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Insecticide resistance gene transmission by insecticide-susceptible insects

Since the conceptualization of sperm competition (Parker 1970) biologists have recognized that sexual selection can proceed post mating at the gametic level. Sperm competition occurs when sperm from two or more males compete for fertilization of a female's ova. This postmating phenomenon is recognized as a cryptic but powerful force in the evolution of reproductive physiology, behaviour and morphology. Males have developed mechanisms to combat sperm competition, such as rival sperm removal (Waage 1979) or sperm displacement (Scott & Williams 1993). However, male reproductive success is also influenced by the female and by the efficiency of sperm competition mechanisms in rival males. Therefore the proportion of progeny fathered by any given male depends on the ability of the male at sperm competition (Parker 1984), on the female sperm choice (Eberhard 1996) and on the sperm precedence pattern of the species (Simmons & Siva-Jothy 1998).

The widespread application of insecticides to control disease vectors and agricultural pests has resulted in the selection of resistance to the toxic effects of insecticides. When insecticide resistance problems arise, any influx of migrant-susceptible adults will tend to dilute the frequency of resistant phenotypes among survivors of previous treatments (Comins 1977; Georghiou 1980; Tabashnik 1990). This is exploited in integrated pest management programmes, which ideally aim to manage susceptibility (Daly & McKenzie 1986). To be able to manage susceptibility effectively, we need to have a good knowledge of the population biology of the pest organism. Most current programmes will often consider the phenotype or genotype of the insects captured and from these data, model resistance gene frequencies in the next generation. This may need to be reconsidered in the light of information gained from sperm competition studies.

The first point to consider is that an insecticide-susceptible female mated with a resistant male may have the capacity to store male resistant genes for extended periods and sire resistant individuals. This will be particularly important if resistance is inherited as a dominant trait, which is the case for numerous insecticide resistance genes and in numerous species (Bourguet *et al.* 1997). We argue that it is important to examine the type of gametes that females store in their spermatheca and the pattern of sperm competition which occurs in a given species ((in)complete first- or last-male sperm precedence, sperm mixing) to estimate the potential decrease or increase of the frequency of resistance genes in the population even in the absence of resistant adults.

Storage of sperm from resistant males would have a small impact in typically monandrous females with low dispersal. Polyandry is the norm in most insect species (Ridley 1988) and the spermatozoa of most males mated to a female participate in egg fertilization to different degrees, depending on mating order. Even if she has only mated once with a resistant male, a polyandrous-susceptible female could still transmit resistance genes many weeks after mating (Haubruge *et al.* 1997). The importance of this will be greater in long-lived, iteroparous females than in short-lived, semelparous ones. The spread of resistance genes through sperm storage will also be more important in highly dispersing species such as stored product insects which spread through the trade of cereals and their by-products (Freeman 1967; Haubruge *et al.* 1997). For instance, in the Red Flour Beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae), females that mated first with a resistant male and then with a susceptible one produced progeny with both phenotypes. Although the susceptible male fathered almost 70% of the progeny, demonstrating last-sperm precedence, resistant individuals were still produced by the female after more than 3 months (Haubruge *et al.* 1997). Thus, when such a female colonizes a new environment, insecticide resistance problems could appear even in the absence of resistant adults.

The second point to consider in resistance management involves fertilization 'by proxy'. Haubruge *et al.* (1999) recently observed that susceptible *T. castaneum* males also participate in the spread of insecticide resistance genes. Males of this species will scrape out the female bursa prior to mating to remove rival sperm from a previous mating. During this process, non-self sperm can attach themselves to the mating male's genitalia and be translocated into the reproductive tract of the next female he mates with. Translocated sperm carrying resistance genes can therefore be transferred from a susceptible male to a susceptible female. This means that a resistant male can fertilize a susceptible female without ever mating with her.

This evolutionary strategy should have an impact on insecticide resistance management. When we delineate the geographical extent and movement of insecticide-resistant insects, resistance detection and monitoring methods are always based on toxicological bioassays. However, because of sperm storage in females and fertilization 'by proxy', this methodology will underestimate the frequency of resistance genes in the population. Molecular tests based on DNA probes provide a much greater degree of resolution and more specific identification of resistance genes in individuals.

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Assaying PHA-induced mitosis: out of control?

Phytohaemaglutinin (PHA, a lectin derived from *Phaseolus vulgaris*) is routinely used as an *in vitro* T-cell mitogen (e.g. Avramis *et al*. 2001; Carloni *et al*. 2001). In this assay PHA is presented to cultured lymphocytes and has the rapid and easily observed effect of stimulating T-cell proliferation. Such T-cell proliferation is almost certainly an important component of the early phase of the acquired immune response in vertebrates: the assay therefore provides a clear, logical connection between the speed/magnitude of an immune response (T-cell proliferation) and host fitness in the face of pathogenic challenge. Not surprisingly this assay has consequently become popular with field ornithologists interested in assaying 'immunocompetence' in the context of ecotoxicology and parasite-mediated sexual selection. However, ecologists use the assay *in vivo* (see Goto *et al*. 1978) and usually perform it by injecting phosphate buffered saline (PBS) into one wing web and PHA dissolved in PBS into the other wing web. A set time after challenge the size of the swellings resulting from both injections are assessed and the measure of PHA-induced swelling is adjusted with reference to the PBS-only swelling.

Smits *et al*. (2000) recently published a technical note in which they made a recommendation for practitioners of the *in vivo* PHA assay. At the core of their thesis is their demonstration that the PHA minus PBS value (i.e. PHA-induced response relative to the PBS control) is strongly correlated with the PHA-only value within birds. Thus, because the immune response towards the PBS plus PHA injection consisted primarily, and consistently, of a response towards PHA in the studies in their analysis, Smits *et al*. (2000) conclude that the PBS-only component of the assay can be excluded from future experiments.

While we agree with their basic analysis we feel strongly that the main purpose of the PBS-only injection has been overlooked. It is the negative control for the procedure. Because the assay can be applied with 'minimal training and without specialized treatment' (Smits *et al*. 2000) it is highly likely to be applied to a variety of different avian models under different ecological conditions by practitioners of different technical aptitude. If such studies drop the negative control, how can those practitioners (let alone reviewers and editors) have an objective assessment of (a) the practitioner's technical ability to conduct the assay and (b) the nature of the response to the manipulation? In short, it is precisely because the negative control is negative that the experimenter(s), and their scientific audience, can interpret the resultant data in the context of the biological predictions that underpin the manipulation.

We have a second concern related to Smits *et al*.'s (2000) assessment of the repeatability of the assay. Smits *et al*. (2000) prudently demonstrate that it is possible to obtain repeatable measurements of the level of wing-web swelling. Since the test can be applied with minimal training and without specialized treatment this is reassuring and implicitly endorses the assay's

wider use. However, in ecological studies repeatability is often assessed for another reason – to quantify within-individual variation (see Boake 1989). Falconer & Mackay (1996) distinguish between 'spatial' and 'temporal' repeatability in this sense, reflecting the two forms of 'special environmental' variation. Thus, an analysis of variance of repeated measurements of the same individuals (taken either simultaneously from different points on the same individual, or with an appropriate time interval between sampling, respectively) is used to partition out the within-individual and among-individual variances. The ratio of the among-individual variance to the total variance provides the repeatability estimate.

In one sense, this level of repeatability analysis may seem of secondary importance compared with the obvious utility of repeatability as a statistical tool for estimating measurement error. For example, where the main aim is to compare mean PHA responses between various experimental and control groups (as in many ecotoxicological studies where experimental groups may be subjected to a particular toxin), an investigator may not be primarily concerned with establishing how consistently different individuals respond to the assay. However, in contrast, the main aim of behavioural ecological studies is usually to quantify population level variation in PHA-induced swelling, using the magnitude of the swelling as a putative correlate of 'immunocompetence' and hence individual quality or fitness (e.g. in relation to female choice for 'immunocompetent' males; Johnsen *et al*. 2000). In such studies it would be misleading to interpret PHA responsiveness as an index of 'immunocompetence' if (unquantified) within-individual variation was high enough to undermine the value of single measurements. Temporal repeatability is particularly relevant in this context, because one is usually interested in the potential of selection to shape immune function as an ecological character. Clearly, since PHA has antigenic – as well as mitogenic – properties, one cannot ignore the potential for acquired responses to confound *in vivo* estimates of temporal repeatability. However, this difficulty does not in itself detract from the need to demonstrate that the object parameter of the assay (T-cell proliferation following exposure to a mitogen) behaves consistently enough within individuals to undergo selection at an ecological level. In this context it should be readily apparent that spatial and/or temporal repeatabilities are of fundamental importance. Even where the main aim of a study is to compare mean PHA responses between different experimental and control groups, spatial and/or temporal repeatability still provides an important base-line from which to interpret results. Without an estimate of these parameters, one would have to assume that within-individual variation was low enough so as not to interfere with the detection of statistically significant differences. Otherwise, the likelihood of Type II statistical errors may be increased considerably (although the detection of significant differences among means would over-ride this concern).

In summary we strongly disagree with the recommendation that negative controls should be abandoned because they show consistently small effects compared to the treatment. Moreover, we recommend that spatial and temporal repeatabilities (rather than just the repeatability of the investigator's measurement technique) for the PHA response are measured in order to set a limit to the biological significance that can be attached to the results from the assay.

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Measurement repeatability and the use of controls in PHA assays: a reply to Siva-Jothy & Ryder

The validity of any scientific technique must never be taken for granted. In our recent paper (Smits, Bortolotti & Tella 1999), we questioned whether a well-established measure of T-cell proliferation in birds

could be improved methodologically. We thus welcome Siva-Jothy & Ryder's (2001) commentary on our paper questioning whether such a previously established protocol should be changed. Their main concerns are the abandonment of the negative control, and the nature of repeatability of measurements. We feel that clarification of the terms 'control' and 'repeatability' is required, and that what we have proposed in simplifying the phytohaemagglutinin (PHA) assay is both valid and valuable for future research.

There is no question that an experiment without a control is not science. The so-called control application of injecting phosphate buffered saline (PBS) into the opposite wing is in no way part of the true requisite control for the objective of ecological or toxicological studies. In Smits *et al*. (1999) we showed seven of our various studies to have a treatment (PCBs, mine tailings, etc.) and hence by inference there were both treatment and control groups (e.g. no exposure to PCBs or mine tailings). The same PHA test was applied to both groups of animals. This is how we understand the scientific method. The terminology 'control' for PBS injection was originally devised to demonstrate that the T-cell proliferation was a response to PHA itself but not to the vehicle (PBS) (Goto *et al*. 1978). As such PBS injection was a valuable and true control in the initial study whose objective was to validate the PHA test. The PBS injection does not in any way therefore allow one to 'interpret the resultant data in the context of the biological predictions that underpin the manipulation'.

Siva-Jothy & Ryder claim that without a negative control within individual birds 'how can those practitioners (let alone reviewers and editors) have an objective assessment of (a) the practitioner's technical ability to conduct the assay and (b) the nature of the response to the manipulation?' Clearly, Smits *et al*. (1999) demonstrate that in determining the 'nature of the response to manipulation' it is not necessary to prove over and over again that PBS is not responsible in provoking skin swelling at the PHA injection site. Hence the continued use of the word 'control' in reference to the PBS injection shows a lack of understanding of how the PHA test is used as a tool in ecological studies.

In contrast to Siva-Jothy & Ryder, we fail to understand how doing both a PHA and PBS injection in any way corrects for technical problems experienced by the practitioner. Simply, two wrongs do not make a right. As Smits *et al*. (1999) demonstrate quite clearly, incorporating the PBS measurement only increases error in estimation of the PHA response. The negative control is also not relevant to Siva-Jothy & Ryder's concern that people with minimal training can apply this test. Our emphasis on minimal training was made because the ability of a person to inject and measure a wing is independent of their understanding of the immune system, just as their ability to measure a wing is independent of a knowledge of aerodynamics. The only tool available for objective assessment of 'the practitioner's technical ability' rests in an evaluation of the repeatability of measurements, and thus we turn to Siva-Jothy & Ryder's second concern.

The term repeatability, as Siva-Jothy & Ryder correctly point out, can have two applications: within-individual variation as may exist in space and time, and variation attributable to measurement error. Smits *et al*. (1999), and most conscientious researchers, use the latter to validate their technical expertise. We reviewed 35 ecological papers published recently (1997–2001) where the PHA assay was used, and found PHA responses to have repeatabilities (r_i) averaging 0·94 (range: 0·70–0·99). Therefore, this objective assessment of ability shows measurement error ($1 - r_i$) is typically only 6% and so the technique can, and has, been applied with an exceptional degree of reliability.

The nature of repeatability in space and time, to understand within-individual variation, was emphasized by Siva-Jothy & Ryder as being important. There are certainly questions where such information is of interest; however, they rest more within the realm of immunology, i.e. where immune function itself is the focus of the study. The PHA test can be used in ecology as a tool for answering questions of a different focus. For example, if we want to study the relationship between prelaying mass and clutch size in a bird, it would not be necessary to document mass of all body parts or mass of the bird in the future. Mass variation is a dynamic process as is immune response. While physiologists may wish to study how mass can vary among organ systems, across seasons and so on, it does not diminish the use of mass as a variable in a simpler form in cross-sectional studies typical of ecology or toxicology.

We could envision how temporal variation in immune function could provide valuable information regarding long-term constancy of immune response, which could be linked to a genetic component. Genetic and environmental components to the T-cell mediated immune response have been well evaluated using cross-fostering experiments (Brinkhof *et al*. 1999; Christe *et al*. 2000; Tella *et al*. 2000a). Alternatively, temporal repeatabilities offer a way of estimating maximum heritability, which in turn fail to disentangle genetic from environmental factors (Falconer & Mackay 1996). Moreover, attempts to quantify such variation have at least three potentially serious problems.

First, there are many confounding variables associated with longitudinal research. Many studies are conducted on nestlings, which show PHA responses different from adults (Martin *et al*. 2001; J. E. Smits & G. R. Bortolotti, unpublished data). Therefore, ontogenetic effects complicate interpretation of

temporal variation. Other variables that would have to be controlled for include seasonal and or age-related patterns in the production of immune-modulating hormones, body condition, and a variety of stressors including temperature, food supply, diet, parasites and social conditions (see, e.g. Dohms & Metz 1991; Lloyd 1995; Duffy *et al*. 2000; Alonso-Alvarez & Tella 2001; Tella *et al*. 2001). There is also the potential for additional artefacts caused by differential stress/habituation caused by repeated handling of the same birds given the immunosuppressive nature of corticosterone. To account for all of these would be a formidable task even in captive birds.

Second, the use of temporally separated tests on the same individuals must consider the artefacts created by injections or other modification of the skin surface (e.g. feather plucking at the injection site). Insertion of the needle plus a space-occupying fluid into the thin and delicate skin of a bird causes some disruption to the subepithelial and hypodermal tissue, and resulting stimulation of fibroblasts leads to changes in the matrix of the tissue and hence one's ability subsequently to measure the PHA response without bias.

Third, the immune system has a memory. The whole idea of using a plant enzyme is that it will be a novel mitogen. Repeated use of the same compound thus invalidates the independence of test points given PHA is no longer a novel antigen and one must account for adaptive aspects of subsequent immunological responses. Responses to second injections of PHA have been larger than the first (Johnsen *et al*. 2000; Alonso-Alvarez & Tella 2001). While Siva-Jothy & Ryder recognize this as a problem, they believe it to be of secondary importance to the need to demonstrate that individuals respond in a consistent manner. We believe their rationale is flawed.

Siva-Jothy & Ryder propose temporal stability of the PHA response as being necessary for studies in behavioural ecology, but not ecotoxicology. We see no scientific justification for their position related to discipline. Furthermore, we disagree that stability is relevant to 'the potential of selection to shape immune function as an ecological character' or how this relates to setting a 'limit to the biological significance' of the results. It appears that the authors do not recognize that natural selection can operate on traits subject to temporal variation. To give an example of mass dynamics again, it is well documented that selection acts on fledging body mass. Heavy fledglings survive the best; information on daily, seasonal or annual fluctuations in mass after that period is irrelevant to that fact. It would not be a surprise in fact if heavy nestlings remained heavy throughout life, just as immune response measured at a single point in time has been correlated (Saino *et al*. Saino *et al.* 1997; González *et al.* 1999; Soler *et al*. 1999) or implicated (Tella *et al*. 2000b) with survival in the future.

Through these arguments, we continue to support our modification of the PHA skin test as a valid research tool in broadly based studies in ecology and ecotoxicology. The simplified method has many advantages both in the application of the test and the interpretation of the results. This test has a valuable role as one of a suite of applications, as it is recognized as measuring one early aspect of the immune response potential.

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