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Removing external DNA contamination from arthropod predators destined for molecular gut-content analysis

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Abstract

Ecological research requires large samples for statistical validity, typically hundreds or thousands of individuals, which are most efficiently gathered by mass-collecting techniques. For the study of interspecific interactions, molecular gut-content analysis enables detection of arthropod predation with minimal disruption of community interactions. Field experiments have demonstrated that standard mass-collection methods, such as sweep netting, vacuum sampling and foliage beating, sometimes lead to contamination of predators with nontarget DNA, thereby compromising resultant gut-content data. We deliberately contaminated immature *Coleomegilla maculata* and *Podisus maculiventris* that had been fed larvae of *Leptinotarsa decemlineata* by topically applying homogenate of the alternate prey *Leptinotarsa juncta*. We then attempted to remove contaminating DNA by washing in ethanol or bleach. A 40-min wash with end-over-end rotation in 80% EtOH did not reliably reduce external DNA contamination. Identical treatment with 2.5% commercial bleach removed most externally contaminating DNA without affecting the detectability of the target prey DNA in the gut. Use of this bleaching protocol, perhaps with minor modifications tailored to different predator–prey systems, should reliably eliminate external DNA contamination, thereby alleviating concerns about this possible source of cross-contamination for mass-collected arthropod predators destined for molecular gut-content analysis.

Keywords: *DNA* decontamination, molecular gut-content analysis, sampling

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Introduction

Molecular gut-content analysis, by immunoassay for prey proteins or by polymerase chain reaction (PCR) assay for prey DNA sequences, is an established and increasingly important approach for tracking arthropod predation in the field (Hagler & Naranjo 1994; Symondson 2002; Cuthbertson *et al.* 2003; Harwood *et al.* 2004, 2007; Juen & Traugott 2007; Kuusk *et al.* 2008; Lundgren *et al.* 2009; Szendrei *et al.* 2010). Molecular gut-content analysis causes minimum disruption to community interactions, requiring only brief periodic intrusions into the field for specimen collection. It is especially useful for situations where predators occupy dense and tangled microhabitats (Harwood & Greenstone 2008), and indispensible if they are in a completely inaccessible space (e.g. Jaramillo *et al.* 2010).

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Ecological research requires large samples for statistical validity, typically hundreds or thousands of individuals, which are most efficiently gathered by mass-collecting techniques. For foliar predators destined for gut-content analysis, these have included sweep netting (Ruberson & Greenstone 1998; Hagler & Naranjo 2005; Harwood 2008), vacuum sampling (Hagler & Naranjo 1994; Chapman *et al.* 2010) and foliage beating onto a net or drop cloth (Sigsgaard *et al.* 2002; Sheppard *et al.* 2004; Fournier *et al.* 2008). Because such methods can cause animals to come into contact with one another and with objects in the environment, they pose the risk that exogenous proteins or nucleic acids could be transferred to the integument of animals under study, thereby compromising later molecular gut-content analysis.

Three routes have been hypothesized (King *et al.* 2008; Greenstone *et al.* 2010) by which mass collection of arthropod predators could lead to external contamination with exogenous material: via material regurgitated by the predators themselves; via material released from prey

that have been broken up during collection; or via insect faeces contacted by the collecting apparatus. Field data may also be compromised by postcollection feeding on prey in the collecting apparatus (King *et al.* 2008). Two experimental field studies have demonstrated explicitly that either or both of the first two routes can lead to external contamination occurring during mass collection employing foliage beating (Greenstone *et al.* 2010) or vacuum sampling (King *et al.* 2011). Contrariwise, a field experiment employing sweep netting (Harwood 2008), and another employing vacuum sampling (Chapman *et al.* 2010), found no evidence of external contamination. Given these results, one can safely conclude that external contamination of mass-collected predators, while not a certainty, presents a significant risk to the molecular ecological study of predation and cannot be dismissed out of hand. The risk of such contamination must therefore be reckoned with whenever gut-content analysis of mass-collected predators is contemplated.

Although the risk of contamination could be assayed on a case-by-case basis, a reliable postcollection decontamination protocol would make this unnecessary. Contaminating DNA might simply be removed from arthropod integument by washing in EtOH. Alternatively, the DNA's integrity could be destroyed by oxidation with a dilute solution of bleach (NaOCl), which is the standard for surface decontamination in nucleic acid research (Prince & Andrus 1992); this approach gave promising results in a pilot study of decontamination in a potato-field predator–prey system (Greenstone *et al.* 2010). Here, we describe experiments, using animals from that same system, designed to test and compare these two approaches to removing externally decontaminating DNA from fed predators destined for molecular gut-content analysis.

Materials and methods

Insects

The spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera, Heteroptera: Pentatomidae), and the spotted pink lady beetle, *Coleomegilla maculata* (De Geer) (Coleoptera: Coccinellidae), are two of the most common predators of larval Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), in North American potato fields (Benton & Crump 1981; Hazzard *et al.* 1991; Heimpel & Hough-Goldstein 1992; Hilbeck *et al.* 1997). The false potato beetle, *Leptinotarsa juncta* (Germar), is often found in or around potato fields on the weedy solanaceous host *Solanum carolinense* L. (Hemenway & Whitcomb 1967), and potato-field predators consume it (McCauley 1992; Weber *et al.* 2006); material from this alternate prey species was therefore used to provide externally contaminating DNA.

The two *Leptinotarsa* species and both predator species used in this research had been in culture for several generations at the time of the experiment; establishment, history and maintenance of the colonies were described previously (Greenstone *et al.* 2010). Neither experimental predator population had ever been exposed to any *Leptinotarsa* species.

Predator feeding and cross-contamination

Second-instar *P. maculiventris* nymphs and third-instar *C. maculata* larvae were housed and fed as previously described (Greenstone *et al.* 2010). Briefly, they were placed into individual plastic Petri dishes, starved for 24 h with water *ad libitum* from a soaked dental wick and offered a single second-instar *L. decemlineata* on which they were allowed to feed until the larva was morphologically unrecognizable. They were then immobilized by placing them in a −20 °C freezer for 10 min, after which 1.0 μL of undiluted supernatant from homogenized fourth-instar *L. juncta* was applied with a micropipette dorsally to the cuticle behind the head. This was the maximum amount that would adhere to these small animals and meant to simulate a worst case for contamination by regurgitant from another predator or by material from a broken-up prey item. The *L. juncta* supernatant was prepared in advance from a large quantity of homogenate to ensure reproducibility of the amount of contaminating DNA and maintained at −20 °C between experiments.

Following contamination, the animals were killed by placing them at −20 °C for 30 min and then removed from the Petri dishes and transferred individually into clean 0.5-mL tubes of ice-cold 80% EtOH and stored at −20 °C to await assay. Any animals that did not feed were discarded from the experiment.

Predator decontamination

The previously fed and contaminated predators were divided at random into two equal-sized treatment groups, designated for 40 min or for overnight exposure to 80% EtOH, or to 20%, 10%, 5% or 2.5% commercial bleach and placed individually, with a clean brush, into clean tubes containing 0.5 mL of the designated ice-cold treatment wash. The tubes were then rotated end-over-end on an orbital rotator at 120 r.p.m. at 4 °C for the prescribed time period. Following treatment, the liquid was evacuated under house suction with a Pasteur pipette, the predators were rinsed by vigorous suspension in ice-cold double-distilled water with a wash bottle, and the rinse water was removed under suction. After two more rinses, the animals were transferred with clean brushes

into individual clean 0.5-mL tubes of chilled 80% EtOH and stored at −20 °C until ready for molecular analysis.

The bleach used in these experiments was a commercially supplied 5.3% (w/v) aqueous solution of NaOCl (The Clorox Co., Oakland, CA, USA).

Molecular analysis

DNA extraction and purification were performed according to the protocols of Greenstone *et al.* (2005). Extracts were subjected to PCR for species-specific *L. decemlineata* and *L. juncta* cytochrome c oxidase subunit 1 (COI) fragments. Primer and amplicon sequences, reagents, cocktail recipes, thermocycling conditions and gel electrophoresis protocols were presented elsewhere (Greenstone *et al.* 2007). Each PCR included five each of three positive (*L. decemlineata*-fed predator, *L. decemlineata* and *L. juncta*) controls, five negative (unfed predator) controls and 1 no-DNA control. Additionally, control PCRs, employing the generic COI primers 'Ron' and 'Nancy' (Simon *et al.* 1994), were conducted on all negative samples to verify that the DNA in the samples was amplifiable. All reactions were set up in a HEPA-filtered work station, using aerosol-resistant tips for all pipetting steps. In addition to the EtOH- and bleach-treated animals, a like number of animals that had been fed, contaminated and stored at −20 °C in 80% EtOH, but not subjected to further treatment, were assayed as controls.

Statistical analysis

All statistical analyses were conducted with PC SAS version 9.2 (SAS Institute, Cary, NC, USA). Data were cast in contingency tables and analysed by Fisher's Exact tests using SAS PROC FREQ. Statistical significance was determined at the 0.05 level.

Results

Treatment with 20% bleach over night caused loss of pigmentation and partial disintegration of many individuals of both predator species, along with loss of most DNA detectability, while 10% bleach, even at 40 min, caused significant loss of *L. decemlineata* (target) DNA detectability (data not shown). Therefore, all further experiments limited bleach treatments to 5% and 2.5%, and overnight exposure was eliminated. Each experiment was begun with equal numbers of animals in the treatments and control, but a few individuals (<10%) were lost when they were inadvertently drawn into the tip of the Pasteur pipette during the removal of the rinse water.

Results for *C. maculata* subjected to 40-min rotation in 5% bleach and 80% EtOH are presented in Table 1. There were no significant differences in the proportions of *L. decemlineata* (target) PCR positives by treatment (P = 0.1394); however, the proportions of *L. juncta* (contaminating) PCR positives were significantly less in bleach and in EtOH than in the controls (overall $P < 0.001$ for comparing the three treatments). Although no differences were found in the proportions of target-positive animals by treatment, sample sizes in this experiment were relatively small, and reduction in the proportion of target-positive assays between the 5% bleach treatment and control (0.56 vs. 0.83) was sufficiently large to warrant concern about the bleach concentration. In further experiments, the bleach concentration was therefore reduced to 2.5%.

Table 1 Numbers of *Coleomegilla maculata* positive and negative for *Leptinotarsa decemlineata* (target) DNA and *L. juncta* (contaminating) DNA following 40-min rotation in 80% EOH or 5.0% commercial bleach, or no treatment. Data with different letters within columns are significantly different

Treatment	*L. decemlineata* positive	*L. decemlineata* negative	*L. juncta* positive	*L. juncta* negative
None (control)	15 a	3	11 a	7
80% EtOH	13 a	3	11 a	5
5.0% Bleach	10 a	8	1 b	17

Results for *C. maculata* subjected to 40-min rotation in 2.5% bleach are presented in Table 2. The proportions of target-positive predators are statistically identical in the treated and control animals (P = 1), while the proportion of contaminated predators is reduced from 0.83 to 0.0 ($P < 0.001$). Data for *P. maculiventris* subjected to both 80% EtOH and 2.5% bleach treatments are presented in Table 3. The proportions of target- positive predators are not significantly different among both treatments and control animals (P = 0.4417), while the proportions of contaminated predators for both EtOH (0.60) and 2.5% bleach (0.06) are significantly lower than for the controls (0.83) (overall $P < 0.001$ for comparing the three treatments).

Discussion

Given our current state of knowledge, the risk of external contamination of predators in the course of mass collec-

Table 2 Numbers of *Coleomegilla maculata* positive and negative for *Leptinotarsa decemlineata* (target) DNA and *L. juncta* (contaminating) DNA following 40-min rotation in 2.5% commercial bleach, or no treatment. Data with different letters within columns are significantly different

Treatment	*L. decemlineata* positive	*L. decemlineata* negative	*L. juncta* positive	*L. juncta* negative
None (control)	14 a	4	15 a	3
2.5% Bleach	12 a	4	0 b	16

Table 3 Numbers of *Podisus maculiventris* positive and negative for *Leptinotarsa decemlineata* (target) DNA and *L. juncta* (contaminating) DNA following 40-min rotation in 80% EOH or 2.5% commercial bleach, or no treatment. Data with different letters within columns are significantly different

Treatment	*L. decemlineata* positive	*L. decemlineata* negative	*L. juncta* positive	*L. juncta* negative
None (control)	35 a	1	30 a	6
80% EtOH	34 a	1	21 b	14
2.5% Bleach	30 a	3	2 c	31

tion is unpredictable. For previously unstudied predator–prey systems, investigators could perform their own experiments to determine whether this risk is significant, and if so, how best to avoid it (King *et al*. 2011). In theory, one could avoid it entirely by individual hand collection (King *et al*. 2008) (but see Greenstone *et al*. 2010), and in some situations, predators are sufficiently exposed and slow-moving to enable efficient hand collection (e.g. Szendrei *et al*. 2010). But restricting oneself strictly to hand-collecting will be impractical in most cases, given the need for large sample sizes and the ubiquity of nimble and flighty predators and cryptic predator–prey interactions. A postcollection, pregut-content-analysis decontamination protocol, which would obviate the need for assessing the risk before hand, would eliminate the potential for false positives because of external contamination during mass collection.

Besides our previous pilot study (Greenstone *et al*. 2010), we know of three previous attempts to remove contaminating DNA from arthropod integument by oxidation prior to assay. In a survey of endosymbionts of psyllids and their parasitoids, Meyer & Hoy (2008) vortexed the insects 'vigorously' for 1 min in 6% bleach to remove externally contaminating microbial DNA, but did not evaluate the effectiveness of the treatment, nor whether it affected their ability to detect endosymbionts. Remén *et al*. (2010) used 3.7% NaOCl in an attempt to remove fungal DNA contamination from the surface of fungus-feeding oribatid mites but achieved only partial success, probably because the DNA was contained in intact fungi that were resistant to removal from the cuticle. In a case more analogous to ours, Linville & Wells (2002) soaked blood-coated calliphorid maggots for 19 h in 20% commercial bleach and could still detect DNA of the vertebrate meal in the crop while completely eliminating the externally contaminating DNA. NaOCl is a potent oxidant, and our strict emulation of Linville & Wells's (2002) protocol was too harsh for our system: the mass of food in a maggot's crop is much greater than that in the gut of an immature *C. maculata* or *P. maculiventris*, and the maggots used in their experiment were relatively large (13–17 mm long), which may have protected both their integument and much of the food mass within from direct attack.

By reducing the NaOCl concentration and exposure time, we were able to oxidize most externally contaminating DNA while sparing target DNA in the gut. Forty min of end-over-end rotation in 2.5% commercial bleach was sufficient to eliminate all (for *C. maculata*; Table 2) or virtually all (for *P. maculiventris*; Table 3) of a large quantity of DNA in undigested insect homogenate applied topically to the integument prior to preservation. At the same time, it did not render target DNA in the gut undetectable, even in these relatively small and delicate animals. However, these animals were killed immediately after feeding on a single prey, whereas field-collected predators will vary in time since feeding and prey number, hence, in the quantity of undigested DNA remaining in the gut (King *et al*. 2007; Lundgren & Weber 2010). Further research is required to determine whether further optimization of the protocol is needed to ensure survival of detectability of prey DNA over the course of digestion in the gut following bleaching to remove external contamination.

This protocol will probably be effective with other species of predators as well, although variations in setation and sculpturing of the cuticle; openings into the digestive, respiratory and reproductive systems; and the extent of sclerotization warrant prior testing and possibly reoptimization for any new system. It is interesting that end-over-end rotation in 80% EtOH reduced external contamination slightly in *Podisus* (Table 3), although not in *Coleomegilla* (Table 1). This suggests that washing in EtOH might be effective in eliminating external DNA contamination in some circumstances. However, the process of washing away DNA is not as direct as oxidizing it with bleach, and provided time and concentration are optimized to protect target DNA in the gut, washing in bleach is more apt to be more effective in eliminating external DNA contamination without compromising gut analysis.

Additional refinements are needed in the water rinsing protocol. We used a wash bottle to produce a powerful stream of water to thoroughly suspend the animals for rinsing. While effective and not unduly harsh in the current instance, this could be destructive to soft-bodied predators such as spiders (J.D. Harwood, personal communication). Further, very small animals can be smashed if forcibly drawn into the tip of the Pasteur pipette during the removal of the rinse water, as happened in a few cases during our study. Our prior use of a 96-cell system (Greenstone *et al*. 2010), in which the rinse water was drawn out under vacuum through a membrane, did not entirely solve this problem, so some ingenuity will be required to recover all animals if they are small.

Our study did not attempt to examine the risk of cross-contamination from postcollection predation on prey within the collecting apparatus. When King *et al.* (2011) released into the field and then immediately suction-collected starved individuals of four spider species and then killed them immediately by placing them onto dry ice, they found that some individuals of two species tested positive for collembolan DNA, while no individuals of the other two species did. This is consistent with feeding in the collector by the first two species, because external contamination should have caused at least some individuals of the other two species to test positive as well. However, sample sizes were small, and the differences among species were not significant, so one cannot be sure that the collembolan DNA positives were because of feeding rather than to external contamination.

The simple and rapid postcollection, pregut-content-analysis decontamination protocol presented here offers an effective solution to the risk of external DNA contamination in mass-collected predators. With this technical advance, molecular ecologists can use mass-collection methods in arthropod predation research while greatly reducing the risk of false positives because of external DNA contamination. Whether, and to what extent, mass collection presents a risk of cross-contamination by postcollection predation in the collecting apparatus, and if so how it can be mitigated or eliminated, remains to be determined.

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Data Accessibility

DNA Sequences: GenBank accessions AY531755; AY613926.'