark grey outline represents the mean plus the shape change that corresponds to an increase of 0.1 units of Procrustes distance in the direction defined by the 1st PLS axis.

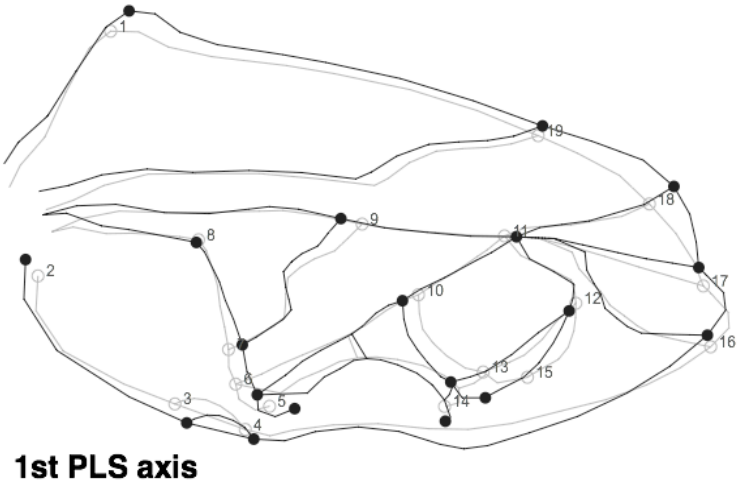


**Figure 5.5**: Shape variance associated with the 1st PLS axis from a pooled with-groups partial least squares regression, pooled within generation, with Procrustes coordinates as block 1 and standardised call traits as block 2. The light grey outline represents the mean shape; the dark grey outline represents the mean plus the shape change that corresponds to an increase of 0.1 units of Procrustes distance in the direction defined by the 1st PLS axis.

**Table 5.6**: The matrix of Pearson correlation coefficients for call structure measures (see text for abbreviations) and pronotum width. Correlations are above the diagonal and *P* values are below (**bold** if significant).

| | P. Width | CPN | TN | ICD | CIPD | DF |
|---|---|---|---|---|---|---|
| **P. Width** | | *0.262* | *0.072* | *0.381* | *0.426* | *-0.386* |
| **CPN** | **<0.001** | | *0.264* | *0.611* | *0.422* | *-0.373* |
| **TN** | **0.048** | **<0.001** | | *0.162* | *0.164* | *-0.049* |
| **ICD** | **<0.001** | **<0.001** | **<0.001** | | *0.547* | *-0.47* |
| **CIPD** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | | *-0.567* |
| **DF** | **<0.001** | **<0.001** | *0.132* | **<0.001** | **<0.001** | |

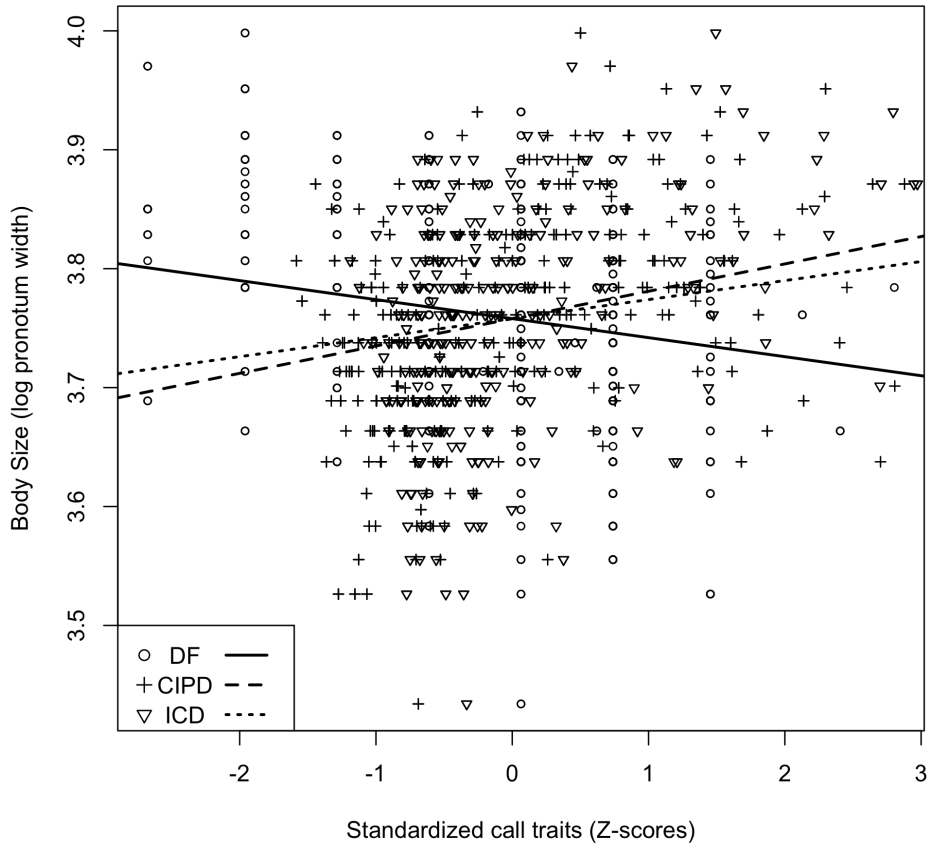


**Figure 5.3**: Plot to show the relationships between call parameters and body size. Note that there is a contrast between the negative association with DF (dominant frequency), and the positive association with the temporal call parameters; CIPD and ICD (chirp inter-pulse duration and inter-call duration). DF, CIPD and ICD were measured in different units (kHz, ms and s respectively), but are presented here as the Z-standardized values used for analysis.

Additionally there was a significant negative association between pronotum width and DF (β=-0.39, S.E.=0.004, $F_{1,528}$=92.5, $P$<0.001, Figure 5.3). All six variables were shown to be widely inter-correlated (Table 5.6).

**Table 5.7**: A summary of previous studies that have examined the relationships between forewing morphology and some aspect(s) of advertisement call in Gryllid crickets.

| Species | Metric of body size | Principle findings | Reference |
|---|---|---|---|
| *Gryllus bimaculatus* | Pronotum width | No associations between calls and body size | Bateman et al., 2004 |
| *Oecanthus nigricornis* | 1st prin. comp. of body measures | Both frequency and pulse duration negatively associated with body size | Brown et al., 1996 |
| *Gryllus bimaculatus* | Pronotum width | No associations between calls and body size | Ferreira & Ferguson, 2002 |
| *Acheta domesticus* | Weight | Chirp pulse number positively correlated with body size | Gray, 1997 |
| *Gryllus campestris* | Initial weight | Frequency decreases with age | Jacot et al., 2007 |
| *Acheta domesticus* | Weight | Nymphal nutritional & immune status positively correlated with harp size | Jacot et al., 2005 |
| *Gryllodes sigillatus* | Body mass | Body size positively correlated with chirp pulse number | Ryder & Siva-Jothy, 2000 |
| *Gryllus campestris* | Body mass | Male mass positively correlated to harp area | Sakaluk et al., 1992 |
| *Gryllus campestris* | Pronotum area | Size positively correlated with harp area. Harp area negatively correlated with frequency | Scheuber et al., 2003b |
| *Gryllus campestris* | Pronotum area | Size positively correlated with harp area. Harp area negatively correlated with frequency | Scheuber et al., 2003a |
| *Gryllus bimaculatus* | Pronotum width | Frequency negatively associated with harp area, harp area positively correlated with body size | Simmons & Ritchie, 1996 |
| *Gryllus bimaculatus* | Pronotum width | Chirp pulse duration negatively associated with male size | Simmons & Zuk, 1992 |
| *Gryllus campestris* | Pronotum width | Frequency negatively associated with harp area, harp area covaries with body size | Simmons, 1995 |
| *Gryllus bimaculatus* | Pronotum width | Chirp repetition rate positively correlated with body size | Simmons, 1988 |
| *Gryllus bimaculatus* | Pronotum area | No associations between calls and body size | Verburgt & Ferguson, 2010 |

## *5.5 DISCUSSION*

Our analyses show differences in call structure and forewing morphology between geographically isolated populations of *T. commodus*, and also between wild-caught and lab-reared generations. The broad variation in both call and morphometric data allowed us to identify dimensions of shape variance that covary significantly with aspects of male call structure. These covariances characterise a novel link between the structure and acoustic performance of the tegmina. Here we discuss the implications of these results in light of sexual selection operating on male call structure.

When animals are reared in common-garden conditions (e.g. Mousseau & Roff, 1995; Mousseau & Howard, 1998; Simmons, 2004), remaining inter-population variation is indicative of inter-population genetic differences. Given the geographical distance between our source populations (Appendix II), they will certainly have experienced different abiotic environmental conditions and thus might be predicted to have diverged through local adaptation. Previous work (Chapter 3) has demonstrated this variation in call structure, but the current study suggests that this divergence appears to be accompanied by a similar pattern of differences in both forewing size and shape. Moreover, the significant interaction between the effects of population and generation in call structure, forewing size and shape, indicates inter-population variation in how expression of these traits are affected by field and lab environmental cues. These different responses to common-garden rearing conditions, expressed in genetically divergent populations, are suggestive of a genotype by environment (GxE) interaction but further work incorporating a more controlled breeding design would be required to verify this conclusively.

### Call Structure and Wing Morphology

Since the tegmina act as a mechanical resonating system, changes in their size or shape have been predicted to affect the frequency of the call produced (Prestwich et al., 2000; Bennet-Clark, 2003). In particular, all else being equal, larger forewings should produce lower frequency calls; and the assumption has been made that body size may therefore be communicated via the frequency of calls (e.g.

Bennet-Clark, 1999). In fact, the relationship that studies typically demonstrate is negatively correlation between frequency and harp area (Simmons, 1995; Simmons & Ritchie, 1996; Scheuber et al., 2003a; Scheuber et al., 2003b; Jacot et al., 2005). These studies support their assumption of frequency signalling body size by showing a positive correlation between harp area and body size, yet a number of other studies (Webb & Roff, 1992; Simmons & Zuk, 1992; Simmons, 1988; Sakaluk et al., 1992; Ryder & Siva-Jothy, 2000; Gray, 1997; Ferreira & Ferguson, 2002; Bateman et al., 2004) have measured frequency and body size but have not found a relationship (Table 5.7).

In contrast, we have demonstrated that call structure does indeed signal body size in *T. commodus*, but frequency is not the only informative feature of the advertisement call. We do find a negative association between the dominant frequency of the call and the body size of the caller, in addition to which there are positive associations between body size and both chirp inter-pulse interval and inter-call duration (Figure 5.3). It is possible therefore, that females may extract reliable information about a male's body size by assessing the structure of his call. However, the signalling of size by dominant frequency may be reinforced or augmented by information conveyed by the temporal aspects of call structure.

We found a weak but significant relationship between forewing size and our five measures of call structure. The strength of the RV coefficient drops from 0.16 to 0.009 when the PLS analysis was re-run with the data pooled by generation, indicating that the majority of the covariance between wing size and calls involved inter-generational variance. This is consistent with our finding that wing size – indicative of general body size – was significantly smaller for the lab-reared generation (though the difference was more marked for some populations than others).

Of the five call structure measures, dominant frequency (DF) had the largest, and only positive, coefficient for the PLS axis describing the call structure - wing size relationship, with the four temporal call traits all having negative coefficients (Table 5.4). Forewing size and harp size are very tightly associated ($R^2 > 0.9$) and so a larger wing will bear a larger resonant area and could be therefore be

expected to emit a lower frequency. However, a larger wing also represents a greater mass for the animal to vibrate, which will presumably displace a larger volume of air. An increase in the values of temporal parameters (lengthening of pulses and intervals) may therefore be the result of a slower duty cycle as the wings are slowed by increased inertia and/or aerodynamic resistance. If this is the case, the escapement of the 'clockwork' cricket (Bennet-Clark & Bailey, 2002; Elliott & Koch, 1985) may provide a mechanism by which temporal and spectral call properties may act as constraints upon each other.

PLS analysis of shape residuals revealed another strongly significant relationship along the 1st PLS axis and call structure, but in common with the wing size PLS, the strength of this relationship was low. The RV coefficient from the first (unstructured) analysis was 0.09, and 0.03 for the structured (pooled by generation) analysis. Despite these low coefficients, the 1st PLS axis nonetheless accounted for the majority of the covariance between wing shape and call structure (respectively; 88% or 60%) in these analyses. As with the case of the previous analyses, the difference in explanatory power between the unpooled and pooled analyses indicates that an amount of the covariance detected involves inter-generational variance. The largest coefficients for call structure parameters in these cases were for CIPD in the initial PLS, and for ICD when the analysis were pooled by generation. In contrast to the PLS analyses of wing size and call structure measures, the coefficient for DF was smaller, the 2nd or 4th largest value respectively. However, DF did have the only negative coefficient in both shape PLS analyses (Table 5.5), with all four temporal coefficients being positive regardless of how the analysis was structured. The most noteworthy feature of the pattern of PLS coefficients is that the loading coefficients for temporal call parameters are opposite in sign to that for frequency in PLS analyses with either wing shape or wing size. This implies that a change in wing size or a shift along the major axis of shape variance would entail opposing changes in the values of spectral and temporal call parameters; either a decrease in DF with an associated increase in temporal measures, or vice versa.

The shape change described by an increase along the principal PLS axes from the two shape analyses is not identical (Figures 5.4 and 5.5), but does share some similarities. In particular, in both cases there is a widening of the wing between the

level of the plectrum and the distal end of the mirror, and the shortening and blunting of the wingtip. A wing that is wider, shorter or blunter at the tip has a reduced aspect ratio (Biewener, 2003) and consequently an increased coefficient of drag. The increased drag could conceivably act to damp the escapement mechanism and thus explain the associated increase in the temporal parameters. A change of +0.1 units of Procrustes distance along the 1st unpooled, though not the 1st pooled, PLS axis (dark grey outline; Figure 5.4) shows a noticeable increase in the size of the harp, which may explain the associated decrease in DF.

Although these mechanisms to explain the covariance between geometric shape and size and advertisement call structure are speculative, it is noteworthy that the patterns of coefficient magnitude and sign are robust across analyses both with and without inter-generation variance. Since the majority of inter-generation variance will be environmental in origin, the persistence of inter-population differences under common-garden conditions shows that there is genetic variation for both call structure and wing morphology traits. Despite this divergence of call structure measures between populations, the call **P** matrix is conserved among populations. One feature of the **P** matrix that is shared by among populations is the pattern of vector loadings for the principal eigenvector ($\mathbf{p}_{\mathbf{max}}$); the loadings for the temporal parameters are opposite in sign to those for DF. The loading for DF is the only positive loading on $\mathbf{p}_{\mathbf{max}}$ for all populations except Smith's Lakes (q.v. Appendix II for populations), for which it is the only negatively loaded trait (Chapter 3). This suggests that the covariance of wing morphology and call structure may play a role in the conservation of the call **P** matrix.

There is a significant directional asymmetry in forewing shape. Given that a number of crickets species have been observed to display 'handedness' in the action of their forewings while calling (Nocke, 1971), it may be that this left-right asymmetry is a common feature among Gryllids. We found an interaction between the effect of side and the effect of generation, but not between side and population effects. Taken together, these results indicate that the left-right asymmetry is more pronounced in wild caught males than by those reared under common-garden conditions in the lab, though all populations express directional asymmetry equally. Although the differences between lab and field environments are many

and largely unmeasured, all six populations have previously been found to be consistently smaller (in terms of both weight and pronotum width) after 3 generations of rearing under common-garden conditions in the lab (Chapter 3). This is consistent with our finding here that lab-reared animals also bear smaller forewings than wild-caught ones. Male body size is assumed to be a fitness correlate, and is selected for by female crickets (Zuk, 1987; Simmons & Zuk, 1992; Simmons, 1995; although not universally; L. F. Bussiere et al., 2006). If, therefore, our smaller common-garden-reared males are less fit or in poorer condition than their wild-caught antecedents, this difference is associated with reductions in both wing size and directional asymmetry, independently of population differences. Thus the generation of smaller, lab-reared males appear to bear more symmetric and smaller forewings than their wild-caught ancestors. This suggests a mechanical function for the directional asymmetry, in contrast to the results found by Simmons & Ritchie (1996) where the fitter male *Gyllus campestris* bore more symmetrical wings with which they produced purer (less frequency modulated) tones.

Even the straightforward-sounding association between male size and call frequency is not simple or ubiquitous. A number of studies have viewed this association in the context of a three-way relationship between body size, frequency and wing/harp size (e.g.; Simmons & Ritchie, 1996; Jacot et al., 2004), but we show here that this too is an over-simplification of the multivariate relationships between multiple call characters, wing size, wing shape and body size. For example; without geometric rationalisation of wing shape, and the separation of size and shape these techniques allow, we could not have detected the conflicting patterns of call covariance between spectral and temporal call properties.

The forewings of male crickets play no role in flight, and so presumably the principal origin of selection thereon is the acoustic preferences of potential mates. Female *T. commodus* express preferences for multiple temporal and spectral call characters, and multiple aspects of the call may therefore be informative. The relationships that exist between wing shape and call structure appear to indicate a comparative lack of variance aligned with the direction (reduction of both

frequency and temporal measures (Brooks et al., 2005; Bentsen et al., 2006)) most preferred by *T. commodus* females. Understanding how wing morphology serves to 'translate' information about male phenotype/condition into call properties may therefore offer researchers the opportunity to link multivariate sexual selection to complex adaptation.


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# CHAPTER 6: Inter-Population Variation in Mate Choice Behaviour under Nutritional Stress in *Teleogryllus commodus*

## *6.1 ABSTRACT*

Sexual traits are often assumed to be condition dependent, and thus informative to the opposite sex. In particular, the condition dependence of male sexual signals has been a subject of intense scrutiny. In part this is due to the prediction that populations where females choose mates based on condition dependent signals can maintain genetic variance in their signals and can thus avoid the lek paradox. By contrast, the influence of condition on female choice has been much less thoroughly investigated, though such condition dependence could have similar consequences for the maintenance of genetic variance. Here we present a study of females mate choice in the field cricket *Teleogryllus commodus.* Females from six genetically divergent populations were reared on either high or low-nutrient diets in order to affect their condition. Once adult, we presented them with synthetic male advertisement calls that differed only in inter-call duration. We then observed diet-induced responses in life-history traits. Animals reared on the low-nutrient diet took longer to develop, but were then larger and heavier at eclosion. Nutritional stress did not affect the direction of population preference functions; shorter inter-call durations are preferred by all females, irrespective of population or diet treatment. Nutritional stress is associated with a reduction in the elevation (intercept) of the preference function for all populations. Female responsiveness was also affected by diet treatment, but in contrast to preference, this effect was population-specific. Though not conclusive, this suggests the presence of a genotype by environment interaction for female responsiveness to male advertisement calls, though not for the direction of their preference for inter-call duration.

**Keywords**: *Teleogryllus commodus*; Advertisement call; Phenotypic plasticity; Preference function

## *6.2 INTRODUCTION*

Condition dependence of secondary sexual traits has been suggested as an escape from the well-known 'lek paradox'; if females consistently select some genotypes over others, these genotypes will spread throughout the population until the genetic variance upon which the choice is based is eroded (Kirkpatrick & Ryan, 1991; Rowe & Houle, 1996; Hine et al., 2004). The genic capture hypothesis (Tomkins et al., 2004) makes the prediction that genetic variation for condition dependent traits ought to be detectable through interaction effects between genotype and environment (GxE interactions). However, the empirical tests of this prediction have tended to focus on the (usually male) sexual signal traits (e.g. Brandt & Greenfield, 2004; Parker & Garant, 2004) rather than the (usually female) mate choice behaviour, which may also be condition dependent (e.g. Hunt et al., 2005; Bakker et al., 1999).

The evolution of sexual signal traits has been the subject of intense theoretical and empirical investigation (Andersson, 1994), and in general abundant phenotypic and genetic variance has been found in the expression of these sexual traits (Moller & Alatalo, 1999). It has been widely assumed that condition dependence is a common feature of sexual traits (Andersson, 1994; Johnstone, 1995) though this may be less generally the case than previously assumed (Cotton et al., 2004). Nevertheless, it is evident that investment in sexual ornaments depends on the investment in other costly traits and behaviours (Hoglund & Sheldon, 1998). There is evidence from several species that condition dependence is involved in the trade-off between costly sexual traits and other major fitness components, such as survival and growth (Kotiaho, 2001; Tomkins et al., 2004; Hunt et al., 2004; Hunt, Bussiere, et al., 2004). Life-history trade-offs may therefore constrain the evolution of sexual signals and vice versa. Despite the effort expended on examining the condition-dependence of male signal traits, and the known costs associated with mate choice (Watson et al., 1998; Gibson & Bachman, 1992), there have been relatively few attempts to measure the relationship between condition and the mate choice decisions of females (Hunt et al. 2005; Brooks & Endler 2001; reviewed by Widemo & Saether 1999 and Jennions & Petrie 1997).

The term 'condition' has been commonly used to encompass those characteristics that reflect the general health and vigour of an individual. However, it is important to acknowledge the distinction between condition and those traits that reflect it (Tomkins et al., 2004) and we shall use condition here to mean the quantity of metabolic resources that an individual is able to allocate to fitness-related traits. Many aspects of a phenotype can be expected to influence the organism's ability to acquire and utilise resources, and condition is therefore likely to be polygenic (Houle, 1998; Houle, 1991). With many loci contributing to it, condition can be thought of as presenting a large 'mutational target' (Houle, 1998); and thus may accumulate genetic variance as fast as that variance is removed by selection. Thus, there is likely to be genetic variance for the capacity of an organism to express condition-dependent traits. If an environmental factor is manipulated, and the rank order of genotypes for the expression of a condition-dependent trait changes between environments, then there is no single 'best genotype' for all environments. As outlined by Tomkins et al. (2004), one way in which genetic variance for condition-dependence would be detectable is through such interaction effects between genotype and environment (GxE interactions).

Mate choice is often the sum of a complex set of female behaviours, including searching, assessment and courtship, and variation in any of these can lead to variation in mating choice decisions. The key for empiricists, as Brooks & Endler (2001) pointed out, is to define behaviours that may be measured reliably while remaining general enough to be generally applicable, rather than specific to the species under investigation. Here we shall use 'choosiness' and 'preference' *sensu* Jennions & Petrie (1997; see also Widemo & Saether 1999) to mean, respectively; the investment in time or effort that a female is prepared to make in mate assessment, and the order in which a female ranks prospective mates. Both these components may be expressed as a function of the male sexual signal evaluated by the female and are referred to as responsiveness and preference functions.

Condition-dependent variation in these functions may have evolutionary consequences at intra- and inter-population levels (Wagner, 1994; Wagner, 1998; Pfennig & Tinsley, 2002; Velez & Brockmann, 2006; Cotton et al., 2006; Welch, 2003). Where individuals vary in the shape of their preference functions or the

strength of their responsiveness, this has the potential to influence the direction and intensity of sexual selection at the level of the population (Jennions & Petrie, 1997; Widemo & Saether, 1999; Cotton et al., 2006). Note that when variation in preference functions exists between individuals, the preference function at the population level may be distinctly different from those of individuals. For example, variation in individual directional linear preference functions can lead to a non-linear population preference function, e.g.; stabilising or frequency-dependent selection modes (Lesna & Sabelis, 1999; Partridge & Hill, 1984).

Sexual signals in the Orthoptera are comparatively well studied. Male crickets display to potential mates acoustically (Zuk, 1987), producing advertisement calls using stridulatory apparatus on the forewings. When the forewings are raised a plectrum on the (typically) left wing engages with a toothed file on the ventral surface of the right wing. As the wings are closed, the movement of the plectrum over the file sets up a vibration in resonant structures of both wings (Bennet-Clark, 2003; Bennet-Clark & Bailey, 2002). Calling in this way is energetically expensive (Hoback & Wagner, 1997), and the condition dependence of these calls has been investigated in a number of species. In *Gryllus campestris*, the rate of calling was found to decline under dietary stress in the lab (Scheuber et al., 2003a), and increase under an augmented food regime in the field (Holzer et al., 2003), but in neither case were individual call structure parameters (e.g. chirp duration, syllable number, dominant frequency) found to change. Similar results have also been published for properties of advertisement calls in two congeners; call rate in *G. lineaticeps* (Wagner & Hoback, 1999), and calling-bout duration in *G. integer* (Hedrick, 2005). While these studies were concerned with adult diet, the effect of nymphal nutrition has also been examined in *G. campestris* (Scheuber et al., 2003b), wherein poor diet during development was associated with advertisement calls that were less attractive due to a higher dominant frequency. This nymphal dietary stress was found not to affect timing-related call structure parameters. In addition to (long-range) advertisement calls, male crickets produce (short-range) courtship calls once a female approaches. However, in both *G. lineaticeps* (Wagner & Reiser, 2000) and *G. texensis* (Gray & Eckhardt, 2001), courtship calls were found to be unaffected by manipulation of adult diet. The courtship call of *G. texensis* was also insensitive to manipulation of nymphal diet (Gray & Eckhardt, 2001). In

general, therefore, it appears that advertisement calls tend to exhibit condition dependence whereas courtship calls do not, but that only certain properties of the call are affected.

This study focuses on the black field cricket *Teleogryllus commodus.* Evidence from this species fits into the general pattern as discussed above. Experimental diet manipulation revealed that higher protein intake was associated with an increase in the time males invested in calling (Hunt, Brooks, et al., 2004), whereas diet-induced condition dependence was not seen in call structure traits (Chapter 4), although an association has been found between one of these traits (syllable duration) and immunocompetence (Simmons et al., 2005). The advertisement call of *T. commodus* males is comparatively complex; beginning with a single chirp sequence, which is followed by a variable number of trill sequences (Bentley & Hoy, 1972; Hill et al., 1972; Appendix III). Female preferences for various parameters of the structure of this call have been studied by a number of groups, and both temporal (Pollack & Hoy, 1979; Hunt et al., 2005) and spectral (Hennig & Weber, 1997; Hill, 1974; Hunt et al., 2005) call properties are known to be important in eliciting phonotaxis in females. A preference for short intervals/high rate of calling is common in acoustic invertebrates (e.g. Jang & Greenfield, 1996; Kotiaho et al., 1996; Wagner, 1996; Hartbauer et al., 2006; Gerhardt & Huber, 2002), and a linear preference for shorter inter-call durations has been measured previously in *T. commodus* (Hunt et al., 2005).

Here we investigated how condition influences female mate choice decisions based on inter-call duration in *T. commodus*; a trait expected to experience directional selection, and known to differ between populations (Chapter 4). The methodology employed here owes much to that used by Hunt et al. (2005; 2004), who manipulated the condition of *T. commodus* from a single population. For this study, hatchlings from six (common-garden maintained) laboratory stock populations were reared on either a high or low-nutrient diet. We measured life-history traits as condition indicators, and then performed 2-way phonotaxis trials in order to measure both female preference between, and responsiveness to, simulated male advertisement calls. This allowed us to test for both effects of condition and differences between populations. Since our stock populations were derived from

wild populations that are known to be genetically divergent, detecting an interaction between these effects would be analogous to a GxE effect for mate choice; an effect that has, to our knowledge, only been demonstrated once before (Rodriguez & Greenfield, 2003).

## *6.3 METHODS*

### Establishment of Stock Populations

The populations used in this study are derived from collections made in February and March of 2007. Approximately 200 field-mated females were taken from each of six sites spanning the southern distribution of *T. commodus*. Populations from Western Australia, South Australia, Tasmania, Smith's Lakes and Kioloa (both in New South Wales), and the Australian Capital Territory are referred to subsequently as WA, SA, TAS, SL, KL and ACT respectively (q.v. Appendix II for details). Stocks were maintained in a constant temperature room at 28° ± 1°C, with a 16:8 hours light:dark regime. Stock animals are kept in 100L ventilated plastic containers with ad lib water and food – 'Go-Cat senior' cat food pellets – and cardboard egg boxes to provide shelter. Our captive populations had been lab reared for 5 generations under common-garden conditions before the start of this experiment, with at least 100 haphazardly assigned breeding pairs per generation. Even in the absence of genetic differences among populations, maternal effects are known to induce adaptive plastic responses that can resemble local adaptation (Agrawal et al. 1999), but after 5 generations of rearing under lab-standard conditions any differences in maternal effects resulting from differences in the habitat of collection ought to have been reduced to a negligible level (Roach & Wulff 1987).

### Diet Manipulation

Experimental animals were collected on the day of hatching, and haphazardly assigned to be raised on one of two diets. Common environmental effects, such as rearing individuals in the same container, have the potential to magnify differences between populations/lines and therefore lead to overestimates of the contribution

of genetics to differences in phenotype (Falconer & Mackay, 1996). In order to avoid this, hatchlings were housed individually in small plastic containers (7 x 7 x 5.5cm), each being provided with a water source and a small cardboard shelter. Thereafter these animals were kept in the same constant temperature room as the stock populations; at 28° ± 1°C, with 16:8 hours light:dark. All the animals within each population were collected at the same time and assigned haphazardly to treatment in order that related hatchlings (sibs or half-sibs) were not grouped within treatment. Food and water were replenished, the container cleaned, and nymph survival recorded on a weekly basis. On reaching the fifth instar, nymphs were checked daily for eclosion. Imagos were weighed on the day of eclosion, and their pronotum widths were measured using a binocular microscope and graticule.

Within each population, 300 individuals were reared; 150 in each of the 2 treatment groups ($n$ = 1800 nymphs). The high-nutrient diet group were fed the same cat food as the stock animals, whereas the low-nutrient diet group were fed a 50:50 mixture (by weight) of cat food and ground oats. Both diets were fed as pellets in order to control for compensatory feeding. Cat food and oats were ground to a fine powder and sieved to remove lumps. Each diet (100% cat food or 50:50 cat food and oats by weight) was then mixed with a small quantity of water to make a paste, which was then spread across a ~1cm thick rigid polymer sheet perforated with holes. After drying for 24hrs at 30°C, the identically sized pellets thus produced could be pushed out from the holes. Food pellets made in this fashion had a mean dry weight of 121 mg. This protocol was simplified from that used by Hunt et al. (2004; 2005) to manipulate resource acquisition. These pellets were stored in sealed containers at room temperature and discarded if any sign of moisture or mould became apparent. Both diets were presented as powder (using a lid of an 1.5ml centrifuge tube as a feeding bowl) for the first 4 weeks because young nymphs can have difficulty breaking the surface of food pellets.

### Phonotaxis Trials

The synthesised calls we played to females were made using SoundEdit 16 (Capps, 1988). From recordings of calling males from all six populations, we calculated mean values for the temporal parameters of the call, in addition to the dominant

frequency (Appendix III). The control call was made using these mean values. We also made 5 test calls by altering a single temporal parameter, the inter-call duration (ICD), of the control call; changing the ICD in each case to –1, +1, +3, +5 or +7 standard deviations from the mean ICD value. Test calls were saved as 1 channel of a stereo AIFF file, with the control call as the other channel in each case.

Females' call preference was tested on day 10 after eclosion. Tests were carried out in a rectangular arena (interior dimensions; 150 x 50 x 35cm) with acoustic insulation foam lining the interior walls. The arena was set up in an insulated room, with the temperature controlled at 28° ± 2°C. Calls were played from a laptop computer using SoundEdit 16 through an two-channel amplifier (Sub-Zero Ice ® 150w) connected to two Euro Tech ® 59-H60.01-02F speakers, one set into each end wall of the arena. Synthesised calls were played back as a continuous loop. Because the ICD differed between test call and control call, the relative start times of the two synthesised calls would change throughout the loop; we therefore assigned the start point haphazardly for each trial in order to avoid any consistent "leader-follower" effects that may otherwise have been present (Greenfield & Roizen, 1993; Minckley et al., 1995; Snedden & Greenfield, 1998; Hunt et al., 2005). Before each testing session, the control call was through the two speakers in turn and the sound pressure-level measured using a digital decibel meter (Tenma ® 72-6635) placed with its probe at the centre of the arena. This allowed the sound pressure-level to be standardised to 75 dB (re 20 µPa); a level consistent with the sound pressure output of a calling male.

Whilst in their rearing containers, *T. commodus* individuals tend to spend most of their time on or in the cardboard shelters we provide, and so we could place each female in turn into the arena by simply lifting their shelter out of their container and placing it on the centre-point of the arena floor. We then placed an upturned rearing container, perforated with 4mm holes, over the animal in its shelter before leaving it to acclimate to its surroundings for 2 minutes. After this period had elapsed, we simultaneously started both calls playing and removed the upturned container. We recorded a female as having expressed a preference once they crossed into one of the semicircular areas (extending 7cm from the centre of each of the speakers) that were marked on the arena floor. Preference was recorded as

a binary factor; 'focal call' or 'control call', and responsiveness was quantified as the latency to choose (seconds). Trials were conducted under red light to minimise disturbance by the observer. If a female had not entered either of the choice areas after 20 minutes, then it was recaptured and returned to the rearing container.

### Statistical Analysis

We used a MANOVA to test for population, diet and population x diet effects on development time, pronotum width and weight at eclosion to confirm the effectiveness of our manipulation. The data for weight and development time were not normally distributed and so were transformed, using log and boxcox transformations respectively before analysis.

Because female preference, recorded as test call or control call, is a binary variable; we used generalised linear models (GLMs) with binomial error structures to test for diet, population and test call effects, and for relationships with life-history variables.

We used an ANOVA to test for differences in latency (time to choose) between diet treatments, among populations, and among test calls, and a GLM to test for relationships with life-history variables. Since the data for latency had an approximately lognormal distribution, they were log-transformed before analysis. Before analysing data for latency, we tested for a relationship between latency and preference using a ordinal logistic regression. All analyses were performed using R (R Development Core Team, 2009).

**Table 6.1**: Numbers of female phonotaxis trials conducted by diet treatment and population.

| Pop. | High-nutrient diet | Low-nutrient diet |
|---|---|---|
| **ACT** | 50 | 52 |
| **KL** | 45 | 50 |
| **SA** | 51 | 54 |
| **SL** | 40 | 50 |
| **TAS** | 51 | 51 |
| **WA** | 51 | 53 |
| Total | 288 | 310 |

## *6.4 RESULTS*

We ran a total of 598 phonotaxis trials (see Table 6.1 for breakdown by population and by diet treatment). A MANOVA of the life-history parameters we measured (weight and pronotum width at eclosion, and development time) showed significant differences between populations (Wilks' $\lambda$ =0.64, $F_{15,1980}$ =23.23, $P$ <0.001), a significant effect of diet (Wilks' $\lambda$ =0.93, $F_{3,717}$ =19.4, $P$ <0.001) and also a significant population-by-diet interaction effect (Wilks' $\lambda$ =0.95, $F_{15,1980}$ =2.71, $P$ <0.001, Table 6.2). Females reared on the low-quality diet emerged significantly later than those fed the high-quality diet, but this extended development time was associated with a significant increase in both weight and pronotum width at eclosion. These effects are plotted as reaction norms for diet in Figure 6.1.

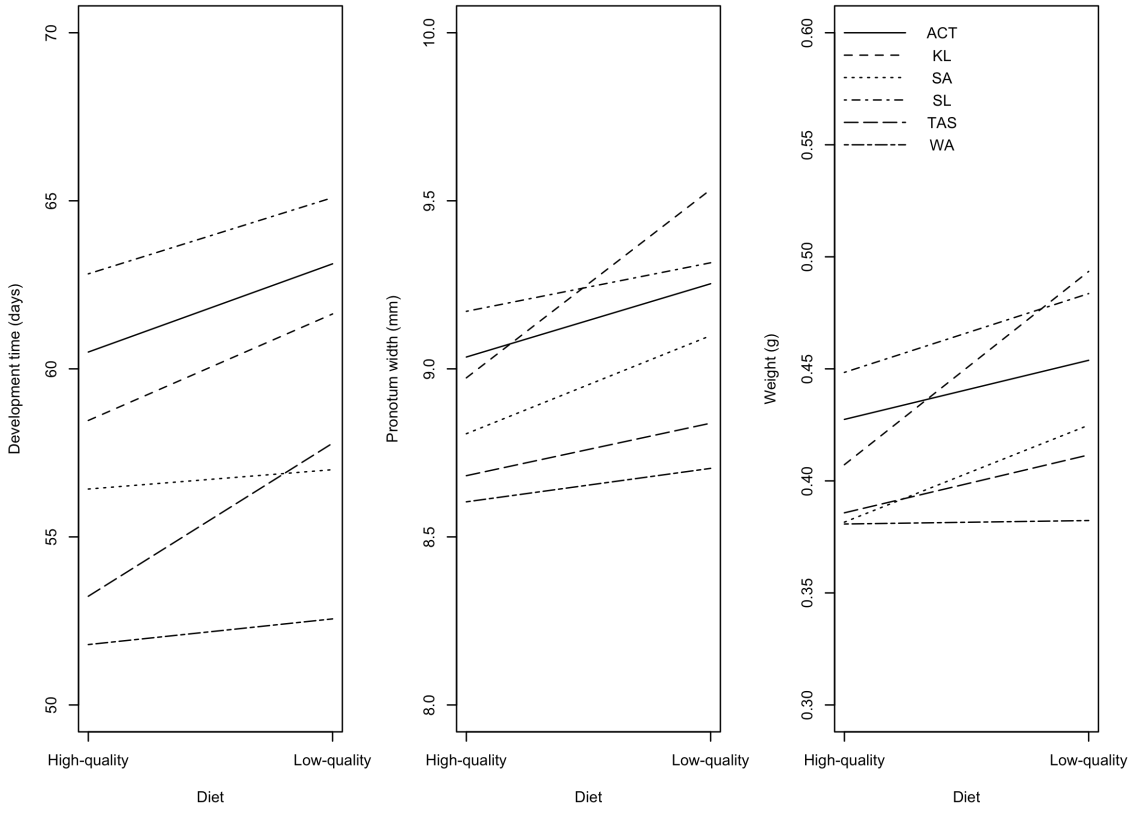


**Figure 6.1:** Reaction Norm plots for the three life-history traits we measured; from left to right; development time (days), pronotum width (mm) and weight (g).

**Table 6.2**: The effect of diet on female life-history traits; trait means (± SE) and ANOVA statistics. Significant *p*-values are in italics.

| | Treatment means | | ANOVA statistics | | | |
|---|---|---|---|---|---|---|
| | Control Diet | Low Nutrient Diet | Sum of Squares | DF | *F* | *P* |
| Pronotum width (mm) | 8.86 (0.03) | 9.12 (0.03) | 12.8 | 1 | 42.0 | *<0.001* |
| Mass at eclosion (g) | 0.40 (0.004) | 0.44 (0.004) | 0.26 | 1 | 45.7 | *<0.001* |
| Development time (days) | 56.8 (0.3) | 59.3 (0.4) | 0.05 | 1 | 17.7 | *<0.001* |
| *N* | 361 | 370 | | | | |

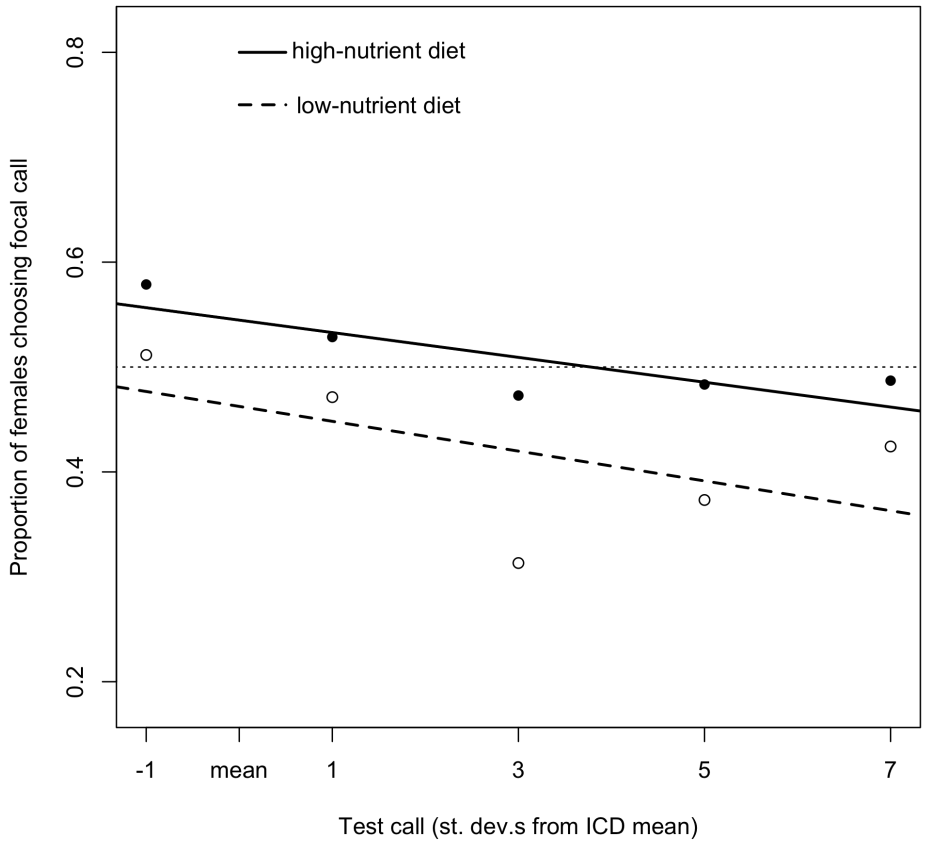


**Figure 6.2:** Preference functions for females reared on both high-nutrient (solid lines and filled circles) and low-nutrient (dashed lines and open circles) diets; points represent cross-population means. The mean ICD value for calls of the males from these populations (0 on the x axis) is marked as "mean". The horizontal dotted line indicates the case where 50% of females prefer each call.

A generalised linear model with a binomial error structure was used to analyse data on preference; with population, diet and test call as main effects, and all possible interactions included. This analysis showed no significant interaction effects, nor a main effect of population on preference, however there were significant effects of test call ($\chi^2$=5.29, df=1, $p$=0.02) and of diet ($\chi^2$=4.07, df=1, $p$=0.04, Table 6.3). These results remained qualitatively unchanged after stepwise model reduction. The preference function is plotted in Figure 6.2.

**Table 6.3**: Results of a GLM on female preference. Since preference is a binary factor (control call or test call), this model used a binomial error structure. Significant *p*-values are in *italics*.

| | Degrees of freedom | Chi-Squared | *P* -value |
|---|---|---|---|
| Population | 5 | 9.25 | 0.10 |
| Diet | 1 | 4.11 | *0.04* |
| Test | 1 | 5.42 | *0.02* |
| Pop x Diet | 5 | 2.12 | 0.82 |
| Pop x Test | 5 | 0.27 | 0.99 |
| Diet x Test | 1 | 0.07 | 0.80 |
| Pop x Diet x Test | 5 | 3.98 | 0.55 |
| Error | 574 | | |

An ordinal logistic regression of latency against preference found no significant relationship ($\chi^2$=0.52, df=1, $p$=0.47). Consequently, we continued to analyse the data for latency using models without a preference effect, i.e.; the following tests relate to responsiveness, independent of which call females chose. We ran a full factorial ANOVA to test for patterns in our data on latency; our model included population, diet treatment and test call as main effects. We found no significant main effects of diet treatment or test call. There was a significant effect of population ($F_{5,574}$ =3.00, $p$=0.01), and there was also a significant 3-way population x diet x test call interaction ($F_{5,574}$ =2.83, $p$=0.02, Table 6.4). These results remained qualitatively unchanged after model reduction. This complex interaction (Figure 6.3) means that a female's responsiveness depended not only on which test call she heard, but also on which population she belonged to and on her rearing diet.

A general linear model was used to test for relationships between latency and life-history traits. The only significant relationship found was a slightly positive one between development time and latency ($R^2$=0.01, $F_{1,596}$ =6.40, $p$=0.01); longer develop times are associated with longer latency times.

**Table 6.4:** Results of an ANOVA to test for differences in latency to choose. Significant *p*-values are in *italics*.

| | Degrees of freedom | Sum of Squares | Mean Squares | F value | *P* -value |
|---|---|---|---|---|---|
| Population | 5 | 7.88 | 1.58 | 3.00 | *0.01* |
| Diet | 1 | 0.42 | 0.42 | 0.80 | 0.37 |
| Test | 1 | 1.42 | 1.42 | 2.69 | 0.10 |
| Pop x Diet | 5 | 1.32 | 0.26 | 0.50 | 0.78 |
| Pop x Test | 5 | 2.32 | 0.46 | 0.88 | 0.49 |
| Diet x Test | 1 | 0.31 | 0.31 | 0.60 | 0.44 |
| Pop x Diet x Test | 5 | 1.49 | 1.49 | 2.83 | *0.02* |
| Error | 574 | 301.78 | 0.53 | | |

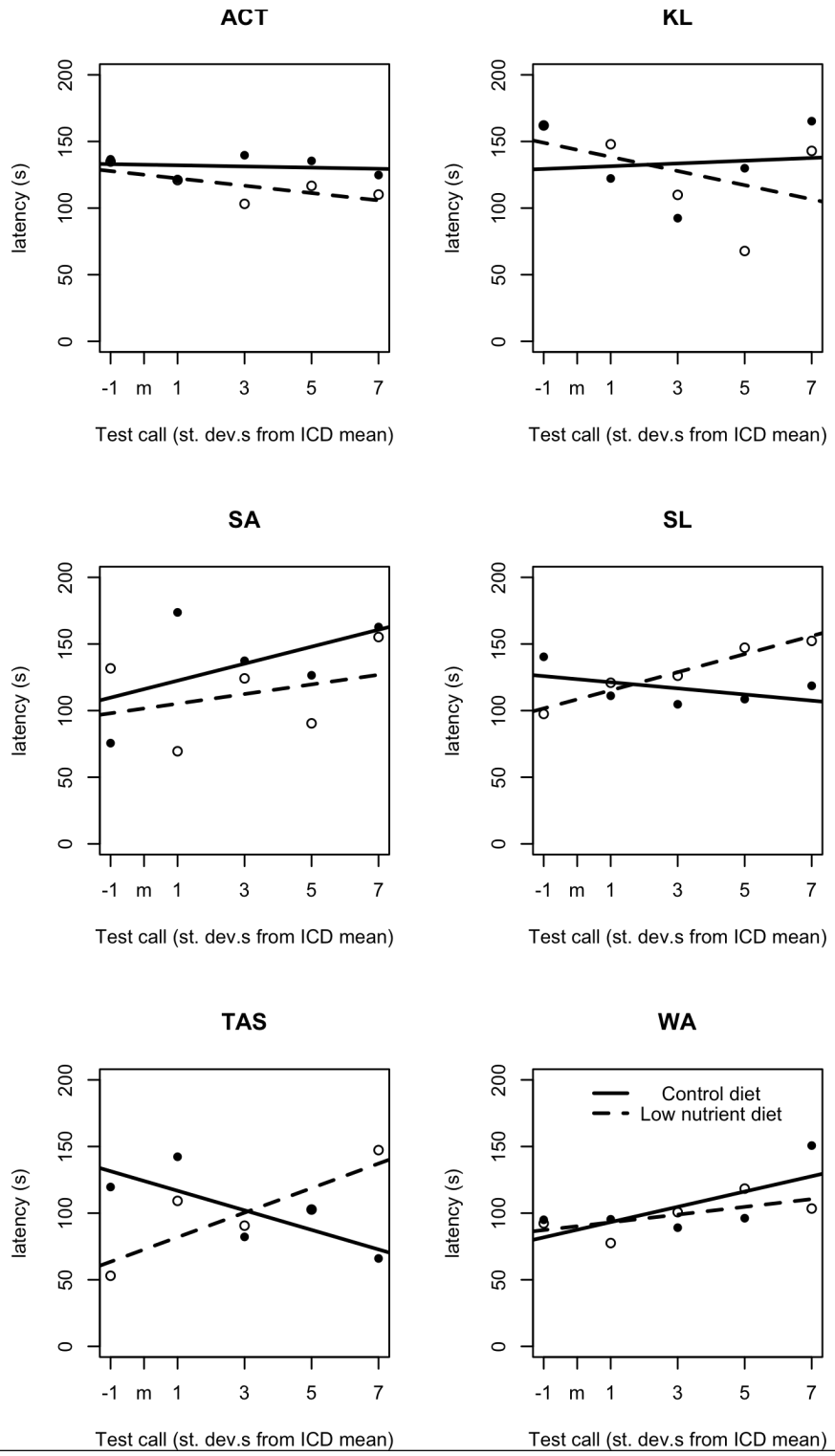


**Figure 6.3:** Plots to illustrate the significant interaction of population x diet x test for responsiveness (latency to choose); Responsiveness functions are plotted for females reared on both high-quality (solid lines and circles) and low-quality (dashed lines and open circles) diets. The mean ICD value expressed by males of these populations (0 on the x axis) is marked as "m". Each graph represents a single population (see text for abbreviations).

## *6.5 DISCUSSION*

Experimentally induced nutritional stress had a significant influence on a number of female traits in the *T. commodus* populations studied here. Not only was life-history affected, females were also observed to adjust the elevation, but not the slope, of their preference function under nutritional stress. Female responsiveness was influenced by a complex test x population x diet interaction.

Our dietary manipulation significantly influenced all the life-history traits we measured in this study, with animals reared on the low-nutrient diet taking longer to reach eclosion, but eclosing larger and heavier than animals reared on the high-nutrient diet (Table 6.3). Experimental nutritional stress has previously been observed to affect the body size of imagos, but this has usually resulted in a reduction, rather than an increase, in adult size (Fox, 1997; Godfray, 1993).

In *T. commodus*, a previous study using different experimental diets found an increase in development time associated with a reduction in mass at eclosion under dietary regimes with lower protein content (Hunt et al., 2005). That study used diets with protein contents of ~45%, ~37% and ~29%. The protein contents of our diets were ~32% (high-quality) and ~22% (low-quality) and so similar in range to the two lower protein diets of Hunt et al. (2005), but there are likely to be differences in the levels of other nutrients between their fish-food based diets and our cat-food based diets.

Compensatory (catch-up) growth has been well documented in a number of taxa (Arendt, 1997), wherein animals escaping nutritional stress grow at an accelerated rate compared to those that did not experience nutritional stress. A few studies have also described compensatory growth to result in over-compensation; in increased body size/mass compared to unstressed individuals (Hayward et al., 1997). The pattern observed here, however, seems more likely to indicate that larval feeding continues until a nutritional threshold is reached (Behmer & Elias, 1999; Fronstin & Hatle, 2008). Since *T. commodus* is univoltine in the wild, with populations maintained over the winter by eggs in diapause, it is reasonable to assume that selection would favour shorter development times, given the nutritional state of the individual. If a minimum level of protein were necessary for eclosion or development to sexual maturity, then females would be expected to

feed more heavily if the available food was lower in protein. Similar behaviour has been observed in *Omocestus viridulus* grasshoppers (Berner et al., 2005), which respond to low-nutrient levels by compensatory feeding to maintain their nitrogen intake. I speculate, therefore, that animals reared on the low-quality diet consumed more food in order to sequester enough nutrients to successfully eclose, storing the excess volume as fat body mass, and thus were more massive at eclosion than those on the high-quality diet, that were able to develop more quickly. Body size is often positively correlated with fecundity in female insects and thus is measured as a fitness correlate (Honek, 1993), hence, although the resulting increase in development time is likely to be maladaptive, an increase in body size may not be.

The association between longer development time and lower responsiveness (longer latency to choose) is the opposite of the association found for the Smith's Lake (SL) population by Hunt et al. (2005). However, although statistically significant, this relationship explains only ~1% of the variance in female responsiveness ($R^2$=0.01) and is therefore unlikely to be biologically significant.

The significant effect of test call on preference indicates that the animals tested did express a non-random preference function; shorter ICD values were preferred (Figure 6.2). A preference for short intervals/high rate of calling is common in acoustic invertebrates (e.g. Jang & Greenfield, 1996; Kotiaho et al., 1996; Wagner, 1996; Hartbauer et al., 2006; Gerhardt & Huber, 2002), and in accordance with our results, this preference has been measured previously in this species (Hunt et al., 2005). The generality of this preference fits well with the hypothesis that females' preference is selecting males based on their call production per unit time (e.g. Bentsen et al., 2006), and thus on either condition or intrinsic metabolic performance (Berg & Greenfield, 2005). Further support for this mechanism comes from a number of studies that have demonstrated positive correlations between calling rate and measures of metabolism in other Orthopterans (Hartbauer et al., 2006; Hoback & Wagner, 1997; Ketola et al., 2009), and from the increased calling effort seen in *T. commodus* males of high condition (Hunt et al., 2004).

Our diet manipulation also significantly influenced the elevation of the preference functions of the animals in this study, as demonstrated by the significant effect of

diet on female preference. The lack of an interaction of test x diet signifies that females reared on the high-nutrient diet expressed the same preference function as those on the low-nutrient diet, though they did so at a reduced elevation (Figure 6.2). That is, females reared on the high-nutrient diet accepted more calls compared to those reared on the low-nutrient diet, irrespective of ICD of the test call they heard. This difference in the intercept, but not the gradient, of the preference function suggests that female choice is costly, and that a female's nutritional state influences her ability to meet those costs.

A number of previous studies in the literature have manipulated diet and revealed a positive association between female nutritional condition and the expression of mate preferences (Brown, 1997; Lesna & Sabelis, 1999; Hebets et al., 2008; Hunt et al., 2005; Hingle et al., 2001). Additionally, other studies have found evidence for an association between female preference and traits used as indices of condition (Jennions et al., 1995; Bakker et al., 1999; Beeler et al., 2002; Bleay & Sinervo, 2007). A considerable amount of research effort has been directed towards the influence of parasites on sexual selection, largely with reference to the hypothesis that male sexual signals may function as an immunocompetence handicap (Folstad & Karter, 1992; Roberts et al., 2004; Hamilton & Zuk, 1982). However, there is also evidence for parasite-mediated condition-dependence of mating preferences; both from correlational (Pfennig & Tinsley, 2002; Poulin, 1994) and manipulative (Lopez, 1999; Mazzi, 2004) studies. This disparate evidence accords with ours in indicating that the strength of female preference can be expected to decrease with declining condition.

The lack of an effect of population shows that the direction of preference is similar among populations, and the lack of an interaction effect of test x population is indicative of the constancy of preference functions among populations. The lack of a significant interaction effect of diet x population means that populations did not respond differently to the effect of diet, and the lack of a 3-way interaction effect of test x diet x population indicates that the preference function does not differ among populations in its response to diet manipulation. To summarise; our animals expressed a consistent preference function, applying linear selection for shorter ICD values, but those females reared on the high-quality diet expressed

this preference function at a greater elevation than those females reared on the low-quality diet.

Our diet manipulation had no independent effect on female responsiveness (latency to choose), but we did find different levels of responsiveness among populations (Table 6.4). However, it would be inappropriate to interpret this in isolation, since population was involved in a significant 3-way interaction with diet and test effects (Figure 6.3). Here we shall characterise the relationship between latency to respond and stimulus (test call in this case) as a "responsiveness function" analogous to preference function (the relationship between preference and stimulus). Couched in these terms, this 3-way interaction can be thought of as an inter-population difference in how the females' responsiveness function is affected by diet. As can be seen in Figure 6.3, the gradient of these responsiveness functions varies considerably between populations, and in some cases between diet treatments also. Given that we know these populations are genetically divergent (Chapters 3 and 4) this interaction is comparable to a GxE-type effect. The source populations for our lab stocks are widely distributed about the southern half of Australia (Appendix II) and can safely be presumed to experience a large range of environmental conditions (e.g. mean annual rainfall data ranges over an order of magnitude from ~71mm to ~750mm, source: Aus. Bureau of Meteorology, www.bom.gov.au), providing ample opportunity for behavioural differences to arise by local adaptation.

Despite drawing research attention for many years, the extent to which mate choice may result in indirect (good-genes) benefits is still unclear (Qvarnstrom et al., 2006; Kokko et al., 2006). The problem at the heart of this debate is the lek paradox: the expected erosion of genetic variance for traits consistently chosen by females (Kirkpatrick & Ryan, 1991; Rowe & Houle, 1996; Hine et al., 2004). We should therefore expect indirect benefits to be small – why should females continue paying the costs of being choosy? One high-profile solution to this problem is the condition-dependence of signal trait (Rowe & Houle, 1996), though there is no a priori reason not to apply similar reasoning to the expression of preference (Jennions & Petrie, 1997). Another concept implicated in the maintenance of genetic variance is that of genes by environment interactions

(Rodriguez & Greenfield, 2003; Hunt et al., 2004), wherein the expression of a given genotype varies across environments. If this interaction is strong enough (ecological cross-over) then no single genotype is superior in all environments and indirect benefits may thus be preserved. Our results are compatible with interpretation under either or both of these conceptual frameworks, but in either case the outcome is that males in disparate populations may be experiencing subtly differing regimes of sexual selection for ICD despite the conservation of the preference function itself.

To conclude, my experiment shows a linear preference function that is conserved among genetically divergent populations, but altered in intensity (i.e. the elevation of the preference function) under diet manipulation. In addition, females' responsiveness function was found to differ between populations. That is, while females from all populations and both diet treatments expressed the same preference, females on the low-nutrient diet accepted fewer calls (irrespective of which they heard), and the latency to express any preference varied among populations.

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# CHAPTER 7: General Discussion

Interactions at various levels of biological organization may contribute to the overall observed pattern of phenotypic integration in an organism. Whether the interactions in question are epistasis between loci within individuals or mechanical interaction of morphological traits, our understanding of the origin and evolution of the phenotype is incomplete if our attention is focussed on single traits. Schluter (2000) cautioned against considering evolvability in a univariate fashion, since even heritable traits that exhibit standing genetic variance may not be able to respond to selection due to covariance with other traits. In light of this, though $V_A$ and $h^2$ may be seen as prerequisites for phenotypic evolution, they are poor measures of evolvability when estimated for single traits. This message has received support from Blows (2007) and Houle (2007), who both advocate the study of adaptation as a multivariate process. The importance of this viewpoint is underlined by my finding in Chapter 2 of more pronounced lines of least evolutionary resistance (LLER's) in life-history or sexually selected traits than in morphological traits (i.e. variance was more evenly distributed among eigenvectors in morphological traits). This difference suggests that LLER's may have more influence on the direction of evolution for life-history and sexually selected traits than for morphology; a difference between trait types that would be undetectable without a multivariate perspective.

The uses of the **P** and **G** matrices in quantitative genetics are based on the assumption that these matrices do not change over evolutionary time. One of the most common uses for **P** and **G** is the multivariate extension of the breeders' equation (Lande, 1979), which is used both to estimate past selection, and to predict the response to known selection. The breeders' equation can be expressed as: $\Delta\bar{z} = \mathbf{G}\mathbf{P}^{-1}\mathbf{S}$, wherein $\Delta\bar{z}$ is the vector of changes in trait means, **G** and **P** are the genetic and phenotypic variance-covariance matrices, and **S** is the vector of selection differentials. There is ample evidence, from both theoretical and empirical studies, to indicate that the structure of that these matrices can and do evolve, both in response to selection and through drift (e.g. Agrawal et al., 2001; de

Oliveira et al., 2009; Eroukhmanoff et al., 2009; Jones et al., 2004; Phillips & McGuigan, 2006; Phillips et al., 2001; Roff, 2000; Roff et al., 1999; Roff et al., 1999). It is therefore important for refining our understanding of phenotypic evolution that we quantify the speed and scale of these evolutionary changes in covariance matrices.

There are formidable challenges in making this determination, however, due to the way that integration (as quantified by the **P** and **G** matrices) interacts with selection. There is evidence that stabilising selection ought to favour the evolution of genetic correlations between traits that reflect their developmental or functional relationships (Cheverud, 1984; Cheverud, 1996a; Jones et al., 2004; Lande, 1979; Lande, 1980). Conversely, disruptive selection ought to disfavour the accumulation of such correlations (Cheverud, 1984; Cheverud, 1996b). However, since the structure of integration can determine which trait combinations are exposed to selection (Pigliucci, 2003; Jernigan et al., 1994), trait covariance matrices are not simply products of selection but can influence the way the individual traits respond to selection.

The strength and stability of patterns of integration have important implications for our understanding of phenotypic evolution. Traditionally, a **P** matrix showing strong integration, with covariances displaying little environmental plasticity, has been interpreted as evidence for a history of multivariate stabilising selection (Armbruster & Schwaegerle, 1996; Schluter, 2000). Once a pattern of integration has evolved, however, it is largely unclear whether we should expect this to act to constrain or facilitate phenotypic evolution (Pigliucci, 2003). Most likely, the answer to this question will depend upon the alignment, or otherwise, of direction(s) of integration with direction(s) of selection (Pigliucci, 2003; Pigliucci & Preston, 2004; Schluter, 1996). The most intuitive way to illustrate this interaction is to visualize the variance around the mean for two traits that covary (Figure 7.1). The ellipses in each plot represent 95% confidence regions about the population mean. From the initial distribution of individual values in Figure 7.1(a), note that in the case of trait elaboration (*sensu* Endler et al., 2005; Figure 7.1(b)) the mean shifts along the direction of greatest variance. By contrast, the change from Figure 7.1 (a) to (c) can be characterized as innovative (also *sensu* Endler et

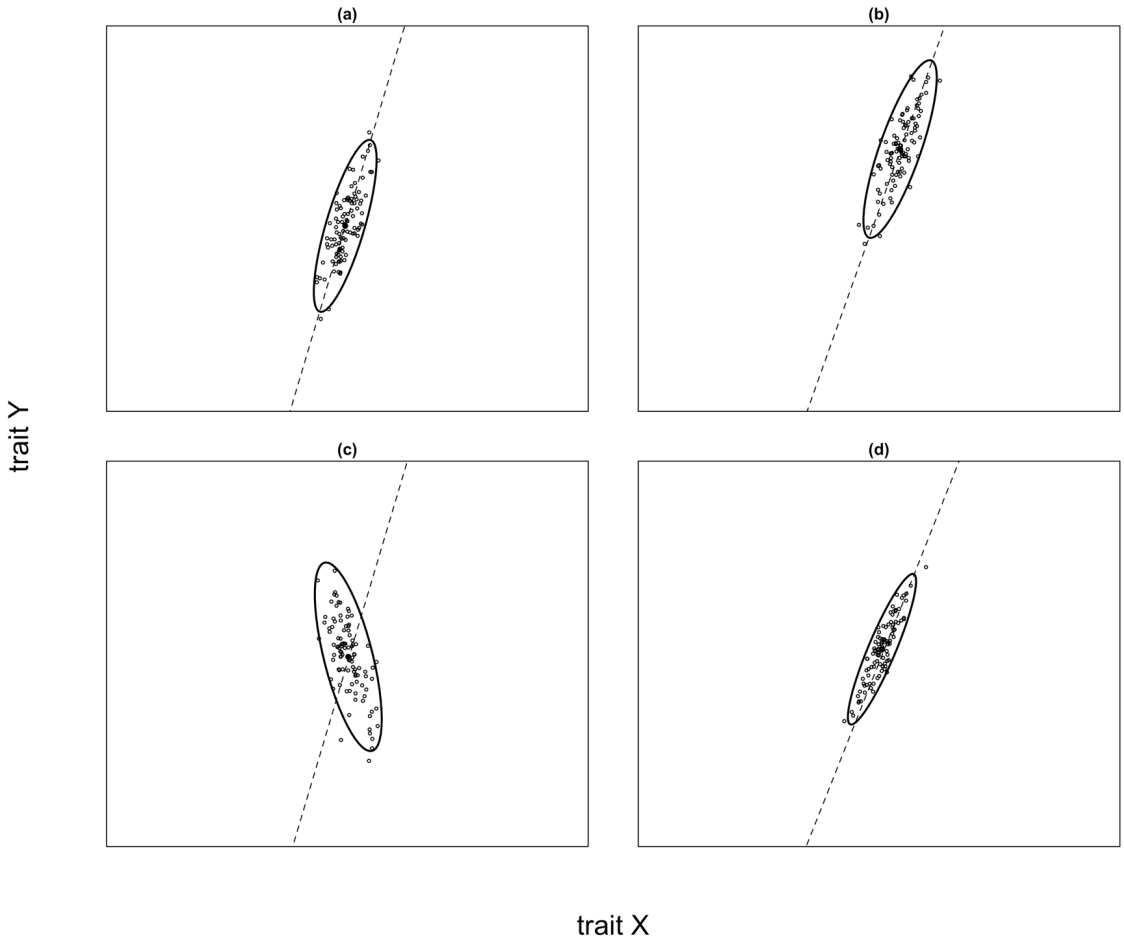


**Figure 7.1**: Characterizing multivariate phenotypic change: Plot (a) represents the distribution of individual values for two correlated traits; X and Y, with the ellipse marking the 95% confidence region around the bivariate mean, and the dashed line representing the principal direction of covariance. Plot (b) shows the condition in which the population mean evolves along the principal direction of covariance. This could be characterized as 'elaboration'. Note that, since the traits covary, this outcome may result from directional selection for an increased value of either trait. Plot (c) shows a change in the population covariance, though the bivariate mean remains unchanged, which could be characterized as trait 'innovation'. Plot (d) demonstrates the results of selection for the reduction of variance in both traits; again the mean remains unchanged. Such a change in trait variance, but not in means or in covariance, could be characterized as 'scalar'.

al, 2005). Note that in Figure 7.1 (c) the population covariances have evolved, and there is now variance present in regions of phenotypic space that were previously inaccessible, even though that the population mean has not changed. Lastly, Figure 7.1(d) shows a change that I shall characterize as 'scalar'; a change in the trait variances, but without a change in either the mean or the covariance structure. I have plotted these changes in terms of trait values, since my aim is to characterize phenotypic change, but it is easy to see that by plotting breeding values for the traits in the same way, one could characterize modes of phenotypic evolution.

The empirical part of this thesis deals with phenotypic measures (or **P**) rather than quantitative estimates (**G**), and therefore I am limited as to what I may infer about call evolution by my experimental design. In Chapter 3, I addressed the constancy of **P** for the advertisement call of *Teleogryllus* commodus among six divergent populations using common-garden rearing. Although 1st and 2nd moment differences (trait means and variances) between populations were detectable in both field-caught and lab-reared cohorts, the structure of **P** was relatively conserved. Chapter 4 addresses the related issue of the plasticity of **P**, specifically related to nutritional stress. I reared hatchlings derived from the same populations under standard laboratory conditions, fed on standard or low-nutrient food, then recorded and analysed the advertisement calls of males from each population reared on each diet. Once again, I found significant 1st and 2nd moment differences between populations, in addition to a similar level of difference being observed between diet treatments. However, **P** retained a common covariance structure among populations and between diet treatments. Thus the effects of both population divergence and diet on **P** can be characterized as a combination of elaborative and scalar changes (corresponding to Figure 7.1(b) or (d)) rather than as innovation (i.e. a change in patterns of covariance structure – Figure 1(c)).

The calls measured for Chapter 3 were recorded from animals derived from a collection made by J. H. in 2002, and those measured from Chapter 4 were recorded from animals derived from a separate collection (made by myself and J. H.) in 2007 from the same locations. I was therefore able to take the opportunity to compare **P** matrices within populations over the intervening 5-year period. The results of these comparisons were qualitatively similar to those shown in Chapters

3 and 4. A MANCOVA of call trait values, using pronotum width as a covariate, revealed significant 1st and 2nd moment differences between collections (Wilks' $\lambda$= 0.002, $F_{5,333}$= 28006.0, $P$ <0.0001) and between populations (Wilks' $\lambda$= 0.742, $F_{25,1238}$= 4.1, $P$ <0.0001), with a significant interaction of collection by population (Wilks' $\lambda$= 0.701, $F_{5,1238}$= 5.0, $P$ <0.0001). Post-hoc ANOVAs revealed that all five call traits differed significantly between collections, and that the collection by population interaction was driven by two traits only; chirp inter-pulse duration (CIPD) and inter-call duration (ICD). Using Roff's (2002) Jackknife MANOVA method, I detected differences in matrix structure between collections, among populations and as an interaction between population and collection (Table 7.1).

**Table 7.1:** The results of a MANOVA on Jackknife pseudovalues for the phenotypic variances and covariances calculated for the common-garden reared cohort from Chapter 3 and the control (high-nutrient) group from Chapter 4. These crickets are derived from independent field collections in 2002 and 2007 respectively.

| | Pillai's Trace | F value | Degrees of Freedom | | P value |
|---|---|---|---|---|---|
| | | | Hypothesis | Error | |
| **Pop** | 4.843 | 673.09 | 75 | 1640 | <0.0001 |
| **Collection** | 0.998 | 12421 | 15 | 324 | <0.0001 |
| **Pop x Collection** | 4.82 | 585.72 | 75 | 1640 | <0.0001 |

As in Chapters 3 and 4 I then characterized these differences using Krzanowski's (1979) geometric matrix comparison method, and detected low to intermediate levels of matrix divergence (Table 7.2(a)). According to both test values, the differences between collections were larger (i.e. smaller sum of **T** eigenvalues, and greater angle between closest eigenvectors) than the differences between diet treatments from Chapter 4, which are themselves larger than the differences between wild-caught and common-garden reared crickets from Chapter 3 (Table 7.2(b)). In spite of these differences, however, none of the contrasts of **P** between collections were significantly different when using the Mantel test of matrix correlation. In addition to this, the results returned by matrix comparisons on Flury's Hierarchy (Common Principal Components or CPC analysis) overwhelmingly indicate shared principal components, with all comparisons

finding matrix equality using the 'jump-up' approach (Table 7.3). It appears, therefore that the plastic changes in matrix structure I detected in response to dietary stress are intermediate in magnitude; less extreme than the inter-population differences in matrix structure I found in Chapter 3, but greater in extent than the within-population differences that exist between collections. This work therefore adds further weight to the claim that **P** for the advertisement call remains largely stable in this species.

**Table 7.2:** (a) The results of my geometric matrix comparisons of **P** within each population for the two different collection dates (2002 versus 2007). Angles are expressed in degrees. See Chapters 3 and 4 for detailed explanations of this method. The lower part of the table (b) compares mean divergence values from this analysis with those from Chapter 3 (field vs. common-garden) and Chapter 4 (high- vs. low-nutrient treatments).

| | Population | F value | Angle between closest eigenvectors |
|---|---|---|---|
| (a) | **ACT** | 1.01 | 15.3 |
| | **KL** | 0.96 | 13.8 |
| | **SA** | 1.15 | 5.46 |
| | **SL** | 1.32 | 7.2 |
| | **TAS** | 0.99 | 19.9 |
| | **WA** | 0.99 | 7.73 |
| (b) means | **between collections** | 1.07 | 11.6 |
| | **field vs lab** | 1.99 | 0.3 |
| | **control vs poor diet** | 1.66 | 5.8 |

In both Chapter 3 and Chapter 4, the majority of differences between populations can be assumed to be genetic in origin, since the environmental contribution to **P** was provided by our controlled laboratory conditions. As discussed in Chapter 4, it is known that maternal effects can lead to the appearance of local adaptation (Agrawal et al. 1999), but this is not a potential source of differences between populations, since experimental animals were drawn from stocks that had been maintained in the lab for at least three generations, and thus any maternal effects ought to be equivalent. Some divergence of the underlying **G** therefore seems

possible, but it would be unwise to assume that this is the case, given there appears to be plasticity enough in the structure of **P** that environmental changes (e.g. diet) can result in changes in **P** that are of a similar magnitude to the present level of population divergence.

**Table 7.3**: The results from between-collections comparisons (2002 versus 2007) of **P** for each population using Common Principal Components analysis.

| | Common PC analysis approach | | |
|---|---|---|---|
| **Population** | **Step-up** | **Model building** | **Jump-up** |
| **ACT** | CPC(3) | equality | equality |
| **KL** | CPC | equality | equality |
| **SA** | CPC | equality | equality |
| **SL** | CPC(3) | equality | equality |
| **TAS** | CPC | equality | equality |
| **WA** | equality | proportionality | equality |

What could explain this stability of **P** matrices, despite 1st and 2nd moment differences? Since the covariances of **P** may be shaped by a number of causes, there are a number of possible explanations; genetic, developmental, mechanical or selective. These are, of course, not mutually exclusive possibilities. One could posit an unchanged **G** underlying our stable **P**, and certainly I would predict that any changes in **G** over a similar range of call divergence would be of lesser magnitude than those found in **P** (simply because additive-genetic variance contributes to both **P** and **G**, yet **P** may be influenced by non-additive and non-genetic factors also). Alternatively, it may be that there are developmental constraints involved in determining the shape of **P**. Such effects could arise from developmental trade-offs among the traits required for song production, such as the forewings, the thoracic musculature connected them and the nervous system that regulates their motion, or between these and non-call-related traits. An effect of this sort has been found in the cricket *Gryllus firmus*, where the thoracic musculature trades off against testis mass (Saglam et al., 2008).

What I referred to as mechanical constraint could arise from covariance between the acoustic parameters of the advertisement call and the morphology of the

forewings used to produce them. An *a priori* reason to expect such covariance comes from the 'clockwork cricket' model (Koch et al., 1988; Bennet-Clark & Bailey, 2002; Prestwich et al., 2000), which predicts a negative relationship between the area of the resonant structures and the frequency of the call produced. Such a relationship has previously been found in field crickets, specifically in *Gryllus campestris* (Simmons & Ritchie, 1996) and in *Gryllus firmus* (Webb & Roff, 1992). To date however, such studies have been limited to testing for bivariate relationships between one call parameter and one morphological metric. In Chapter 5 I used geometric morphometrics techniques and partial least squares regression (PLS) to extend this approach, and test for relationships between elements of forewing shape and geometric size, body size and acoustic parameters of the advertisement call. This revealed a very strong allometric relationship between body size and wing size and a modest multivariate relationship between wing size and a PLS axis defined by the measured acoustic parameters. Larger wings were associated with reduced dominant frequency and increased values for the other four focal call parameters; all of which are timing-related. When I examined wing shape, the analyses revealed a relationship (also of modest intensity) defined by the principal PLS axis of covariance with call structure. Spectral (i.e. dominant frequency) and temporal (i.e. trill number, chirp inter-pulse duration, inter-call duration and chirp pulse number) call traits were found to have loading of opposite sign on this axis also, and the associated shape vector could be characterised as a change in aspect ratio (i.e. a change in the ratio of breadth (chord) of the wing to wing length). In the clockwork cricket model the catch-and-release of the plectrum on one wing by the file on the opposing wing is suggested to act as an 'escapement' (Bennet-Clark & Bailey, 2002; Elliott & Koch, 1985), regulating the duty cycle of the open-close motion of the wings. Both wing size and aspect ratio have the potential to influence the wings' aerodynamic drag and therefore, the speed with which the wings may be moved during calling. Since spectral and temporal parameters covary in opposite directions with both size and shape variance, my findings are consistent with the hypothesis that the escapement mechanism of the clockwork cricket allows temporal and spectral call properties to act as constraints upon each other.

A fourth possibility is that the covariance structure of advertisement calls is maintained by selection. Multivariate sexual selection on calls in *Teleogryllus commodus* has been measured, both in the lab (Brooks et al., 2005) and under natural conditions (Bentsen et al., 2006). These authors used conventional selection analyses (Lande & Arnold, 1983) to estimate the vector of linear selection gradients ($\beta$) and the matrix of non-linear selection gradients ($\gamma$), and then performed a canonical rotation of $\gamma$ (Phillips & Arnold, 1989) to extract the major axes of the multivariate response surface. In both cases significant multivariate stabilizing selection was found along multiple axes, and this would fit the hypothesis of selection acting to maintain the structure of **P**. However, Bentsen et al (2006) also found multivariate significant disruptive selection acting on one axis, and both they and Brooks et al (2005) also found significant directional selection acting on multiple major axes. These two pieces of conflicting evidence suggest that the relationship between selection and the stability of **P** is unlikely to be clear-cut. Furthermore, Brooks et al (2005) report significant linear selection acting to reduce inter-call duration (ICD), a result which is supported by my finding (in Chapter 6) of a negative preference function for ICD expressed by females from all six of my study populations (i.e. all females, irrespective of population, showed a similar preference for reduced ICD). In addition to the lack of inter-population difference in the preference function for ICD, I also show in Chapter 6 that diet, though it did influence female choosiness, had no significant effect of the slope of the preference function for ICD. However, although this evidence suggests that some elements of selection on advertisement calls may be common among populations, it would be unwise to assume that the multivariate patterns of selection are equivalent, especially in light of the complexity of the selection found in these previous studies (Bentsen et al., 2006; Brooks et al., 2005) and the genetic divergence I demonstrate between populations (Chapter 3).

Indeed, the integration of call structure in my study populations may be constraining the response to this linear selection. To demonstrate this, I pooled call data across populations from the control group in Chapter 4 and calculated a single estimate of **P**; this was justified because of the stability of **P** observed across populations. Diagonalization of **P** reveals that ~98% of the variance present can be described by the first two eigenvectors (Figure 7.2(a)). In Chapter 6, I calculated

the preference functions for ICD for each population and showed that the slope of the negative preference function (i.e. selection for reduced ICD) did not differ across populations or with diet (Figure 7.2(b)). The females in the control group for this experiment were drawn from the same populations and reared under identical conditions to the males whose calls I recorded for Chapter 4. Once the **P** matrix has been diagonalized, it becomes clear that the first two eigenvectors ($\mathbf{p}_{max}$ and $\mathbf{p}_2$) encompass very little variance in ICD. The relative contributions (i.e. factor loadings) of ICD to $\mathbf{p}_{max}$ and $\mathbf{p}_2$ were -0.005 and 0.006 respectively. This can be illustrated in Figure 7.3 where ICD is plotted against dominant frequency (DF) and $\mathbf{p}_{max}$ and $\mathbf{p}_2$ are represented as the primary and secondary axis of the ellipse. I plotted ICD against DF because Hunt et al (2005) had previously reported a stabilizing (negative quadratic) selection gradient for DF, with a peak at 3.96 kHz. Since this selective peak is lower than the grand mean DF in this dataset (4.06 kHz), I make the assumption that, in combination with the negative linear gradient I measured in Chapter 6, there is a bivariate selective peak indicated by the direction of the arrow from the bivariate mean. Although this is certainly not conclusive, I suggest that the alignment of $\mathbf{p}_{max}$ and $\mathbf{p}_2$ at an acute angle with the 2-dimensional trait space defined by DF and ICD may act to constrain these populations from responding to the measured sexual selection favouring a reduction of ICD values. However, further work is needed to measure multivariate selection operating on all call traits, and to relate this to **P** and **G**. This work is currently underway in the Hunt research lab.

It must be reiterated that the empirical work contained in this thesis deals with phenotypic integration and the **P** matrix. The reason for this was logistical; the estimation of **G**, particularly with any degree of accuracy, is a considerable empirical undertaking, and as such proved to beyond the time and resources available to me during the course of this studentship. That is not to say that this diminishes the utility of this work. Indeed, some authors have suggested that working with **P** as a proxy for **G**, in addition to providing a way to make quantitative genetic inferences where **G** is impossible (or prohibitive) to estimate, may offer a more precise estimate of the structure of **G** when the two matrices are proportional (Arnold & Phillips, 1999; Phillips, 1998; Cheverud, 1988). Their rationale for this is that **P** may be estimated much more accurately than **G**, since

sampling error can be expected to scale with the inverse of the sample size (Steppan et al., 2002) which is the number of individuals when calculating **P**, but

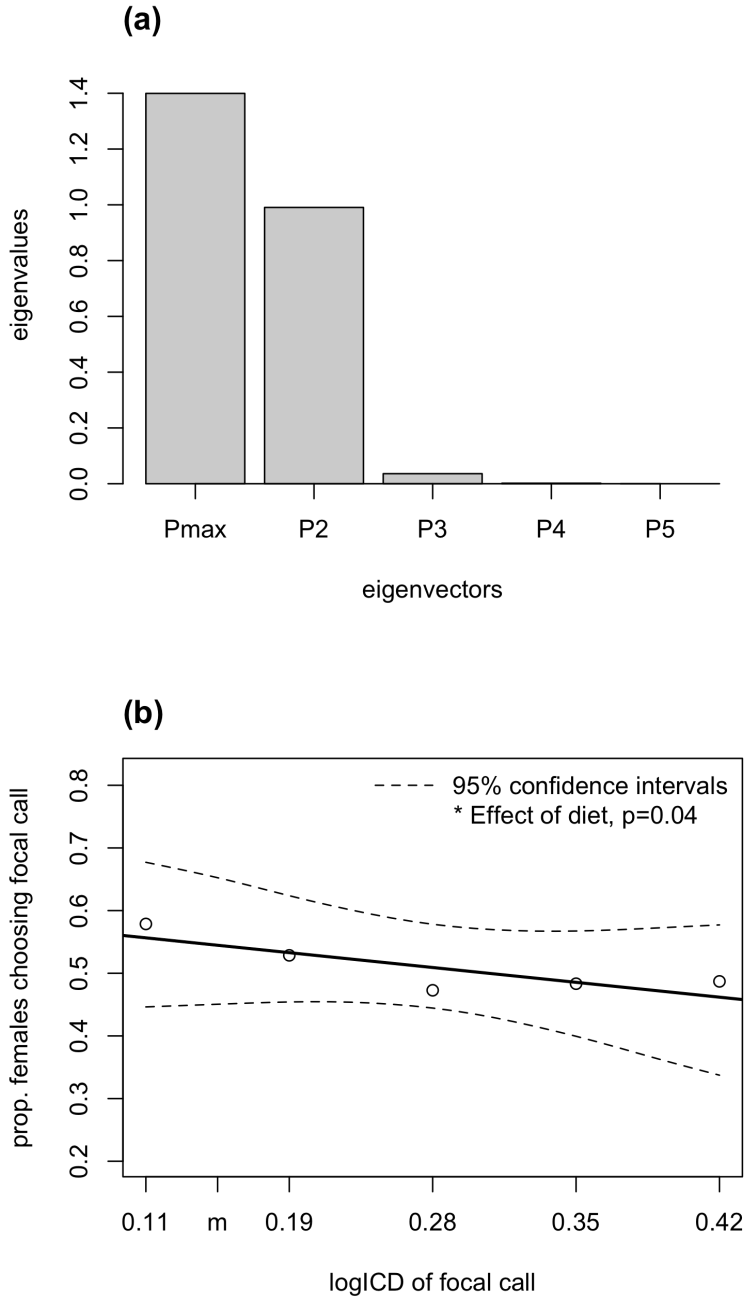


**Figure 7.2**: (a) Scree plot of the distribution of variance among the eigenvectors of the advertisement call **P** matrix, calculated from all 6 populations under standard lab conditions (Chapter 4). Note the small eigenvalues associated with vectors $\mathbf{p}_3$ to $\mathbf{p}_5$; approx. 98% of variance is to be found cumulatively on $\mathbf{p}_{max}$ and $\mathbf{p}_2$. (b) Preference function for inter-call duration (Chapter 6), with x-axis labels expressed as log values for ease of comparison to Figure 3. The mean ICD value of male calls is marked 'm'.

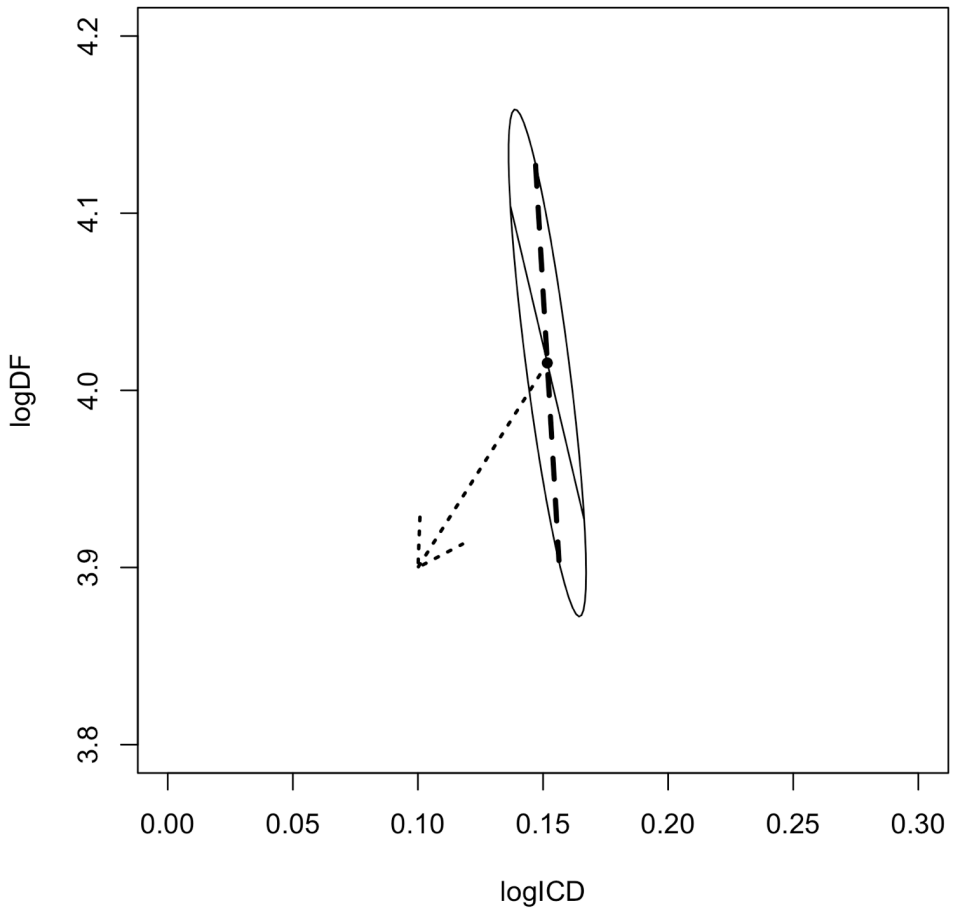


**Figure 7.3**: Trait covariance as a potential constraint on call evolution. Dominant frequency (DF) and inter-call duration (ICD) for all six populations under standard lab conditions are plotted on a log scale. The ellipse represents the distribution of variance summarised by **P**, the point at the centre of the ellipse is the inter-population bivariate mean. The axes of the ellipse represent the first two eigenvectors of **P** (comprising a ~98% of the variance present in **P**), with the dashed line representing $\mathbf{p}_{max}$ and the solid line representing $\mathbf{p}_2$. Note that although $\mathbf{p}_{max}$ and $\mathbf{p}_2$ are orthogonal, their axes do not appear to be at right angles in this figure; this distortion is due to their being projected from the 5-dimensional space occupied by **P** into the 2-dimensional subspace defined by the traits ICD and DF. The dashed arrow is directed from the bivariate mean, towards a y-intercept (i.e. a 0 value of ICD; since the preference function in figure 2(b) has a negative gradient) at 3.96 kHz (the selective peak for DF reported by Hunt et al (2005).

the number of families when calculating **G**. Of course, since the **P** matrix can be thought of as the sum of **G** and all other sources of covariation, there are certainly plenty of reasons that the two may be different (Willis et al., 1991), particularly if **E** (the environmental variance-covariance matrix) is not similar in structure to **G** (Arnold & Phillips, 1999). Nevertheless, the correlations between **G** and **P** reported in the literature are very high (Waitt & Levin, 1998; Houle, 1992), frequently high enough to fit the hypothesis that apparent difference was due only to sampling errors (Roff, 1997). My finding (Chapter 2) of no difference in the strength LLER's between **G** and **P** matrices is complementary to this reasoning. Intuitively, the correlation of **G** and **P** seems to be highest for those traits that have high heritabilities; i.e. when **G** makes up a large proportion **P**. Since the call traits that have been the primary focus of this thesis have moderately high heritabilities, (ranging in $h^2$ from 0.2 to 0.65 (Hunt et al., 2007)) **P** may be a good proxy for **G** in this case. In order to test this supposition I performed a Mantel test, CPC analysis and geometric matrix comparison between the all-populations pooled **P** calculated for animals derived from my 2007 collection above and the **G** estimated by Hunt et al (2007) for animals derived from the Smith's Lakes (SL) population. The Mantel test showed no significant difference between **P** and **G**, the CPC analysis indicated common principal components or equality, and the geometric comparison indicated moderate differences in alignment (Table 4). The magnitude of these results is well within the range of inter-population divergence for **P** as discussed above. It would appear therefore, that for advertisement call in this species at least, **P** and **G** are similar enough to be indistinguishable by some tests and the use of **P** as a proxy is justified.

A great deal of the previous work on the topics of integration and constraint has been concerned with the **G** matrix (e.g. Arnold et al., 2008; Blows & Hoffmann, 2005; Eroukhmanoff, 2009; Rice, 2008; Jones et al., 2003), and doubtless making accurate population-level estimates of **G** for the *T. commodus* advertisement call would be a worthwhile endeavor. Not least because partitioning out the contributions of **G** and non-additive-genetic sources of covariance would allow an estimate to be made of **E**, which could then be compared with both **P** and **G**; a comparison which has not yet been made in a complex behavioural trait to my knowledge. Another potentially fruitful (and elegant) way forward would be to use

artificial selection to determine the role of integration as facilitator or constraint. One could select along the principal eigenvector ($\mathbf{p}_{max}$) in one sub-population, and along orthogonal vector(s) in other sub-population(s). Of course elegance does not imply facility, and such an experiment would be formidable to say the least, since multiple traits would need to be measured for each individual in each generation, and experimental sub-populations would need to be large enough to retain the majority of the genetic (co)variance present in the founder population.

**Table 7.4**: The results of comparisons between the all-populations **P** from my 2007 collection and the **G** estimated for animals derived from the Smith's Lake population (as published in; Hunt et al., 2007). See above and Chapters 3 and 4 for detailed explanations of these analyses.

| **Matrix Comparison** | test statistics/outcomes | | |
|---|---|---|---|
| | F value | Angle between closeest eigenvectors | |
| **Geometric method** | 1.14 | 15.9° | |
| | no. iterations | 1-tailed *p* | 2-tailed *p* |
| **Mantel test** | 10,000 | 0.64 | 0.73 |
| | step-up approach | model building approach | jump-up approach |
| **CPC analysis** | CPC(3) | equality | equality |

My results in Chapter 2 demonstrate the importance of investigating the multivariate context of a particular trait of interest, since all lines of least evolutionary resistance are not equivalent. Collectively, my results from Chapters 3, 4 and 5 emphasise the utility of **P** as a measure of integration of complex traits. I show the structure of **P** for advertisement calls in *T. commodus* to be remarkably stable, despite the trait means of the **P** components varying between populations, over time and between diet treatments. This robust conservation of covariance structure suggests that integration is likely to act as a constraint on the evolution of call structure in this species. Additionally, the link I uncovered between call structure and wing morphology indicates the potential for aspects of morphology

to contribute to the pattern of integration amongst behavioural traits, such as advertisement call parameters. Viewed in the context of the current literature in evolution and genetics (in particular; Houle, 2007; Blows, 2007; Schluter, 1996; Hunt et al., 2007), results such as mine illustrate the value of a multivariate approach to the study evolution. Given the potential gains to be made in our understanding, the perspective offered by a multivariate approach deserves to be seen as a key tool in our study of phenotypic evolution.

# APPENDICES

## *APPENDIX I*

Reference list of sources from which **P** and/or **G** matrices were drawn for the dataset analysed in Chapter 2:


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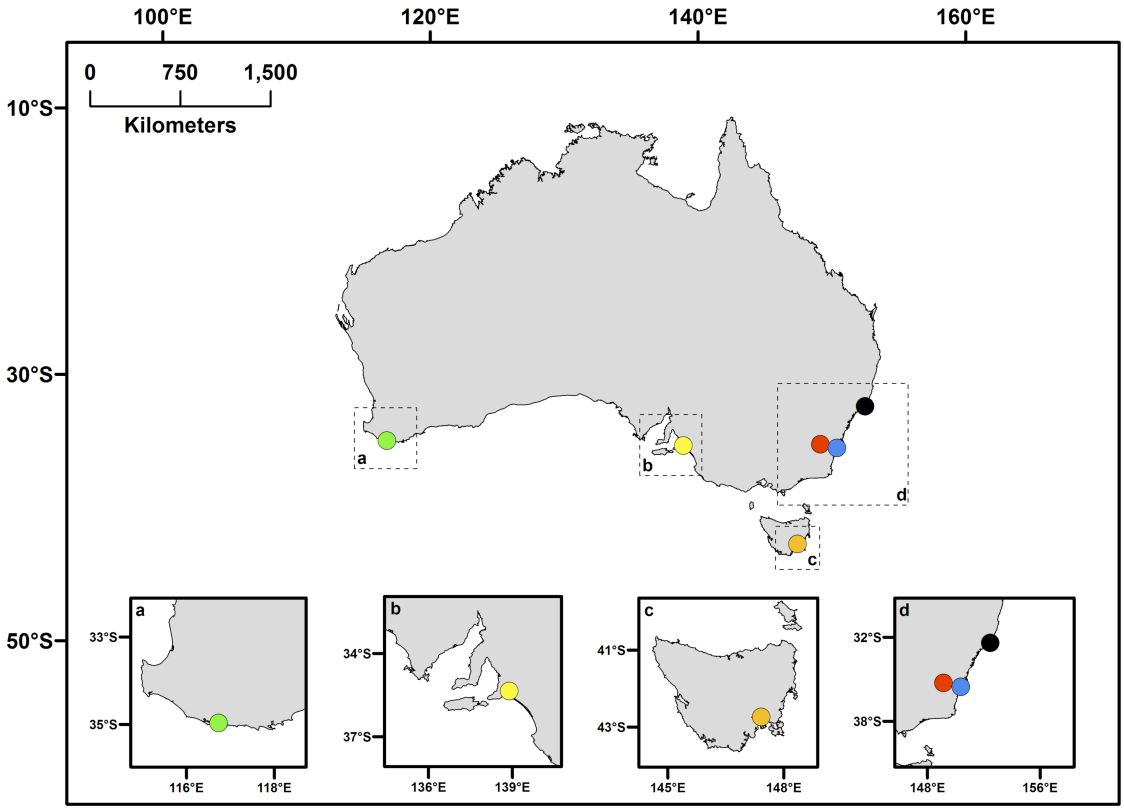
## *APPENDIX II*

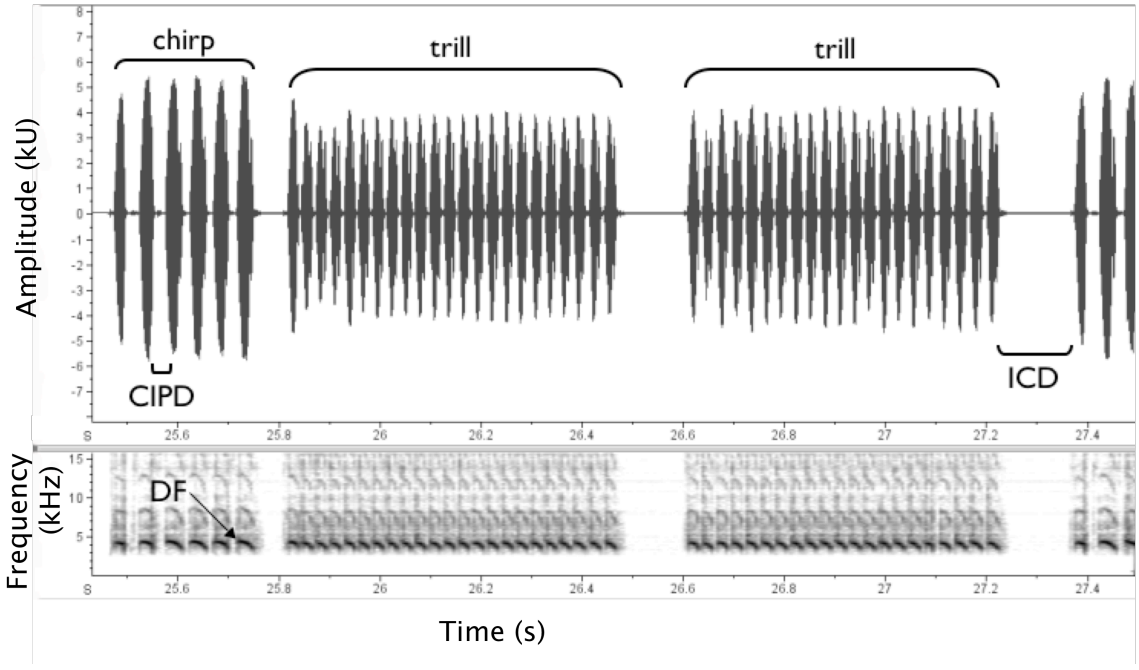
The locations from which animals were collected to establish the laboratory populations from which study animals were drawn for the work described in this thesis. Note that both Hunt's 2005 collection (Chapter 7) and the Hunt & Pitchers collection of 2007 were made from these same locations.




| Population | Colour code | Location of collection | | Coordinates | |
|---|---|---|---|---|---|
| ACT | RED | Canberra | Aus. Capital Territory | 35.2°S | 149.1°E |
| KL | BLUE | Kioloa | New South Wales | 35.5°S | 150.3°E |
| SA | YELLOW | McLaren Vale | South Australia | 35.2°S | 138.5°E |
| SL | BLACK | Smith's Lakes | New South Wales | 32.2°S | 149.1°E |
| TAS | ORANGE | Richmond | Tasmania | 42.7°S | 147.5°E |
| WA | GREEN | Walpole | Western Australia | 34.9°S | 116.7°E |

## *APPENDIX III*

The advertisement call produced by male *Teleogryllus commodus*. This call was recorded using the apparatus described in Chapter 4. The upper part of the figure is an oscillogram, the 'units' displayed on the y-axis (kilo-units, kU) are the actual sample values in the signal, which are proportional to the sound pressure recorded, but this is a relative measure only since the sound pressure at the microphone was not standardised. The lower part is a spectrogram with a y-axis in kilohertz (kHz). The traits measured from the advertisement call are: chirp inter-pulse interval (CIPD) and inter-call duration (ICD) measured in seconds; dominant frequency (DF) measured in kHz; chirp pulse number (CPN) and trill number (TN) were counted (6 and 2 respectively in this figure).



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