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Unnecessary roughness? Testing the hypothesis that predators destined for molecular gut-content analysis must be hand-collected to avoid cross-contamination

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Abstract

Molecular gut-content analysis enables detection of arthropod predation with minimal disruption of ecosystem processes. Mass-collection methods, such as sweep-netting, vacuum sampling and foliage beating, could lead to regurgitation or rupturing of predators along with uneaten prey, thereby contaminating specimens and compromising resultant gut-content data. Proponents of this 'cross-contamination hypothesis' advocate hand-collection as the best way to avoid cross-contamination. However, hand-collection is inefficient when large samples are needed, as with most ecological research. We tested the cross-contamination hypothesis by setting out onto potato plants immature *Coleomegilla maculata* and *Podisus maculiventris* that had been fed larvae of either *Leptinotarsa decemlineata* or *Leptinotarsa juncta*, or unfed individuals of these predator species along with *L. decemlineata* larvae. The animals were then immediately re-collected, either by knocking them vigorously off the plants onto a beat cloth and capturing them *en masse* with an aspirator ('rough' treatment) or by hand-searching and collection with a brush ('best practice'). Collected predators were transferred in the field to individual vials of chilled ethanol and subsequently assayed by PCR for fragments of cytochrome oxidase I of *L. decemlineata* and *L. juncta*. Ten to 39 per cent of re-collected fed predators tested positive by PCR for DNA of both *Leptinotarsa* species, and 14–38% of re-collected unfed predators contained *L. decemlineata* DNA. Overall levels of cross-contamination in the rough (31%) and best-practice (11%) samples were statistically different and supported the cross-contamination hypothesis. A pilot study on eliminating external DNA contamination with bleach prior to DNA extraction and amplification gave promising results.

Keywords: molecular gut-content analysis, sampling

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Introduction

Molecular gut-content analysis, by immunoassay for prey proteins or polymerase chain reaction (PCR) assay for prey DNA sequences, is an established and increasingly important approach for tracking arthropod predation in the field (Hagler *et al.* 1992; Hagler & Naranjo 1994; Symondson 2002; Harwood *et al.* 2007; Juen & Traugott 2007; Kuusk *et al.* 2008; Lundgren *et al.* 2009). Among other virtues, molecular gut-content analysis causes minimum disruption to ecosystem processes, requiring only brief periodic intrusions into the field for specimen collection (Harwood & Greenstone 2008).

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Ecological research requires large samples for statistical validity, on the order of hundreds or thousands of individuals, which in turn calls for use of 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 (Clark *et al.* 1993; 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).

In a review of 'best practice' for molecular gut-content analysis, King *et al.* (2008) strongly caution against the use of such 'harsh' methods to collect predators, suggesting that they are likely to lead to external contamination by material regurgitated by the predators themselves or

by material released from prey that have been broken up during collection. Although such a scenario is plausible, we consider it to be speculative: a 'cross-contamination hypothesis' requiring rigorous testing before it can be accepted as an element of best practice. Published tests have compared predation frequencies in free-living predators collected by rough and best-practice methods (Harwood 2008; Chapman *et al.* 2010). Here, we test the hypothesis with an experiment in which small, soft-bodied predators with known feeding histories are placed into the field with or without soft-bodied prey, re-collected by a protocol designed to enhance the likelihood of cross-contamination and assayed by PCR for DNA of prey they had not consumed. Because feeding mode may affect the likelihood of regurgitation, we included both sucking and chewing predators in the experiment.

Materials and methods

Insects

The spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera, Heteroptera: Pentatomidae), and the spotted pink ladybug, *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); they also differ in feeding mode, sucking and chewing, respectively (Greenstone *et al.* 2007). The false potato beetle, *Leptinotarsa juncta* (Germar), was employed as an additional potentially contaminating prey species, because its plant host is often found in or around potato fields (Hemenway & Whitcomb 1967) and potato-field predators consume it (McCauley 1992; Weber *et al.* 2006). We used second-instar prey and second- or third-instar predators to ensure fragility.

The two *Leptinotarsa* species had been in culture for at least eight generations at the time of the experiment, and *C. maculata* and *P. maculiventris* had been in culture for at least ten and at least 135 generations, respectively, at the time of the experiment; establishment and maintenance of the colonies were described elsewhere (Coudron *et al.* 2002; Greenstone *et al.* 2007). Neither experimental predator population had been exposed to any *Leptinotarsa* species.

Predator feeding for cross-contamination experiment

Forty-eight hours before the start of the experiment, second-instar *P. maculiventris* nymphs and third-instar (because seconds were not sufficiently voracious) *Coleomegilla maculata* larvae were placed into individual plastic Petri dishes (3.5 × 1.0 cm; Fisher Scientific, Pittsburgh, PA, USA) each supplied only with a water-soaked dental wick. These were placed on laboratory bench tops that had been previously wiped with full strength commercial bleach (5.25% NaOCl) and covered with clean bench paper, at ambient temperature and photoperiod; *P. maculiventris* had been starved for an additional 24 h during shipment from TCC's laboratory in Columbia, Missouri to Beltsville, Maryland where the experiments were performed. On the morning of the experiment, all animals were separated into three groups, with each animal to be offered a single second instar of *L. decemlineata* or *L. juncta*, or no food. A different investigator was responsible for transferring larvae of only one *Leptinotarsa* species into the Petri dishes. The animals were given 2 h to feed; any that had not fed by then were removed from the experiment. All food was then removed from those animals that had fed, and the Petri dishes containing the animals were immediately transported to the field. All animals consumed at least one-half of the larva they had been offered.

Field plot set-up for cross-contamination experiment

The experiment was conducted twice, on 2 July and 16 July 2009, in two 0.3-ha fields about 300 m apart at the Beltsville Agricultural Research Center (39°01′N, 76°55′W) that were seeded with potato ('Kennebec,' 2 tonnes/ha, 76 × 30 cm spacing) on 22 May and 3 July 2009. Seven to 10 days before the cross-contamination experiments, when the plants had reached *c.* 20 cm in height, individual plots 3 rows wide and 8 m long were laid out. The plants were extensively hand-searched, and any insects observed were removed manually from the plants, which were then covered with spunbonded polypropylene cloth (Agribon; Polymer Group Inc., Charlotte, NC, USA) with the edges sealed by earth, to prevent colonization by any of the predator or prey species to be used in the experiment. Very few insects were discovered during the hand-search and were limited to a few egg masses and adults of *L. decemlineata*.

The experiment was conducted on three 0.1-ha plots, two of which received the rough treatment and one the best-practice treatment (described in the following). The plots were subdivided into three 5 × 10 m subplots placed randomly within each main plot; each subplot contained one of the following feeding treatments, with a total of ten predators in each: second-instar *P. maculiventris* nymphs, half of which had been fed *L. decemlineata* larvae and the other half *L. juncta* larvae; third-instar *C. maculata* larvae, half of which had been fed *L. decemlineata* larvae and the other half *L. juncta* larvae; and equal numbers of unfed *P. maculiventris* nymphs and *C. maculata* larvae in the presence of first- and

second-instar *L. decemlineata* larvae that had been introduced into the plots by stapling leaflets containing 1-day-old egg masses from the colony to the undersides of leaves in the upper third of the plants 5 days previously. Three egg masses were placed on each of four plants equally spaced down the row. The first two treatments examined the possibility of contamination by regurgitation in sucking and chewing predators, respectively; the third examined the possibility of contamination by a ruptured potential prey animal. The experiment was replicated over the two dates, which served as a blocking factor.

After the cover was removed from the sub-plot, the ten predators in each feeding treatment were individually placed with a brush on the upper surfaces of leaves in the upper third of ten individual plants within the middle row of the sub-plot. After all ten had been emplaced, they were immediately re-collected within 5 min, in the same order they had been emplaced, by one of two methods: best-practice, in which each animal was picked up with a brush and placed into a 1.5-mL microcentrifuge tube of chilled 70% EtOH; and rough, in which the plants were vigorously beaten with a wooden stick in an attempt to knock all predators off the plants and onto polyester beat cloths arrayed on the ground beside the plants (Mack & Smilowitz 1980); the insects were recovered from the cloths *en masse* with an aspirator (model 1135P; BioQuip, Gardena, CA, USA) that was emptied into a 1000-mL beaker. Each animal was then picked up with a brush and transferred into an individual microcentrifuge tube of chilled 70% EtOH. To stop digestion in the predators' guts and maximize preservation of the DNA (Weber & Lundgren 2009), the sample tubes, each containing 0.7 mL of 70% EtOH, had been stored at −20 °C for 24 h, and then transported to the field in bench-top coolers certified to remain at or below −10 °C for at least 2 h at ambient temperatures (Stratagene, La Jolla, CA, USA). The animals were returned to the laboratory in the coolers within 1 h of collection and immediately transferred to a −20 °C freezer to await DNA extraction and PCR.

A single brush was used to emplace the animals of each treatment onto the plants. In the single-predator treatments, animals fed *L. decemlineata* larvae were alternated with those fed *L. juncta* larvae as they were emplaced on the plants to promote co-mingling during collection. For the rough method, the beat cloths had been used for *L. decemlineata* predation research for the previous two consecutive field seasons; a single brush was used to remove each aspirator collection from the beaker into the individual EtOH vials; and the same aspirator and beaker were used for the entire experiment.

The time elapsed between removal of the predators from the laboratory to be emplaced on the plants until all collected animals were placed into chilled EtOH was less than 2 h, well below the half-lives of detectability of DNA from a single *L. decemlineata* egg within gut contents of *P. maculiventris* nymphs and *C. maculata* larvae: 50.9 and 7.0 h, respectively (Greenstone *et al.* 2010). We assume that half-lives for *L. juncta* DNA are of the same order, because the PCR amplicons differ in size by only 5 bp (Greenstone *et al.* 2007) and half-life varies approximately inversely with amplicon size (Zaidi *et al.* 1999; Agustí *et al.* 2000; Chen *et al.* 2000).

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 oxidase I fragments. Primer and amplicon sequences, reagents, cocktail recipes, thermocycling conditions and gel electrophoresis protocols were presented in Greenstone *et al.* (2007). Each PCR included 5 positive (*L. decemlineata*-fed and *L. juncta*-fed) controls for each predator species, 5 negative (unfed) controls for each predator species and 1 no-DNA control. Additionally, control PCRs, employing the generic CO I 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. Amplified DNA bands from re-collected animals that scored as cross-contaminated were sequenced and compared to sequences previously deposited in GenBank (AY613926 and AY531756; Greenstone *et al.* 2007) to verify that they were not false positives.

External decontamination experiment

We performed a separate experiment to explore the feasibility of eliminating external DNA contamination by oxidation with NaOCl at 0.53% (1:10 v/v commercial bleach), the standard for surface decontamination in nucleic acid research (Prince & Andrus 1992). Second-instar *P. maculiventris* and third-instar *C. maculata* from the same colonies as the field experiment were placed in individual Petri dishes, starved for 24 h and offered a single second-instar *L. decemlineata* larva and allowed to feed until the larva was morphologically indistinguishable as a larva. They were then immobilized by placing them in a −20 °C freezer for 10 min, after which 1.0 μL of supernatant from homogenized fourth instar *L. juncta* was applied to the cuticle. The animals were killed by returning them to the freezer for 30 min, then transferred individually into clean 0.5-mL tubes of 80% EtOH and stored at −20 °C.

On the following day, the animals were divided at random into five equal-sized groups designated for

0-, 1-, 2-, 5- or 10-min exposure to 0.53% NaOCl and placed individually, with a clean brush, into individual cells of a Bio-Dot™ apparatus (Bio-Rad, Richmond, CA, USA) equipped with a positively charged nylon membrane (# 1-209-992; Roche Applied Science, Mannheim, Germany). For the 0-minute treatment, the cells were filled with 0.5 mL chilled 80% EtOH, and the apparatus was mixed on an orbital rotator at 120 RPM for 5 min. For all other treatments, the cells were filled with chilled 0.53% NaOCl and rotated at 120 RPM for the prescribed number of minutes. Following treatment, the apparatus was evacuated under suction and the cells, with the individual predators still in them, washed four times with a wash bottle containing double-distilled water, with the apparatus evacuated after each wash. 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 DNA extraction and PCR.

Statistical analysis

All statistical analyses were conducted with PC SAS version 9.2 (SAS Institute, Cary, NC, USA). Analysis of variance procedures were conducted with PROC MIXED. A split plot model was used, with collection method (two levels: rough and best practice) as the main unit factor and predator species (three levels: *P. maculiventris*, *C. maculata*, and unfed) as the split unit factor. The simple effects of one factor at a specified level of the other factor were assessed with a SLICE option in an LSMEANS statement. Proportions involving the combinations of collection method, predator configuration and date were compared with the use of Fisher exact tests applied to contingency tables. Frequencies of *L. juncta* DNA positives and negatives in results of the external decontamination experiment were compared using chi-square tests applied to contingency tables conducted with PROC FREQ. The probability for statistical significance was set at 0.05 for all comparisons.

Results

Our field experiment demonstrated that best-practice collecting could not eliminate cross-contamination entirely in our system, but it was significantly reduced compared with that occasioned by the rough method. We did not detect differential rates of cross-contamination by predator treatment or date, possibly because of small sample sizes or low statistical power inherent in the experimental design. However, we did find molecular evidence for cross-contamination by predator regurgitation and general environmental contamination with prey DNA.

Field experiment

Results of the molecular assays of field-collected animals are presented in Table 1. There was no evidence of contamination of predators by exogenous prey material on 16 July, possibly because of the very low egg mass hatching rate, 20–30%, on that date, but it affected three of the ten unfed animals collected in the best-practice treatment and four of the five unfed animals collected in the two rough treatments on 2 July, when hatching rates ranged from 75% to 90%. In the fed-predator treatments, one of 20 *Podisus maculiventris* nymphs and one of 13 *Coleomegilla maculata* larvae recovered by the best-practice method

Table 1 Proportions of predators recovered in the field experiments showing cross-contamination by polymerase chain reaction for DNA of *Leptinotarsa decemlineata* and *Leptinotarsa juncta*. Cross-contamination for the *Podisus maculiventris* and *Coleomegilla maculata* treatments is indicated by the presence of DNA from both *Leptinotarsa* species in one individual. Cross-contamination for the unfed treatments is indicated by the presence of *L. decemlineata* DNA in a predator. There were two rough plots and one best-practice plot

Collecting Method	Feeding treatment	2 July 2009	16 July 2009	Proportion cross-contaminated overall
Best practice	Unfed	3/10	0/4	3/14
	P. maculiventris	1/10	0/10	1/20
	C. maculata	0/9*	1/4	1/12†
Rough Plot 1	Unfed	1/2	0/5	1/7
	P. maculiventris	0/4	1/6	1/10
	C. maculata	0/6	5/7	5/13
Rough Plot 2	Unfed	3/3	0/5	3/8
	P. maculiventris	0/3	3/5	3/8
	C. maculata	0/6	4/5	4/11
Totals	Best practice	4/28†	1/18	5/46†
	Rough	4/22	13/33	17/55

*We could not amplify DNA from one of the ten animals recovered in this sample.
†Only animals with amplifiable DNA were included in the statistical analysis.

contained DNA of both prey species; this was verified by sequencing (data not shown). For those collected by the rough method, 4 of 18 recovered *P. maculiventris* nymphs and 9 of 24 *C. maculata* larvae contained DNA of both prey species, which again was verified by sequencing. All but one of the animals scoring negative for the two *Leptinotarsa* species produced correct-sized bands when subjected to PCR with primers 'Ron' and 'Nancy' (Simon *et al.* 1994). That animal, a fed *C. maculata* from the best-practice treatment, was also not amplifiable with species-specific *C. maculata* primers (Greenstone *et al.* 2007) and was dropped from the analysis (Table 1).

The relationships of a factor to categorical binary responses (e.g., cross-contamination or recovery) were assessed initially for specified values of the other factors. The configuration of predators within a treatment – *C. maculata*, *P. maculiventris* or unfed – did not affect the proportion of individuals cross-contaminated when rough and best-practice treatments were compared by ANOVA (Table 2). On 2 July, animals in the unfed configuration collected by the rough method were statistically more likely to be cross-contaminated than those in the fed single-species predator treatments; the reverse was true on 16 July. The proportions of cross-contamination associated with the collection practices (best practice vs. rough) were not significantly different when compared by Fisher exact test for any of the various combinations of date and species configuration. However, when treatments were collapsed (Table 1; Totals), animals collected by the rough method were almost three times as likely to be cross-contaminated as those collected by best practice. This difference was highly significant and in the direction predicted by the cross-contamination hypothesis (chi-square = 5.9; $P = 0.008$, one-tailed test).

Table 2 Results of split-model analysis of variance of field experiment, with collection method (rough and best practice) as the main unit factor and predator species configuration (Unfed, *Podisus maculiventris* and *Coleomegilla maculata*) as the split unit factor

Main unit	Split unit	DF_{Num}	DF_{Den}	F	$P > F$
Best practice		2	10	0.50	0.622
Rough		2	10	0.36	0.705
	Unfed	1	5.01	4.43	0.089
	P. maculiventris	1	5.01	2.77	0.157
	C. maculata	1	5.01	0.34	0.586

Recovery rates of the emplaced animals by the two collection methods are given in Table 3. With the almost significant ($P = 0.064$) exception of *C. maculata*, the proportions of animals re-collected by best-practice and rough methods were statistically different, with $P < 0.001$ overall by Fisher exact test regardless of the predator configuration or date. However, we detected no significant differences in predator configuration in the proportion collected by the rough and best-practice methods. As noted in Table 3, field mishaps on 16 July reduced the number of collected animals available for molecular gut-content analysis.

External decontamination experiment

Results of the molecular assays of animals from the external decontamination experiment are presented in Table 4. The two predator species did not differ in the proportion positive for *L. juncta* DNA by time of immersion in bleach (chi-square = 0.029–1.840, $P > 0.175$). For *C. maculata*, the proportion positive differs significantly with time of immersion (chi-square = 22.257, $P < 0.0002$),

Table 3 Numbers of predators emplaced and recovered in the experiments; E = emplaced, R = recovered. There were two rough plots and one best-practice plot

Collecting method	Feeding Treatment	2 July 2009 E/R	16 July 2009 E/R	E/R, overall
Best practice	Unfed	10/9	10/10*	19/20
	Podisus maculiventris	10/10	10/10	20/20
	Coleomegilla maculata	10/10	10/10*	20/20
Rough Plot 1	Unfed	10/2	10/6	8/20
	P. maculiventris	10/4	10/7	11/20
	C. maculata	10/6	10/7	13/20
Rough Plot 2	Unfed	10/3	8/5†	8/18
	P. maculiventris	10/3	10/5	8/20
	C. maculata	10/6	10/6‡	12/20
Totals	Best practice	30/29	30/30	59/60
	Rough	60/24	58/36	60/118

*Six of the animals in each of these collections were inadvertently mis-labelled and lost to the molecular gut-content analysis.
†Two of the 10 animals scheduled for emplacement were inadvertently placed into the wrong plant row and lost from the experiment.
‡One of the animals recovered in this sample was inadvertently smashed and not subsequently subjected to PCR.

Table 4 Numbers of predators fed *Leptinotarsa decemlineata* and externally contaminated with *Leptinotarsa juncta* homogenate subsequently testing positive and negative by polymerase chain reaction for *L. juncta* DNA. Treatments followed by the same letter are not statistically different at $P = 0.05$ by likelihood ratio chi-square test

Species	Bleach treatment	Negative	Positive	Totals
Podisus maculiventris	0-min – b	1	24	25
	1-min – a	9	16	25
	2-min – a	7	18	25
	5-min – b	5	20	25
	10-min – a	5	17	22*
Totals		27	95	122
Coleomegilla maculata	0-min – d	1	36	37
	1-min – c	11	26	37
	2-min – c	12	25	37
	5-min – d	3	34	37
	10-min – c	12	25	37
Totals		39	146	185

*Three animals from this treatment were inadvertently trapped and smashed in the Bio-Dot™ apparatus and lost from the experiment.

with the 1-, 2-, and 10-minute treatments having lower proportions positive than the 0-minute treatment. For *P. maculiventris,* the proportion positive also differs significantly with time of immersion (chi-square = 9.651, $P < 0.05$), with the 1-, 2-, and 10-minute treatments having lower proportions positive than the 0-minute treatment, and the 5-minute treatment statistically not different from the 0-minute treatment. By contrast, all but one of the 122 *P. maculiventris* nymphs, and two of the 185 *C. maculata* larvae, were PCR-positive for *L. decemlineata*, indicating that the bleach treatment did not prevent detection of target DNA in the gut.

Discussion

The 40% to 65% of emplaced animals recovered by beat sampling in this study may be the first such published estimate and contrasts with the 98% recovered by hand-collection. Both estimates, however, are probably high compared with standard sampling conditions, because all animals had been emplaced, on the upper surfaces of leaves, just minutes before collection, affording them little time to seek cover. In simultaneous comparisons, beating, vacuum sampling, and chemical fogging were the most efficient tools for biodiversity sampling, i.e., they collected the largest number of species (Moir *et al.* 2005). Whether they would be similarly ranked for most efficiently collecting individuals of a given species is not known.

In the only other published tests of the cross-contamination hypothesis, Harwood (2008) and Chapman *et al.* (2010) found no significant differences in the proportions of spiders positive for prey molecular markers in sweep-netted or vacuum-collected, respectively, vs. hand-collected samples. The animals were all collected in the same aspirator and transferred to individual chilled vials, but the aspirator was a simple tube with a screen at the end rather than a reservoir as in our case, so that the animals could be collected singly and then blown into the sample tube (J. Harwood, personal communication). Therefore, there were fewer opportunities for cross-contamination than in the present study.

The predator densities used in our experiment ranged from 8 to 20 times those normally found in Maryland potato fields (Z. Szendrei, personal communication) which, along with other aspects of the design, produced a worst-case risk of cross-contamination by co-mingling of regurgitants of predators fed different species, and of unfed predators with ruptured prey. Making the field protocol more stringent, for example by using decontaminated beat cloths, individual rather than group collection from the beat cloth, a separate decontaminated brush for every individual collected or a single-animal aspirator design, would reduce the likelihood of cross-contamination.

The incidence of cross-contamination in the best-practice samples, 11% (Table 1), is disconcerting, given that hand-collection is the gentlest method available and has been widely used (Cuthbertson *et al.* 2003; Harwood *et al.* 2004; Read *et al.* 2006; Juen & Traugott 2007; Kuusk *et al.* 2008; Szendrei *et al.* 2010). The possibility of technical error in our field experiment, such as using an inadvertently contaminated brush, cannot be ruled out. Indeed, the use of brushes to handle predatory mites appears to promote cross-contamination (J. Lundgren, personal communication). This could be easily tested by repeating the best-practice experiment with separate decontaminated or never-before-used brushes to transfer each animal to EtOH. While it is conceivable that wild immature *P. maculiventris* or *C. maculata* with different feeding histories could have migrated into the experimental plots under the cloth covers, the strict protocol employed in emplacing and re-collecting the experimental animals, and their uniformity in size, made collecting wild animals in error extremely unlikely. Finally, although cross-contamination during sample handling in the laboratory can never be ruled out, the feeding protocol employed, in which surfaces were decontaminated beforehand with bleach and a single investigator used clean brushes to handle larvae of only one *Leptinotarsa* species, and the stringent procedures used for DNA extraction and amplification make this highly unlikely.

A striking finding was the absence of external contamination in unfed predators on a date with low *L. decemlineata* hatching rates, and a high incidence of external contamination in unfed predators on the date when hatching rates were high. As is usual with early instars in unmanipulated potato fields, these larvae were found aggregated near the egg masses from which they had hatched. As they were purposely placed in the same plant rows from which the predators were collected, the probability of contact during foliage beating was enhanced. Nevertheless, no *Leptinotarsa* eggs or larvae were aspirated off the beat cloth when predators were collected. Also, feeding by predators on other predators was never observed during their short residence in the 1000-mL container into which aspirators were emptied prior to preservation. These observations strongly implicate contamination by contact with *L. decemlineata* larvae or larval faecal material on the plants during collection.

The contrast between our findings and those of Harwood (2008) and Chapman *et al.* (2010) may involve differences in the biology of predators and prey in the systems. Linyphiid and tetragnathid spiders may be less apt to regurgitate when roughly handled than immature pentatomids and coccinellids. Most strikingly, larvae of *L. decemlineata*, like those of many other chrysomelids, are notoriously filthy, generally covered with faeces and smearing it on foliage, thereby providing a ready source of DNA-spiked material to contaminate externally any animal that comes in contact with it during collection. The high egg-hatching rates in the 2 July experiment and the resultant larval faecal contamination may explain the high cross-contamination rates on that date. This suggests that whether or not rough collecting methods should be excluded from best practice has no one correct answer. Rather, the answer may be system specific.

Treatment with bleach reduced the proportion of externally contaminated (*Leptinotarsa juncta* DNA-positive) animals that had fed on *L. decemlineata* for all exposure periods, but did not eliminate it entirely. For both predator species, only the 5-minute treatment differed from the other bleach treatments (chi-square = 22.257, $P < 0.0002$ for *C. maculata*; chi-square = 9.6512, $P < 0.05$ for *P. maculiventris*). Soaking in NaOCl has proven effective for removing contaminating DNA from vertebrate bones and teeth (Kemp & Smith 2005). In the only published research similar to ours, Linville & Wells (2002) soaked externally contaminated calliphorid maggots for long periods of time in 1.6% NaOCl and could still detect DNA of the vertebrate meal in the crop. However, the mass of food in a maggot's crop is much greater than that in the gut of most arthropod predators, so the same conditions might not be appropriate. Optimal times and concentrations to destroy external DNA without affecting amplifiability of DNA within the gut would need to be determined, but if effective, this treatment would banish the spectre of cross-contamination and permit a variety of 'rough' collecting methods to be used with confidence. Our initial results are promising, and experiments to refine the procedure are ongoing.

Overall, our results demonstrate that published 'best practice' collecting methods (King *et al.* 2008), in which predators are individually collected into EtOH with clean instruments, may significantly minimize the risk of external contamination with exogenous DNA but, depending on the predator–prey system, not eliminate it entirely. The best hope for that at this time may lie with post-collection external decontamination treatments now in development.

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This study reports collaborative research on natural enemy–pest interactions in agroecosystems, led by Matthew Greenstone. The research was made possible by Donald Weber's expertise in predator and pest rearing and field ecology, Thomas Coudron's in predator rearing, and Mark Payton's in experimental analysis.