The use of inbreeding to assess the genetic component of condition underlying GEIs in sexual traits

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INTRODUCTION

It is widely believed that females use variation in sexual ornaments and courtship behaviour of males to assess the environmental and genetic quality of potential mates. There are a multitude of studies showing how environmental variation affects the expression of male sexual traits (e.g. Cotton et al. 2004a, c, Bonduriansky and Rowe 2005, Eraud et al. 2007, Punzalan et al. 2008, McGuigan 2009). However, genetic analysis remains a rarity, and the interaction of genetic variation with the environment seems scarcely to have been addressed. There is a need to understand the extent to which phenotypic responses are contingent upon genotype, and whether the genetic signal is amplified or scrambled in different environments or under environmental stress. Without such information it will be hard to interpret what males are signalling and how females benefit from their mate choice.

In this chapter, we discuss the use and potential of inbreeding as a means to study genotype by environment interactions (GEIs). Fundamental to studying sexual trait GEIs is our ability to define, measure and manipulate both the environmental and genetic states of individuals. In principle, there is little problem in deciding how to measure environmental variables (e.g. food quality, temperature, parasite load) or in predicting their likely consequences for the environmental conditions experienced by individual organisms. Inbreeding provides similar opportunities for the manipulation of genetic condition. Inbreeding increases genomic stress (i.e. homozygosity) within individuals and is typically associated with a directional decrease in fitness. It thus constitutes an empirical tool that can be used to generate individuals that differ predictably in their average genetic quality. This surmounts a major problem with other types of genetic technique that lack any *a priori* way to define the quality of particular genotypes. Put simply, inbreeding offers a simple and controlled way to experimentally manipulate genetic condition.

We first critically review how inbreeding can be used to study the GEIs of sexual traits. In the context of this book, inbreeding-by-environment interactions are simply one form of genotype-by-environment interaction. We then consider relevant empirical work that has used inbreeding to determine the extent to which sexual traits suffer from genetic stress, and ask whether evidence from inbreeding studies supports the hypothesis that the degree of genetic condition dependence is more marked in sexual traits. Finally, we relate these findings to the potential for using inbreeding to evaluate GEIs in sexual traits. Our review points to the need to take account of environmental variation when assessing genetic variation. This seems particularly important for laboratory based studies in which many aspects of the environment can be varied systematically along with the degree of inbreeding. Such an approach has the potential to identify whether variation in sexual traits is linked to genetic variation in condition and also how this genetic component interacts with environmental variation in condition.

Sexual traits and genetic condition

Females are known to express mate preferences for males with highly exaggerated sexual traits, even when there are no obvious direct benefits. In this case, it is thought that females receive genetic (i.e. indirect) benefits (Andersson, 1994). There has always been scepticism about this proposal as selection erodes additive genetic variance in fitness and consequently reduces the genetic benefits likely to flow from mate choice. This problem is referred to as the 'lek paradox' (Borgia, 1979) and has been debated for decades (Taylor and Williams, 1982, Kirkpatrick and Ryan, 1991, Iwasa and Pomiankowski, 1995, Kokko et al., 2003, Kotiaho et al., 2001). However, sexually selected traits show relatively high amounts of additive genetic variation compared to non-sexual traits (Pomiankowski and Møller, 1995). A variety of mechanisms have been proposed for maintaining suitable genetic variance that could be signalled through male sexual traits (Radwan, 2008). The importance of condition-dependent expression in sexual traits has attracted considerable attention as a general resolution to the lek paradox. This hypothesis proposes that sexually selected traits have evolved strong condition dependence during their evolutionary exaggeration. This follows from the handicap theory assumption that such traits are costly to produce so that only individuals of a good condition are able to afford the cost of producing a larger sexual traits (Zahavi, 1975, Pomiankowski, 1987, Grafen, 1990, Iwasa et al., 1991).

Condition is predicted to have high and persistent additive genetic variance because it is likely to be dependent upon a range of morphological, physiological and life-history traits, such that it sums genetic variation across many loci (Pomiankowski and Møller, 1995, Houle, 1992, Rowe and Houle, 1996, Tomkins et al., 2004). Condition has often been empirically interpreted as the nutritional state or the body size of an individual, which has the beneficial consequence of permitting simple estimation. A widely held view is that condition refers to the "pool of resources" available to be allocated to sexual traits when traded off against other fitness enhancing traits (Rowe and Houle, 1996) although it is less obvious how this can be measured. A more abstract view is to describe condition in terms of residual reproductive value as used in life history models (Williams, 1996). This latter view is insightful because then any state or trait could contribute to condition (leaving open the somatic processes involved). For example, it seems essential to include the extent of parasite infection as a source of "condition" (Hamilton and Zuk, 1982).

So male sexual traits that have gained condition-dependent expression during their evolutionary exaggeration through sexual selection, are expected to also have high and persistent additive genetic variation (Iwasa and Pomiankowski, 1994). Such traits will recruit and amplify allelic variation from many loci across the genome (Pomiankowski and Møller, 1995, Rowe and Houle, 1996). In contrast, non-sexual traits that are less costly to produce and under a regime of more static stabilising selection, are predicted to evolve much weaker condition-dependent expression. Indeed, many morphological traits are expected to favour condition-independent expression through increased developmental buffering against environmental or genetic stress (de Visser et al., 2003). Non-sexual traits are therefore likely to be dependent upon far fewer loci relative to sexual traits.

Studies of environmental variation

Many studies of condition dependence have indirectly assessed these ideas by manipulating environmental conditions, on the assumption that environmental and genetic stress are likely to have similar consequences for sexual traits. A variety of environmental stressors have been investigated, for example, heat stress (Rashed and Polak, 2010, Bjorksten et al., 2001, Eraud et al., 2007), dietary stress (Cotton et al., 2004a, Bonduriansky and Rowe, 2005, Punzalan et al., 2008, McGuigan, 2009, 2004c) and parasite load (Zuk et al., 1990, Thompson et al., 1997, Costa and Macedo, 2005). This approach is appropriate because environmental stress is likely to represent an important source of variation in condition in the wild. In addition, in many contexts, the signalling of male condition has value whether variation between males originates from genetic or environmental differences in condition.

Several studies have explicitly looked at environmental stress to infer whether male sexual traits signal genetic quality (for example, (Kotiaho et al., 2001, Bonduriansky and Rowe, 2005, Tschirren et al., 2003). The assumption made is that environmental differences in condition have similar consequences to genetic differences in condition. This may be correct if environmental and genetic factors both affect the same physiological pathways involved in development and resource allocation among traits. Increased availability of resources would then be expected to have the same consequences on sexual trait development as would an increased quality of genes. However, there is no *a priori* necessity for environmental and genetic variation to contribute the same effects on development or resource acquisition and hence on sexual trait growth and size. Also it is essential to investigate how environmental and genetic factors interact in trait development. As such, there is a need to directly investigate the genetic basis of condition-dependent expression in male sexual characters (Cotton et al., 2004b, Tomkins et al., 2004).

Studies of genetic variation

One of the main approaches used to investigate the genetic basis of condition dependence is to measure the genetic variation (or better, the additive genetic variation) in both condition and the sexual trait, and test the genetic covariance between the two. If sexual traits are condition-dependent, and represent large mutational targets, one would expect a strong positive genetic correlation between the sexual trait and condition. So far, tests investigating this genetic covariance have produced mixed results. Kotiaho et al. (2001) found a strong positive genetic covariance between male courtship rate and male condition in the dung beetle, *Onthophagus taurus*. Positive genetic correlations between the sexual trait and male condition have also been identified in the lesser wax moth, *Achroia grisella* (Brandt and Greenfield, 2004) and the red junglefowl, *Gallus gallus* (Parker and Ligon, 2007). However, in the zebra finch, *Taeniopygia guttata*, Birkhead et al. (2006) found that even though the male sexual trait (beak colour) was positively correlated with the immune response, no genetic covariation was found between male condition and the sexual trait. Similarly, Hughes et al. (2005) failed to find genetic covariation between the area of orange spots and the condition proxy of body size in the guppy *Poecilia reticulata*.

Although trait values of additive genetic variation are in theory simple to measure (for example, by using a half-sibling mating design, Green (2001a), p169), reliable and unambiguous measures of condition are difficult to make. This is because it is quite opaque how to make a true measure of condition nor is it obvious how to quantify low through to high values of condition.

For example, consider the frequently espoused idea that condition is defined by the mass of an individual after controlling for body size (Kotiaho, 1999, Jakob et al., 1996b, Green, 2001b, Speakman, 2001, Schulte-Hostedde et al., 2001, Schulte-Hostedde et al., 2005, Birkhead et al., 2006, Green, 2001a). The most common method used is to regress body mass against a linear index of absolute body size. The residuals from such a plot provide a measure of the fat reserves, and are assumed to correspond to the pool of resources available for investment. An individual with a positive residual is assumed to be in a better condition than an individual with a negative residual (Jakob et al., 1996a). However, body mass/body size residuals are not condition, they are just a proxy for it (and note, should really be referred to as “body condition”). Green (2001a) reviewed the use of residual measures of condition and proposed a number of key assumptions for their use: 1) that the relationship between mass and body size (or the body size indicator, BSI) is linear, 2) that condition is independent of BSI and the BSI is independent of mass, and 3) that the measure of the BSI accurately indicates overall body size. Green (2001) concluded that these assumptions are violated in many, maybe even all, studies that were reviewed.

It would be interesting to demonstrate that the extent to which body mass/body size residuals actually correlate with fitness, or a major component of fitness in a range of different organisms, to assess the relevance of this measure. In some systems it is well known that fatness in part reflects risk aversion that is more typical of low quality individuals, contrary to this simplistic formulation (Witter and Cuthill, 1993, Cuthill et al., 2000). In addition, there are many other possible components to consider as relevant to the notion of condition. The most widely studied possibility is immune system activity (Birkhead et al., 2006). There seems no compelling reason to assume that this will simply be a correlate of residual mass/body size. Interestingly, studies using different residual measures of condition have produced different results depending upon the measure used (Schulte-Hostedde et al., 2005, Birkhead et al., 2006, Jakob et al., 1996a).

Another important assumption is that body mass/body size residual measures of condition correlate with reproductive success, and this correlation is the same under all environmental circumstances. This seems highly unlikely. Animals are typically adapted to a range of environmental circumstances that impact on the aspects of their condition. We know of many cases where this results in different strategies associated with male signalling to females during courtship, for example between different rhinoceros and stag beetle morphs (Emlen and Nijhout, 2000). This means that residual mass/body size ought to be assessed across a range of realistic environmental conditions, those that typify natural environmental variation for the species in question, and then correlated with fitness measures. The generality is that simple expectations from the genetics of body condition indexes need to be treated with caution.

The use of inbreeding to infer the genetics of condition-dependent traits

A potential way of improving measurements of condition is to use inbreeding to impose genetic stress (Cotton et al., 2004b, Tomkins et al., 2004). This has the merit that it avoids having to think too hard about what condition might mean. Successive rounds of brother-sister mating (or other forms of inbreeding) cause inbreeding depression, which can be simply measured as the reduction in mean trait values of sexual and non-sexual traits. There are a number of ideas about the causes of inbreeding depression, the most prominent of which is the dominance hypothesis (Charlesworth and Charlesworth, 1999, Roff, 1997). This predicts that the standing genetic variation in fitness in an outbred population is made up of many mildly deleterious, recessive or partially recessive alleles. Any particular deleterious allele is likely to be rare, but a large fraction of the genome is likely to harbour low frequency recessive alleles. Under inbreeding, homozygosity will increase, resulting in the random fixation of some of these deleterious alleles leading to inbreeding depression.

Regardless of the mechanism, all traits are likely to suffer some degree of inbreeding depression. However, condition-dependent sexual traits are predicted to suffer greater inbreeding depression. As condition is supposed to be affected by a relatively large proportion of the genome, and each locus might have a more significant effect on trait values, it represents a large target for the detrimental effect of inbreeding (Rowe and Houle, 1996, Tomkins et al., 2004, Pomiankowski and Møller, 1995). So condition is expected to suffer from marked inbreeding depression. Likewise traits with strongly condition-dependent expression are also expected to carry a higher mutational load and suffer from marked inbreeding depression. In contrast, traits less dependent upon condition are expected to be affected by fewer loci and to suffer less from inbreeding depression. This allows inbreeding to be used as a method of manipulating individual genetic condition in a graded manner as a function of the inbreeding coefficient, without the need to directly define and quantify what is meant by condition. Adopting inbreeding also avoids the need to use proxy measures, like residual mass or body size, with their attendant problems as outlined above.

Designing inbreeding experiments

The use of inbreeding is not a panacea for the investigation of the genetics of sexual traits. Like other approaches it comes with useful properties but also some caveats. What is clear from the literature survey described below is that, in this context, few researchers have taken the possibility of using inbreeding seriously and the field remains in its infancy. We undertook this review for several reasons. First, whilst several authors have promoted the use of inbreeding to study the genetics of sexual traits (Cotton et al., 2004b, Tomkins et al., 2004), no assessment of the data has been undertaken. Second, investigations using inbreeding depression to investigate the genetics of condition-dependent traits have produced inconsistent results, and the potential reasons for this variation need to be highlighted. Third, many studies using inbreeding suffer from poor experimental design and there is need to clarify what is required to obtain reliable outcomes.

Choice of control traits

A major problem in the empirical literature on testing condition dependence is that both sexual and non-sexual traits are expected to show condition-dependent expression to some extent (Cotton et al., 2004b). So it is not sufficient to show sexual trait condition dependence. It is also necessary to compare sexual and non-sexual traits, and demonstrate that the former are more sensitive to changes in condition. We need to apply this logic to studies using inbreeding. Simply showing that sexual traits suffer from inbreeding depression is not especially informative. It is important that the extent of inbreeding depression in the sexual trait is assessed relative to other traits that are either not subject to sexual selection, or subject to demonstrably weaker sexual selection.

One possibility is to compare the male sexual trait with the homologous trait in females (if present). However, the female trait may itself be subject to sexual selection, for example, by male mate choice (Bonduriansky, 2001). In addition, the male and female traits may be dependent upon the same underlying genetic architecture and consequently the female trait may be condition-dependent due to genetic correlations between the sexes (David et al., 2000, Falconer and Mackay, 1996). Another restriction for this comparison is that the male trait may have evolved to be a signal used by females because ancestrally it was a trait sensitive to condition (Cotton et al., 2004c). In this case, both male (sexual) and female (non-sexual) traits may show enhanced condition dependence simply because this is the ancestral state of that trait (Cotton et al., 2004c). Consequently further non-sexual comparison traits are needed.

An alternative approach is to compare sexual traits with non-sexual traits that are not sexually dimorphic. This has the benefit that comparisons can be made of traits within the same sex and the same individual. This eliminates inter-sexual and inter-individual differences present when comparing male and female traits. However, non-sexual traits are not all equivalent, and themselves may vary in the degree to which they are sensitive to condition. Some may be subject to strong stabilising selection and so have evolved canalization, and a degree of insensitivity to environmental and genetic variation. So there is a need to compare similar types of traits, on similar biological scales (e.g. morphological, colouration, behaviour, acoustic). Unfortunately, some sexual traits do not have any obvious non-sexual counterparts. For example, it is often difficult to identify suitable non-sexual comparators for courtship behaviour.

The magnitude of inbreeding depression in sexual traits may also be compared to life-history traits (defined as traits subject to trade-offs between development and reproduction, Tomkins et al. 2004). Life-history traits have relatively high genetic variance and are predicted to be dependent upon many underlying traits, such that they sum genetic variation over many loci (Houle, 1992). Therefore condition-dependent sexual traits are similar to some extent to life history traits, and both are expected to suffer from similar high inbreeding depression (Rowe and Houle, 1996, Tomkins et al., 2004).

Finally, where a species has multiple sexual traits, these can be compared. For example, mate choice in *Drosophila melanogaster* is dependent upon morphological traits (Ng and Kopp, 2008), behavioural traits (Talyn and Dowse, 2004) as well as pheromones (cuticular hydrocarbons (Foley et al., 2007). There is an extensive literature on the nature of multiple sexual ornaments and their likely signalling values. These traits may differ in the extent to which they are subject to sexual selection by female mate preference, and in the way that they react to environmental or genetic stress (Candolin, 2003, Iwasa and Pomiankowski, 1994). For example, in the piophilid fly, *Prochyliza xanthostoma*, one sexually dimorphic trait (male head length) was larger in high condition males, whereas other sexually dimorphic traits (male head width, antenna length and fore tibia length) were not (Bonduriansky and Rowe, 2005). Comparing inbreeding depression in these different sexual traits may prove illuminating, for example in addressing the question of whether there is a connection between the strength of mate preference and the degree of condition dependence. Usually there is a need to include a non-sexual trait in such an analysis in order to benchmark responses against which the sexual traits can be compared.

Overall, this brief analysis suggests that it is preferable that several different sexual and non-sexual traits are investigated, and that prior information is used when interpreting the pattern of inbreeding depression in different traits.

Controlling for body size covariation

Many sexual and non-sexual traits correlate with body size (Bonduriansky, 2007). This is true of morphological and behavioural traits. As there is a large body of empirical evidence showing that body size suffers from inbreeding depression (Pante et al., 2001, Thrower and Hard, 2009, Holt et al., 2005, Mandal et al., 2005), it is important that the effect of inbreeding on sexual traits is tested both before and after controlling for body size (Prokop et al., 2010). This will then show whether the genetic basis underlying the sexual (or non-sexual) traits is a general size-related response or is independent of the general decline in body size.

Outbred controls

The consequences of inbreeding are typically assayed by comparing inbred with outbred individuals. Often the effects of inbreeding are measured in inbred lines produced over successive generations. In this design, control lines are ideally run alongside the inbred lines in each generation (Lynch, 1988). This allows inbreeding depression to be assessed independently in each generation relative to the outbred control line(s). It removes the false assignment of inbreeding depression when fluctuations in trait values across generations occur for other reasons.

An alternative approach is to compare inbred individuals with the base population from which they were derived (Aspi, 2000). This has the advantage that less effort needs to be expended maintaining outbred lines. However, it is less useful because inevitably the base population is not subject to the same experimental procedures as the inbred lines. Therefore differences can potentially arise simply through selection and drift that arise from inadvertent differences in housing (e.g. due to density, competition). A further option is to generate outbred individuals after (or during) the period of inbreeding, by crossing different inbred lines (Bellamy et al., 2013). This regenerates high levels of heterozygosity in the outbred individuals to enable comparisons between individuals with the same history of selection. This approach removes the need to spend effort maintaining an outbred stock similar to the inbred lines.

All of these approaches suffer from the inevitable pressure of selection that occurs both in inbred lines and outbred controls. The generation of inbred lines inevitably involves a reduction in the scope of female choice, because the experimenter places a breeding male and female together. Inbreeding thus causes relaxed sexual selection due to absence of mate preference (Whitlock and Agrawal, 2009). Hence part of any "inbreeding depression" that is observed might be due to relaxation of sexual selection, rather than to genetic stress arising from the random fixation of alleles. However the reverse may occur. Typical inbreeding procedures require the generation of several sub-lines in each generation, from which one is chosen to produce the next generation (Lynch and Walsh 1997). Choosing one sub-line will generate selection in favour of life history values that are likely to maintain the sub-line (e.g. female fecundity, male fertility). If these are genetically correlated (positively or negatively) with the sexual and non-sexual traits under examination, then the latter are likely to be subject to evolutionary change.

Experimental scale

Several considerations of scale have been emphasized for the proper experimental design for studies of inbreeding (Lynch, 1988): 1) the number of inbred lines, 2) the number of generations of inbreeding, and 3) the number of inbred and outbred offspring collected for comparison. These aspects are likely to be constrained by the individual life-histories of species and the practical limits of keeping populations of animals under controlled conditions. Guides to the design of inbreeding experiments, often recommend that a large number of inbred lines (e.g. >20 (Keller and Waller, 2002), >100 (Lynch, 1988)) need to be monitored over several generations (with more needed to characterise weaker effects). We note that this calls for a scale that may be well beyond the feasible logistical limits for many species and certainly is far greater than that typically used by the studies reported here.

The motivation for adopting a large scale for line replication is to reduce the sampling variance of parameter estimates (e.g. of the degree of non-additivity). A more modest scale can nonetheless prove to be illuminating if the differences between sexual and non-sexual traits are large. But this can't be assumed. Many studies report no differences between sexual and non-sexual trait after inbreeding (see below). But if the chosen scale is insufficient, then such a pattern could just reflect the lack of statistical power to detect differences.

GEI

It has been pointed out that the magnitude of inbreeding depression can be highly sensitive to the level of environmental stress (Fox and Reed, 2011, Armbruster and Reed, 2005). It is expected that inbred individuals are more sensitive to environmental stress because homozygosity for deleterious recessive alleles will likely degrade the ability of individuals to cope with poor environmental conditions. In the context of this book, such inbreeding-by-environment interactions constitute one form of genotype-by-environment interactions.

It has frequently been claimed that the negative effects of inbreeding are stronger under higher levels of environmental stress (Roff, 1997). Recent meta-analyses of the mean fitness of inbred and outbred individuals under environmental variation, confirm that inbreeding depression in fitness increases with the magnitude of environmental stress (Fox and Reed, 2011, Armbruster and Reed, 2005). But these studies also reveal that there is context dependence, and variable responses under different environments. Some of the observed variation in outcome may be due to the problems of statistical power mentioned above, but this is unlikely to account for all of the variation observed. Possible underlying explanations of this include strong effects of genetic background, non-additive genetic effects and the influence of varying selection (Fox and Reed, 2011, Armbruster and Reed, 2005). These studies point to the need to take account of environmental variation when assessing genetic variation in sexual traits. This seems particularly important for laboratory based studies in which many aspects of the environment can be varied systematically along with the degree of inbreeding. This gives the potential to identify how sexual traits respond to genetic as well as environmental variation in condition and the nature of the genotype-by-environment interaction.

METHODS

We surveyed the published literature, restricting the search to studies that tested whether male sexual traits suffer from inbreeding depression. We used the electronic search engine Web of Knowledge (www.wok.mimas.ac.uk) using the search words ‘inbreeding depression’ AND ‘trait’ OR ‘(male) ornament’ OR ‘(male) sexual trait’ OR ‘non-sexual trait’ OR ‘condition dependence/dependent’ (date of search; April 2012). Some further studies were identified from the cited literature within papers. We only considered studies in which there was experimental manipulation of inbreeding under controlled conditions, and where some aspects of the male sexual ornamentation or courtship were measured. We excluded those in which proxy measures of inbreeding were reported (e.g. multi-locus heterozygosity, population size). Where only general aspects of mating were recorded, like mating propensity or mating speed, the studies were omitted. When the object of the study was to record male antagonistic interactions, or traits associated with them, these studies again were omitted.

For all relevant studies we recorded: 1) the secondary sexual traits measured (both morphological trait and courtship behaviour); 2) whether non-sexual control traits were also measured – non-sexual traits were only considered as such if they were of the same kind and similar biological scale as the sexual trait; 3) the presence or absence of inbreeding depression in each sexual and non-sexual trait; 4) whether the magnitude of inbreeding depression was greater in the sexual trait; 5) whether control of body size was included in the study; 6) the number of generations of inbreeding imposed; and 7) the number of levels of environmental stress used.

We used this survey to test the following three central predictions: 1) sexual traits should suffer from inbreeding depression; 2) sexual traits should still suffer from inbreeding depression after controlling for body size scaling; 3) non-sexual traits may also suffer from inbreeding depression, but this should be of a smaller magnitude than seen in sexual traits. In addition, in species with multiple sexual traits, we consider whether there is variance in the amount of inbreeding depression exhibited by different traits.

RESULTS

In total, the literature search yielded 310 hits, from which 16 studies tested inbreeding depression in male sexual traits in animals (Table 1). All of these investigations were laboratory based. The studies are summarised in Table 1. A small number of studies looked at inbreeding depression of male sexual traits in the wild, but in general did not satisfy all our inclusion criteria. The search also identified a number of studies that investigated the effect of inbreeding depression in plant flower size, which could be considered equivalent to a sexual trait in animals (Andersson, 1994). However, we do not consider these studies further in this review. Below we discuss these studies, classified by taxa, and then make some general observations.

Fish

Guppy

The guppy *Poecilia reticulata* is the fish species that has been most extensively used to study inbreeding depression in male sexual traits (5 studies). Females discriminate between males based on body size, colour patterns and courtship displays (Houde, 1997). An experiment tested for the effect of two generations of full-sibling inbreeding on sexual and non-sexual traits in two populations from the Aripo and Paria rivers in Trinidad (Sheridan and Pomiankowski, 1997). Inbred and outbred males were measured for three different pigmentations; melanin, carotenoid and iridescent pigments, of which the carotenoid pigment had previously been shown to be under female mate selection in both populations (Houde, 1988). In line with expectations, the Paria population showed evidence of inbreeding depression for the size of the area of carotenoid pigment (a sexual trait), but not for the areas of the melanin and the iridescent pigments (non-sexual traits). The number of coloured spots also showed inbreeding depression. In contrast the Aripo population only showed evidence of inbreeding depression in melanin pigmentation. These results persisted after controlling for body size scaling, which also showed inbreeding depression.

This work was developed by testing for inbreeding depression in sexually selected morphological and behavioural traits using three generations of full-sibling matings and larger sample sizes (van Oosterhout et al. 2003). They used a different population from the Tacarigua river in Trinidad, and treated all aspects of the male's colouration pattern as subject to sexual selection. They found a significant decline in body size and in the relative size of the area covered by melanin and carotenoid spots (sexual traits). There was no loss in colour pattern area between generations 1 and 2, but there was inbreeding depression between generations 2 and 3. Different components of male mating behaviour such as the time males spent courting females, the number of high-intensity displays, the duration of sigmoid displays and charging behaviour also showed evidence of inbreeding depression. However, no non-sexual behavioural traits were measured nor was there a comparison with an outbred control.

A further experiment used one generation of full-sibling inbreeding to produce inbred guppies, founded from an introduced population from Australia (Mariette et al., 2006). Compared to an outbred control, there was strong inbreeding depression in many aspects of male courtship including display rate, the time spent following females, the number of successful matings and the number of post-copulatory jerks, but not in gonopodial thrusting. Once again, there was no comparison with non-sexual behavioural traits. In contrast to previous studies, no inbreeding depression was found in male sexual traits (melanin and carotenoid pigments) or in body size. However, the sample size was very small, limiting the power of the analysis.

None of the studies above considered how environmental variation might alter the response to inbreeding. However, the introduced population from Australia exploited by Mariette et al., 2006 was used in a later study in a GEI design (Zajitschek and Brooks, 2010). This study is unique in combining inbreeding with more than one level of environmental stress. Individuals were generated using two generations of full-sibling matings and were reared and maintained in either a laboratory (low stress) or semi-natural (high stress) environment. There was inbreeding depression in two male colouration pigments – melanin and iridescent, but not in two others – fuzzy black and orange. All of these pigments were taken to be part of the male sexual ornamentation, so the response to inbreeding in sexual traits was patchy. Inbreeding depression was only evident in the high stress environment, not in the benign environment. In contrast, male mating attractiveness which was assessed in a mate choice assay, did not show evidence of inbreeding depression in either environment.

This study (Zajitschek and Brooks, 2010) reveals that environmental stress may exaggerate the effect of inbreeding depression that would otherwise be masked in a benign environment. Although this is the only investigation of GEI inbreeding interactions in sexual traits, it has to be interpreted with caution. The semi-natural environment used to exert high stress was not a controlled environment and varied in many aspects, so it is not easy to identify the environmental source that contributed to high stress. Likewise, two levels of environmental stress (laboratory vs. semi-natural) is a limited approach if one wishes to capture the directionality of environmental dependence in inbreeding depression (Cotton et al., 2004a).

Overall, the work on guppies is mixed. Some elements of the male sexual colouration show inbreeding depression, but the pattern is inconsistent across populations. Some studies explicitly used non-sexual traits for comparison, but this approach was ignored by others. There was also variable opinion about whether all or only some elements of the male colouration should be taken to be sexual traits. Sample sizes vary enormously both in the number of inbred lines analysed and the number of individuals assessed per line. A possible explanation for the conflicting results is that female mate preference may vary markedly between different populations in separate streams, and even within parts of the same stream if those parts are subject to different predation pressures (Brooks, 2002).

Other fish

Two other fish species, sticklebacks and killifish, have been used to study inbreeding depression in male secondary sexual traits. Female sticklebacks (*Gasterosteus* *aculeatus*) prefer to mate with males who have bluer eyes (Bakker and Rowland, 1995) and display a more intense red-coloured throat colouration during courtship (Bakker and Milinski, 1993). The effect of inbreeding on these sexual traits and on life history traits (fertility traits and survival) was tested using individuals inbred for 1 or 2 generations of full-sibling matings (Frommen et al., 2008). Inbreeding caused reduced fertilisation success, hatching success and survival relative to outbred individuals. However, inbred and outbred individuals did not differ in male throat or eye colour. No non-sexual traits were measured. So this study provides no evidence that sexual traits show heightened inbreeding depression.

A further study investigated inbreeding depression in male mating behaviour in the least killifish, *Heterandria Formosa* (Ala-Honkola et al., 2009). Males court females by approaching them rapidly and thrusting their gonopodium towards the female. Male success correlates with the number of gonopodial thrusts (Ala-Honkola et al., 2009, Bisazza and Pilastro, 1997). The authors used one generation of full-sibling mating and found that inbred individuals performed fewer gonopodial thrusts. Body size also declined with inbreeding although no associations were found with thrusting number or rate. Again, no non-sexual traits were measured, so this study only provides weak evidence that sexual traits suffer from heightened inbreeding depression.

Birds

Zebra finch

In the zebra finch (*Taeniopygia guttata*), females discriminate between males depending on courtship song, beak colour and plumage colour (Collins and tenCate, 1996, Simons and Verhulst, 2011a, Holveck and Riebel, 2007). The effect of inbreeding on these male sexually selected traits was tested using females as a control, as they are subject to weaker sexual selection (Bolund et al., 2010). Inbred and outbred individuals were generated from one generation of mating for 12 full-sibling or outbred families and measured for morphological, life history and sexual traits (song rate, beak colour and plumage colour).

Inbreeding had strong negative effects on several morphological traits in adulthood (Bolund et al., 2010). Inbred males and females less than 100 days old were smaller and had lower body mass, although the effect was not present in older adult males. The amount and rate of song by males directed towards females was less in inbred individuals, but not the amount of undirected song. Syllable rate also showed inbreeding depression but other song elements did not. There were no female control song traits with which to compare male song. Beak colour was also negatively affected in inbred males, but there was also inbreeding depression in female beak colour. Only one (flank plumage) of four components of male plumage was significantly affected by inbreeding. Inbred males were less attractive to females in mate choice chamber tests. Overall, these results provide mixed support for the hypothesis that sexual traits show heightened condition dependence. If different sexual traits in males have different signalling roles, as appears to be the case in the zebra finch (Simons and Verhulst, 2011b), one would expect them to vary in the amount they suffer from inbreeding depression. But this was not explicitly considered in the design of these experiments.

Insects

Drosophila

Surprisingly few studies have investigated the effect of inbreeding on male sexual traits in *Drosophila*. In *D. melanogaster,* studies have reported on inbreeding depression in male mating ability, but these studies do not specify what aspects of male behaviour of morphology are associated with the inbreeding depression. An early study reported that male mating ability was lower in inbred males (after up to 18 generations of inbreeding) when in competition with outbred control males (Sharp 1984). Another investigation used flies from lines isogenic for chromosome 2 compared to flies from crosses that were heterozygous for chromosome 2 (Miller et al., 1993). All inbred lines suffered reduced male mating ability. Most of the lines (4/6) also showed reduction in the ability to initiate courtship and copulation. A further study in *D. simulans* again provided evidence of reduced male mating ability of inbred flies (comparing full-sib inbred flies with crossed family outbred flies), measuring time to mating as a measure of male attraction (Okada et al., 2011).

In *D. montana*, males produce a courtship song characterised by a train of polycyclic sound pulses. Females show a mating preference for the highest frequency of pulses per train, but other pulse train characteristics do not appear to affect female choice (Aspi and Hoikkala, 1995, Hoikkala and Suvanto, 1999, Ritchie et al., 1998). 20 generations of full-sibling mating were used to produce highly inbred lines, and tested whether male pulse frequency suffered more from inbreeding depression using comparison with laboratory strains as outbred controls (Aspi, 2000). Consistent with our hypothesis, only male pulse frequency was significantly affected by inbreeding depression, but in contrast six other aspects of the male song pulse train did not show evidence of inbreeding depression.

Crickets

Male black field crickets (*Teleogryllus commodus*) attract females by producing a long-distance call and females prefer to mate with males with the strongest calling effort (Bentsen et al., 2006). One generation of full-sibling inbreeding was used to investigate nightly calling effort and a number of call parameters shown to influence attractiveness (Drayton et al., 2007). Inbred and outbred families were generated from 20 inbred lines. Evidence for inbreeding depression in the sexual trait was mixed. There was no effect on calling effort. Of the five call parameters measured, three were subject to inbreeding depression (chirp interpulse interval, intercall interval duration and the number of pulses per chirp) and two were not (the number of trills and dominant frequency). Inbreeding was not associated with a decline in body mass, but was with four out of six life-history traits (egg hatching success, nymph survival and male and female survival).

In decorated crickets (*Gryllodes sigillatus*), a similarly mixed pattern was observed after seven generations of inbreeding (Ketola and Kotiaho, 2010). In this case, trait measurements were measured each generation and regressed against the inbreeding coefficient. There was again no effect on calling effort (number of calls, total time spent calling). But there was inbreeding depression in call parameters. Inbreeding decreased first call bout duration and mean call bout duration, and increased the time to start calling (latency). The *T. commodus* and *G. sigillatus* studies demonstrate that sexual traits vary in their susceptibility to inbreeding depression, but do not provide evidence that sexual traits show heightened inbreeding depression relative to non-sexual traits.

Stalk-eyed flies

Stalk-eyed flies are characterised by their eyes being located on stalks protruding from their sides of their heads. In many species, males have larger eyespans than females as a result of female mate preference (Cotton et al., 2006, Wilkinson and Reillo, 1994, Hingle et al., 2001) and male-male competition (Panhuis and Wilkinson, 1999, Small et al., 2009). In line with predictions, a one generation full-sibling inbreeding study in the stalk-eyed fly *Teleopsis dalmanni* found that male eyespan (a sexual trait) decreased more than female eyespan (a non-sexual trait) (Prokop et al., 2010). However, after controlling for body size, no significant effect in inbreeding was observed in either male or female eyespan. This result shows that the sexual trait does not suffer from heightened inbreeding depression.

A further study used 11 generations of full-sibling inbreeding to generate highly inbred lines of a different stalk-eyed fly species, *Diasemopsis meigenii* (Bellamy et al., 2013). They found that over the period of repeated inbreeding, the male sexual trait (eyespan) decreased more than the control traits of male wing length and the homologue female eyespan. After the period of controlled inbreeding, the inbred lines were crossed to generate inbred and outbred families. There was a considerable response in outbreeding in terms of rebound in trait values. Amongst outbred flies, male eyespan increased in size more than male wing length, but about the same level as female eyespan after controlling for body size changes. These results are consistent with the theory that the male sexual trait suffers more from inbreeding depression than non-sexual traits.

Wolf spider

In the wolf spider, *Hygrolycosa rubrofasiata* males court females by drumming their abdomens on the floor. Male drumming rate is condition-dependent and females tend to mate with males with higher drumming rates (Mappes et al., 1996). In addition, male mobility is positively associated with male mating success (Kotiaho et al., 1998). The effect of population size on male drumming rate and mobility was examined in 39 isolated natural populations (Ahtiainen et al., 2004). Under controlled laboratory conditions males from smaller populations had reduced drumming rates but did not differ in mobility, suggesting inbreeding depression in one male sexual trait (drumming rate) but not the other (mobility). Although no comparisons were made to non-sexual traits, body condition and survival also showed evidence of inbreeding depression associated with smaller population size. Given that individuals from different populations were measured under a common environment, it suggests that differences between populations were genetic, although there was no independent measure of genetic variation to establish that the relevant difference truly reflected inbreeding depression.

DISCUSSION

Do secondary sexual traits suffer from inbreeding depression?

Based on the simplest interpretation, our review of the literature provides strong support that male secondary sexual traits suffer from inbreeding depression. Collectively, 15 out of 16 studies reviewed (Table 1) report inbreeding depression in male sexual traits. Lack of support for this hypothesis was found in only one study (Frommen et al., 2008). This high rate of positive results suggests that sexual traits do indeed suffer from inbreeding depression.

Several studies looked at multiple sexual traits (e.g. male ornament and courtship behaviour) in a single species, and found that while inbreeding depression was identified for at least one of the sexual traits, it rarely was identified in every sexual trait (Table 1). Likewise, a range of studies looked at multiple finer scale components of single sexual traits and found that only some components suffered from inbreeding depression. Treating each trait or component as an independent entity, 25 out of 40 sexual traits showed inbreeding depression. The remaining 15 traits/components did not show evidence of inbreeding depression.

In addition, there appeared to be considerable variation in the extent to which individual sexual traits suffered from inbreeding depression. For example, the several studies of guppy colouration are inconsistent in their reporting of inbreeding depression (Table 1). We have refrained from making a formal comparison of the effect sizes found in different studies because there is considerable variation in the experimental design used, and making such a compendium figure would be of dubious validity. However, it seems reasonable to conclude that there is high heterogeneity in the response of sexual traits to inbreeding, and this ought to be the subject of further investigation. From Table 1 there appears no obvious pattern in comparisons of morphological traits, song, colouration and behaviour – there is simply insufficient data to be confident in drawing conclusions. This heterogeneity is to be expected if different sexual traits (or components of a single sexual trait) have different signalling roles and so vary in their degree of condition dependence. But we remain ignorant of whether this is the main or a significant factor.

Does inbreeding depression in sexual traits persist after controlling for body size?

Body size is usually related to major components of fitness, so it is a trait that is expected to suffer from inbreeding depression (Pante et al., 2001, Thrower and Hard, 2009, Holt et al., 2005, Mandal et al., 2005). Since male sexual traits often correlate with body size (Bonduriansky, 2007), they too might show inbreeding depression simply because of this association. If sexual traits are particularly sensitive to genetic stress, we might expect that they will show inbreeding depression after control for body size, as has been shown in several studies using environmental stress to investigate condition dependence (Cotton et al., 2004b).

Only one study found that inbreeding depression in the sexual trait was not independent of body size scaling, bringing out the importance of this control (Prokop et al., 2010). However, a large number of studies did measure body size in some manner, almost half did not (8 out of 16). This seems odd given the attention control of body size variation has received in several previous commentaries on the signalling role of sexual traits (Cotton et al., 2004b). Of particular concern are studies of behaviour where only 1 out of 8 studies took account of body size, and it appears that there is an implicit assumption that correlations with body size variation don't exist. If a sexual ornament or behaviour does not correlate with body size this should be demonstrated rather than being assumed. This picture raises concern that some examples of inbreeding depression in sexual traits may just reflect the overall association with body size.

Do sexual traits suffer greater inbreeding depression than non-sexual traits?

Sexually selected traits are predicted to have evolved condition-dependent expression and so be dependent upon a greater number of loci relative to non-sexual traits (Rowe and Houle, 1996, Tomkins et al., 2004, Pomiankowski and Møller, 1995). They should therefore constitute larger targets for the negative effects of inbreeding and suffer from heightened inbreeding depression compared to non-sexual traits. Using genetic stress, 4 studies support this claim (Sheridan and Pomiankowski, 1997, Zajitschek and Brooks, 2010, Aspi, 2000, Bellamy et al., 2013, Drayton et al., 2007) but 2 do not (Prokop et al., 2010, Bolund et al., 2010, van Oosterhout et al., 2003). This pattern contrasts with an abundance of literature using environmental stress that demonstrate that sexual traits are more condition-dependent than non-sexual traits (Cotton et al., 2004b, Bonduriansky and Rowe, 2005, Boughman, 2007, Kemp, 2008, Siitari et al., 2007, Eraud et al., 2007, Punzalan et al., 2008, McGuigan, 2009, David et al., 1998). Overall the evidence from inbreeding studies only weakly supports the idea that male secondary sexual traits respond strongly to genetic stress.

The lack of evidence for heightened inbreeding depression in sexual traits needs to be considered in the light of aspects of experimental design. A key issue is scale . In the studies reported here, the number of inbred lines typically assessed was small, rarely more than 20 lines (Table 1). The number of individuals per line that were assayed was also often very few (Table 1). In order to reliably measure inbreeding depression in the future it will be essential to assay many lines (Keller and Waller, 2002, Lynch, 1988). Although logistical constraints will inevitably limit what is possible in any given study species, the lack of support for greater inbreeding depression in sexual traits in many experiments could just be a consequence of the lack of statistical power.

A related problem is that the full impact of the negative effects of inbreeding may not be apparent after just one generation of inbreeding. To reveal the effects of inbreeding, repeated brother-sister matings may be required. For example, in the guppy *P. reticulata*, the effect of inbreeding depression on male pigmentation was negligible after one generation, but was revealed after two generations (van Oosterhout et al., 2003). Repeated inbreeding also has the advantage of allowing data on several generations of inbred individuals to be analysed together (after including appropriate statistical controls to account for repeated measures), charting the response in traits to increases in the *f*-value. It takes of the order of 10 generations to raise the probability of identity at a locus to greater than 90% (Falconer and Mackay, 1996). Such an approach has great promise but has only been attempted in one study (Bellamy et al., 2013), and proved highly illuminating. Note however that repeated brother-sister mating will lead to fixation of the majority of recessive deleterious alleles and subsequent rounds of brother-sister mating may have limited response (Falconer and Mackay, 1996). In addition, in long-term experiments mutational input and selection are likely to make increasing contributions to the response (Garcia-Dorado, 2012). These factors may limit the value of repeated inbreeding in the context of secondary sexual traits.

Does inbreeding reveal condition-dependent GEIs?

Finally, the theme of this book cannot be ignored. Environments vary in the way in which they interact with genetic variation. We know from many previous studies that sexual traits are more sensitive to environmental variation and that in some cases, the genetic signal is amplified under environmental stress (Cotton et al., 2004b). Previous work has also shown that the effects of inbreeding depression can be magnified in relatively stressful environments (Armbruster and Reed, 2005). However, nearly all of the studies here were carried out under standard laboratory conditions, which most likely are equivalent to minimal environmental stress.

A single study tested for inbreeding depression on male sexual traits under different levels of environmental stress. In the guppy *P. reticulata*, rearing inbred males in more stressful environments revealed greater inbreeding depression in pigmentation, but not in courtship behaviour (Zajitschek and Brooks, 2010). It may well be that genetic differences between sexual and non-sexual traits are typically weak or non-existent when masked by benign environmental conditions. In large part it remains to be investigated whether genetic differences between traits are revealed when environmental and genetic stress levels are elevated.

CONCLUDING REMARKS

There is a pressing need for quantitative investigations of the GEIs of condition-dependent sexual traits (Cotton et al., 2004b, Tomkins et al., 2004). We know far more about the sensitivity of sexual traits to environmental stress than to genetic stress and very little about the interaction between the two sources of stress. Inbreeding is potentially a powerful tool to remedy this gap in our knowledge. Unlike other genetic approaches, it requires neither direct manipulation nor even a precise definition of condition. It also gives a scale on which genetic stress can be evaluated, through the simple assumption that on average, inbred animals suffer greater genetic stress which increases as a function of the inbreeding coefficient.

The potential of inbreeding to investigate condition-dependent genetic expression of male sexual traits was recognised many years ago (van Oosterhout et al., 2003, Sheridan and Pomiankowski, 1997), promoted in an influential review article (Tomkins et al., 2004) and has been increasingly used in recent papers (Bellamy et al., 2013, Zajitschek and Brooks, 2010, Prokop et al., 2010, Okada et al., 2011, Bolund et al., 2010, Ketola and Kotiaho, 2010). However, the number of experimental studies remains tiny relative to the explosion of attempts to measure environmental condition dependence. As well as being few in number, the relevant studies are often poorly designed, lacking proper control traits, with small sample size and only a few generations of inbreeding. In order to capitalise on the potential of inbreeding in this context, it will be necessary to employ more rigorous experimental designs on a far more ambitious scale.

The greatest omission in the field is the lack of exploitation of GEI designs. It has been shown repeatedly that sexual traits are highly sensitive to environmental stress and is some cases that greater environmental stress amplifies genetic differences

(David et al., 2000, Cotton et al., 2004b). Significant insights are likely to be gained by coupling inbreeding as a scaled increase in genetic stress with a graded range of controlled environments that differ step-wise in stress (e.g. changes in temperature, diet, toxins). Thus far only one study has used inbreeding to provide a source of genetic stress in each of two environments that are distinctly different in stress (Zajitschek and Brooks, 2010). Far more studies using this approach are needed.

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Table 1. Inbreeding depression in sexual ornamentation and courtship behaviour

Species		Number of generations of inbreeding	N	L	Sexual trait(s)	ID in sexual trait(s)	Non-sexual trait(s)	ID in non-sexual trait(s)	ID Sexual trait > ID non-sexual trait[1]	BS control	Reference
Guppy	*Poecilia reticulata*	1-2	14-24	6	Carotenoid pigment (Paria)	Yes	Melanin/iridescent pigments	No	Yes	Yes	Sheridan & Pomiankowski (1997)
					Carotenoid pigment (Aripo)	No	Melanin/iridescent pigments	Yes (mel) No (irid)	No	Yes	
	Poecilia reticulata	3	~13	14	Courtship behaviour	Yes	-	-	-	No	van Oosterhout et al. (2003)
					Carotenoid and melanin pigments	Yes	-	-	-	Yes	
	Poecilia reticulata	1	2-4	12	Courtship behaviour	Yes, most components	-	-	-	No	Mariette et al. (2006)
					Carotenoid and melanin pigments	No	-	-	-	Yes	
	Poecilia reticulata	3	~20	20	Attraction in mate choice assay	No	-	-	-	No	Zajitschek & Brooks (2010)
					Several colour pigments	Yes, melanin /iridescence in stressful env. No, fuzzy black /carotenoid	Area of tail	No	Yes	Yes	

Stickleback	*Gasterosteus aculeatus*	1-2	-	14-30	Colouration	No	-	-	-	No	Frommen et al. (2008)
Least killifish	*Heterandria formosa*	1	~1-2	15	Mating behaviour	Yes	-	-	-	Yes	Ala-Honkola et al. (2009)
Zebra finch	*Taeniopygia guttata*	1	6	12	Male song	Yes, song directed at females and syllable rate	-	-	-	No	Bolund et al. (2010)
					Male beak colour	Yes	Female beak colour and morphological traits	Yes	No	No	
					Male plumage	Yes (1), No (3) elements	Morphological traits	Yes	No	No	
					Attraction in mate choice assay	Yes	-	-	-	No	
Fruit flies	*Drosophila melanogaster*	3-18	25	5-21	Male mating ability	Yes	-	-	-	No	Sharp (1983)
	Drosophila melanogaster	Isogenic lines	~16-80	6	Male courtship behaviour	Yes	-	-	-	No	Miller et al. (1993)
	Drosophila simulans	1	50	2	Mating behaviour	Yes	-	-	-	No	Okada et al. (2011)
	Drosophila montana	20	10-80	2	Song frequency	Yes	Song train characters	No	Yes	No	Aspi (2000)
Cricket	*Teleogryllus commodus*	1	~1-2	20	Calling effort	No	-	-	-	Yes	Drayton et al. (2007)
					Call structure	Yes (3), No (2) elements	-	-	-	Yes	
	Gryllodes sigillatus	7	50	1	Call effort	No	-	-	-	No	Ketola & Kotiaho (2010)
					Components of call	Yes (3) elements	-	-	-	No	

Stalk-eyed flies	*Teleopsis dalmanni*	1	-	24	Male eyespan	Yes, but No after body size control	Female eyespan, male wing length	No	No	Yes	Prokop et al. (2010)[2]
	Diasemopsis meigenii	11	~10-200	>100	Male eyespan	Yes	Female eyespan, male wing length	Yes	Yes	Yes	Bellamy et al, in prep
Wolf spider	*Hygrolycosa rubrofasciata*	NA	50-130	4	Drumming Rate	Yes	-	-	-	No	Ahtiainen et al. (2004)[3]
					Mobility	No					

Table footnote.

1. We report “Yes” when there was inbreeding depression in the sexual trait but not in the non-sexual trait.

2. Sexual and non-sexual traits showed inbreeding depression, but not after taking account of body size.

3. Populations grouped into 4 size classes taken to be equivalent to degree of inbreeding.

List of acronyms:

N - Number of inbred individuals assayed per line;

L - Number of inbred lines assayed;

ID - inbreeding depression;

BS - body size;

NA - not applicable to this study.

In the body of the table, the term ‘–’ indicates that the parameter in the corresponding heading was either not measured or not described in a given study.