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Nondestructive sampling of insect DNA from defensive secretion

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Abstract

Nondestructive techniques to obtain DNA from organisms can further genetic analyses such as estimating genetic diversity, dispersal and lifetime fitness, without permanently removing individuals from the population or removing body parts. Possible DNA sources for insects include frass, exuviae, and wing and leg clippings. However, these are not feasible approaches for organisms that cannot be removed from their natural environment for long periods or when adverse effects of tissue removal must be avoided. This study evaluated the impacts and efficacy of extracting haemolymph from a defensive secretion to obtain DNA for amplification of microsatellites using a nondestructive technique. A secretion containing haemolymph was obtained from *Bolitotherus cornutus* (the forked fungus beetle) by perturbation of the defensive gland with a capillary tube. A laboratory experiment demonstrated that the sampling methodology had no impact on mortality, reproductive success or gland expression. To evaluate the quality of DNA obtained in natural samples, haemolymph was collected from 187 individuals in the field and successfully genotyped at nine microsatellite loci for 95.7% of samples. These results indicate that haemolymph-rich defensive secretions contain DNA and can be sampled without negative impacts on the health or fitness of individual insects.

Keywords: Coleoptera, forked fungus beetles (*Bolitotherus cornutus*), haemolymph, microsatellite analysis

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Introduction

Nondestructive sampling of DNA for genetic analyses is a vital component of many studies in the fields of conservation, ecology and evolution. Biologists increasingly rely on genetic sampling to infer patterns of dispersal, estimate lifetime fitness and assess long-term population viability in endangered species. Although nondestructive techniques of obtaining DNA have been developed in a variety of taxa (e.g. Broquet *et al.* 2007; Caudron *et al.* 2007), a common sampling technique in insects often involves killing the individual. This approach could alter the dynamics of populations and is unacceptable for use in protected species, museum specimens, studies that involve key individuals or long-term studies of lifetime fitness (Ribble 1992; Holehouse *et al.* 2003; Valade *et al.* 2009; Monroe *et al.* 2010).

There are a variety of techniques of sampling DNA that are nonlethal to insects. One noninvasive technique involves collection of frass (excreted insect waste) or exuviae (remains of the exoskeleton). This methodology is most useful in organisms that have at least one stage of their life cycle that produces a large quantity of frass, such as caterpillars, or when individuals are likely to frequently shed their exoskeleton (Feinstein 2004; Watts *et al.* 2005). These collection methods, however, require large amounts of time to observe moulting or involve isolating individuals from others in the population and from their natural environment (Monroe *et al.* 2010). When individuals cannot be taken out of their environment or when spending a large quantity of time is impractical, wing and leg clippings have been taken in such a way that damage resembles natural wear and tear (Hamm *et al.* 2010; Monroe *et al.* 2010). In studies involving wing or leg clippings, survivorship, mating behaviour or other animal behaviours such as pollen collection or flight were not severely affected (Starks & Peters 2002; Holehouse *et al.* 2003; Chaline *et al.* 2004; Vila *et al.* 2009; Koscinski *et al.* 2011). While tissue removal is a valuable approach in some systems, these techniques may be inappropriate in certain cases (for example, museum specimens) or with certain insects of interest that may more heavily rely

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on these body parts for reproduction, feeding or movement. In these situations, a nondestructive technique is essential.

One technique to obtain DNA without removal of tissue is by drawing haemolymph. In bumble bees, pointed capillary tubes were used to extract haemolymph from the thorax; however, survivorship was low (Moret & Schmid-Hempel 2000; Holehouse *et al.* 2003). In addition to the risk of high mortality, haemolymph extraction from the thorax may not be feasible if the thorax is inaccessible or impenetrable, as it is in many species of beetles. Extraction of haemolymph by stimulating defensive response may be an alternative to tissue removal. Defensive secretions often contain haemolymph in species such as in froghoppers (*Prosapia* sp. n.), sawfly larvae (*Athalia rosae*) and armoured ground crickets (*Acanthoplus discoidalis*) (Peck 2000; Muller *et al.* 2001; Bateman & Fleming 2009). The study of these organisms could benefit from a nonlethal and nondestructive technique of DNA sampling (Peck 2000; Muller *et al.* 2001; Bateman & Fleming 2009).

The goal of this study was to develop a nondestructive technique that could use insect defensive secretions as a source of DNA. In this study, 0.2–4.0 μL of a defensive secretion were drawn from individual *Bolitotherus cornutus* (forked fungus beetle). We assessed the impact of this collection on survivorship, defensive behaviour and reproductive output in a laboratory setting. We also evaluated the feasibility of this protocol on a large number of individuals by collection of defensive secretion from a wild population.

Methods

Study species

Bolitotherus cornutus, the forked fungus beetle (Panzer 1794; Coleoptera: Tenebrionidae), primarily lives on logs infected with three species of bracket-forming fungi: *Ganoderma applanatum*, *Ganoderma tsugae* and *Fomes fomentarius* (Liles 1956; Pace 1967). These beetles are long lived (up to 8 years; H. M. Wilbur, personal communication).

Legs are essential not only for movement and adherence to the substrate, but also for mating, in which males use all six legs to grip females during courtship and mate guarding (Conner 1988; Formica *et al.* 2011; K. M. Benowitz, E. D. Brodie III & V. A. Formica, unpublished). Like most coleopterans, *B. cornutus* have two wings under hardened elytra that make wing tissue inaccessible without injury. These beetles normally display a defensive gland with two bulb-like tips at the end of the abdomen that surface from under the anal sternite when subjected to warm mammalian breath (Conner *et al.* 1985; Fig. 1). Normally, these glands secrete a defensive volatile that decreases predation by small mammals such as mice

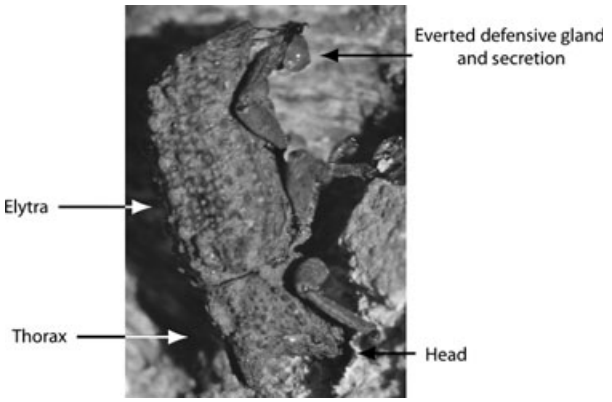

Fig. 1 A disturbed male *Bolitotherus cornutus* everting his defensive glands (indicated by arrow). While this particular male was not measured, a male with his horn length to body size ratio would be ~9 mm in length. The drop of secretion on his gland is ~0.5–1 μL. The photograph was taken by Stan Malcolm.

(Conner *et al.* 1985; Holliday *et al.* 2009; Tschinkel 1975a,b). Living *B. cornutus* range from 6.8 to 13.7 mm in length (mean = 10.9 ± 0.10 mm) and weigh 0.11 ± 0.005 g (unpublished).

Collection of defensive secretion

A volume of defensive secretion was collected from all beetles by stimulating the defensive behaviour. In our procedure, beetles were blown on gently by researchers, stimulating the eversion of the defensive gland and reflexive bleeding. Upon gland eversion, a capillary tube (Drummond Scientific Company 1–5 and 10 μL) was placed on the surface of the gland. If the secretion was not immediately drawn into the capillary tube, the gland was gently abraded with the tip of the capillary tube. This procedure continued until at least 0.2 μL of secretion was obtained. Beetles varied in their opening of the anal sternite and expression of the gland. In a few cases, anal sternites had to be gently forced open. Contents of the capillary tubes were then blown into a tube containing 50 μL of prepared lysis buffer (Promega DNA IQ System) for storage.

Laboratory experiment: impacts on survivorship and reproduction

Forty wild-caught males and 40 wild-caught females were captured in May and June of 2010 around the property of Mountain Lake Biological Station (Pembroke, Virginia). The beetles were kept at 23 °C with a 12L:12D light cycle and given *G. tsugae ad libitum* throughout the experiment. The collected individuals were equally divided into treatment and control groups. Because one goal of the experiment was to assess the effect of

defensive secretion collection on egg laying, we ensured that mean female body size was equal in both groups in case there was a size effect on fecundity, as is often seen in insects. Each female was then randomly assigned a male, making a total of 40 pairs. Haemolymph was collected from treatment pairs in the method described above. Control pairs were handled identically except that the area surrounding the anal sternite and abdomen was rubbed with a similar sized capillary tube without contacting the defensive gland or internal organs. Both treatment pairs and control pairs of beetles were placed in individual containers in an incubator on a 12L:12D photoperiod at 23 °C. Survivorship of individuals and gland eversion were checked every 7–8 days for 3 weeks. Gland eversion (whether a beetle everted its gland or not) was tested by blowing air over the subject to determine if the treatment affected beetles' responses to predators; neither the gland nor the anal sternite was physically disturbed with any instrument at this time. At the end of the 3 weeks, the light cycle was switched to 6D:18L to induce reproductive activity. During the following 2 months, the number of eggs was recorded and all eggs were collected twice for each pair. Males and females were considered reproductively successful if the female laid one or more eggs during the trial period. There is no previous evidence suggesting that female *B. cornutus* lay unfertilized eggs; additionally, a small sample ($N = 9$) of larvae were hatched from eggs in both treatments, suggesting that egg counts are an appropriate measure of successful mating.

Very little mortality was observed in either group, so Fisher's exact test was used to test for effects of treatment on survival (number of individuals alive at the end of 3 weeks) and for the effect of sex on survival in both the treatment and in the control group. Fisher's exact test was also used to compare groups for differences in gland response. A generalized linear model with a Poisson distribution was used to determine if the total number of eggs produced over a 2-month period by each pair differed between treatment groups. One pair of beetles in the treatment group escaped from its container during the experiment and was excluded from this analysis.

Feasibility of collection and PCR amplification in a wild population

During June and July 2011, all adult beetles were collected from a single population (i.e. 187 individuals from one fallen tree with fungi) on the property of Mountain Lake Biological Station in Pembroke, Virginia. Each beetle was individually labelled and defensive gland secretions were obtained using the method described above. All secretion samples were immediately stored in 50 μL of prepared lysis buffer and extracted using Promega DNA IQ System (Promega 2010). Each beetle was removed from the population and returned within 48 h owing to the nature of our labelling method. In cases where field collection of haemolymph is preferable, capillary tubes, prepared lysis buffer and samples can be easily transported and stored at room temperature for a number of days. The DNA IQ system was designed for use in human forensics and works well for extracting DNA from samples with small original volumes. Previous attempts to use other methods of extraction that did not include a filter or magnetic step failed to produce PCR product, suggesting that a compound in the defensive secretion may inhibit the PCR reaction. We followed the methods from the Promega Small Sample Casework Protocol with some minor modifications: we used 25 μL of the sample and lysis buffer mixture in each reaction, and used half the recommended amount of all reagents (Promega 2010). DNA was eluted in 40 μL of elution buffer. The concentration of DNA yielded by these techniques was determined using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA) with the Qubit dsDNA HS Assay Kit and was found to be 0.33 ± 0.06 ng/μL. While these concentrations are low compared to DNA collected with body parts or whole body extractions (0.95 ± 0.47 ng/μL), the amount of total extracted DNA was sufficient to run ~40 PCR reactions (using the methods described below). We also tested 11 samples with known amounts of sampled haemolymph and regressed the resultant DNA concentration on the amount of haemolymph collected. We estimated the cost including pipette tips and other plastics at $1.53 US per individual (DNA IQ kit alone = $1.01 per individual).

All samples were run in one microsatellite multiplex reaction using the Qiagen Multiplex PCR kit (Qiagen 2010; Table S1, Supporting information) – we followed the protocol in the accompanied handbook, except that we reduced the total volume of the reaction to 10 μL, ultimately using 1 μL of the eluted DNA solution. The PCR contained nine primer pairs (Table S2, Supporting information; V. A. Formica, R. A. Johnson & E. D. Brodie III, unpublished). All individuals were extracted and run in the PCR once. Fragment analysis was completed by GeneWiz Inc. (South Plainfield, NJ, USA) using Applied Biosystems 3730xl DNA Analyzers, and peaks were analysed using Peak Scanner software (version 1.0; Applied Biosystems Inc., Foster City, CA, USA). An extraction was considered a failure if the multiplex reaction failed to produce any peaks that could be confidently called by a trained researcher. An extraction was considered low quality if three or more of the nine loci failed to produce readable peaks. These failures may be due to reaction problems other than the quality of the DNA; however, to be conservative during our feasibility analysis, we attributed all failed individuals or loci to the DNA extraction.

Results

In the laboratory experiment, mortality did not differ by treatment (Fisher's exact test, $P = 0.117$) or sex within either the treatment (Fisher's exact test, $P > 0.999$) or control groups (Fisher's exact test, $P > 0.999$). Gland response did not differ by treatment (Fisher's exact test, $P = 0.103$) or sex within either the treatment (Fisher's exact test, $P > 0.999$) or control groups (Fisher's exact test, $P = 0.482$). Finally, the number of eggs laid by a pair did not differ between treatment and control pairs (Control = 2.2 eggs ± 0.57 SE; Treatment = 2.0 eggs ± 0.59 SE; $\chi^2 = 0.615$; d.f. = 1.76; $P = 0.433$; Table 1).

For our feasibility study in the field, we collected and sampled 187 adult *Bolitotherus cornutus*. Four samples suffered complete failure in the multiplex reaction, yielding a success rate of 97.8%. Another four samples had three or more loci that could not be confidently called and were considered low quality, bringing the total to eight from 187 samples with failure or low quality yields from our method (95.7% success rate). DNA concentration covaried positively with the amount of haemolymph collected ($R^2 = 0.61$, $F_{1,9} = 14.34$, $P = 0.004$; Fig. 2).

Table 1 Mortality of males and females in the treatment and control groups used in contingency tables for Fisher's exact tests reported in text

Treatment group	Sample size	Total dead	Mean weeks alive ± SE
Male control	40	1	2.9 ± 0.08
Female control	40	0	3.0 (all survived)
Male treatment	40	3	2.9 ± 0.06
Female treatment	39	2	2.9 ± 0.07
Combined control (male and female together)	80	1	
Combined treatment (male and female together)	79	5	

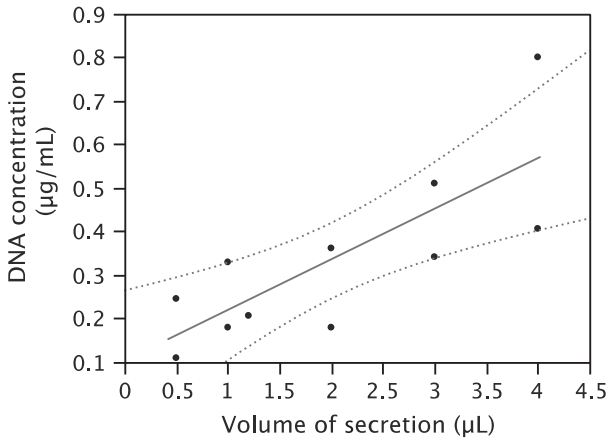

Fig. 2 Relationship between the concentration of the eluted DNA solution and the volume of defensive secretion collected from the everted defence gland. The solid line is the linear regression and the dotted lines are the 95% confidence curves.

Discussion

Mortality, defence response and reproduction of *Bolitotherus cornutus* were not negatively affected by extraction of the defensive secretion for DNA. The resulting DNA was of high enough quality for any future studies and was easily obtainable from over 180 individuals that were never permanently removed from their natural environment. Nondestructive sampling of *B. cornutus* using this method will allow us to collect DNA for population structure studies, parentage analyses and to assess lifetime fitness with no detriment to the study subjects or populations.

The majority of mortality in this study (all but one male and one female) occurred to individuals in the first sampling period, in which two groups of five males and five females were used in each of the control and treatment groups (a total of 20 beetles used). Mortality of individuals in this sampling period was most likely the result of perfecting the techniques to open the anal sternite and place the capillary tube in the correct position near the gland for extraction.

This study suggests that haemolymph-rich defensive secretions may provide high-quality DNA for other insects, especially those species with defence secretions from glands or that exhibit reflex bleeding, such as other beetle species (e.g. the tenebrionid *Lubprops tristis*), all termite species or the armoured ground cricket (*Acanthoplus discoidalis*) (Tschinkel 1975a,b; Bateman & Fleming 2009; Abhitha *et al.* 2010; Sobotnik *et al.* 2010). In conclusion, the extraction of haemolymph from a defensive secretion can be used to successfully amplify microsatellites from a large number of individuals, facilitating further genetic studies using insects.

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Data accessibility

PCR reagents, conditions and temperatures uploaded as Table S1 (Supporting information).

Microsatellite primers: NCBI Probe Database ID numbers – 12324345–12324358. These data are uploaded as Table S2 (Supporting information).

Survivorship, gland health and egg laying data: DRYAD entry doi:10.5061/dryad.75pp8f32.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 (A) Reaction conditions for PCR and (B) PCR cycle protocol used to evaluate haemolymph sampling and DNA extraction techniques.

Table S2 Microsatellite primer information submitted to NCBI Probe database, including primer sequence and repeat motif of the loci.

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