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REVIEW

Advances in molecular ecology: tracking trophic links through predator–prey food-webs

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Summary

1. It is not always possible to track trophic interactions between predators and prey by direct observation. This is especially true when observing small or elusive animals with cryptic food-web ecology. Gut and/or faecal analysis can sometimes allow prey remains to be identified visually but is only possible when a component of the diet is resistant to digestion. In some cases there are no solid remains, and when there are it can lead to bias in interpretation of prey choice.

2. Numerous invasive and non-invasive methods have been developed to characterize predator–prey interactions but two principal areas dominate 'molecular' research. These are reviewed under the headings of monoclonal antibodies and DNA-based techniques.

3. Early 'molecular' studies of predator–prey food webs were dominated by the development of monoclonal antibodies. These methods continue to be used for mass-screening of field-collected arthropods for insect-specific proteins.

4. The application of species-specific primer design, polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLP), DNA cloning and sequencing, comparative sequence analysis (e.g. BLAST; basic local alignment search tool), high-resolution gel electrophoresis, Temperature/denaturing gradient gel electrophoresis (TGGE/DGGE) and automated fragment analysis with fluorescent probes is reviewed. The development of molecular techniques for use in predator–prey studies is primarily limited by their cost and the development of new procedures and equipment that complement them.

Key-words: DNA techniques, faeces analysis, gut content analysis, monoclonal antibodies, predator–prey interactions

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Introduction

In order to understand interaction pathways within complex food-webs, it is necessary to characterize encounter frequencies between each constituent part of the web, and describe the consequence of these interactions. Determination of encounter frequencies is, in general, relatively straightforward. Sampling protocols are routinely designed for monitoring both vertebrate and invertebrate populations, but measuring trophic connections between species can be difficult, especially in highly mobile and/or cryptic organisms. Video technology can provide information on the spectrum of prey attacked by predators (or those predators consuming sedentary prey) (e.g. Merfield, Wratten & Navntoft 2004), but for most generalist predators, information is limited to those interactions in the view of the camera (which may constitute a small fraction of total predation events). The collection of samples from the field, and subsequent post-mortem analysis of gut or faecal samples, is highly advantageous in that predation events are allowed to occur naturally, without interference or bias towards certain prey types (Sunderland 1988; Harwood & Obrycki 2005).

In the nineteenth century, visual identification of gut-contents revealed feeding preferences of coleopteran predators (Forbes 1883). Many species, however, feed by liquid ingestion and/or some prey may be unidentifiable, thus favouring detection towards hard

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parts of the exoskeleton. For example, a coccinellid feeding on pollen or aphids may contain many identifiable remains, but the same individual feeding on Lepidoptera eggs may yield no predation data owing to the intake of liquefied and digestible food. Despite these difficulties, valuable information can be gathered and gut dissection has enabled the identification of prey remains from museum specimens (Webb *et al*. 2000). Similarly, the dissection of faecal samples provides information on indigestible prey passing through the stomach (e.g. Burger *et al*. 1999) but predation events upon small and soft-bodied organisms are underestimated. Accurately qualifying trophic connections within complex food-webs is only possible by post-mortem gut-content analyses which rely on biochemical, analytical or molecular detection systems.

Techniques to study the interactions between predator and prey communities have become increasing complex as they attempt to address the imbalance created by visual identification. These include radioisotope labelling, the application of stable isotopes, electrophoretic detection of prey isozymes, the detection of prey pigments by chromatographic analysis and the detection of prey proteins using polyclonal antibodies (reviewed by Sunderland 1988, 1996; Pierce & Boyle 1991; Greenstone 1996; Symondson 2002). Current predator–prey studies, however, tend to rely on monoclonal antibody and/or DNA-based technology, which allow accurate and rapid detection of prey remains within predator guts or faecal samples. Although molecular detection systems have a wide application beyond predator–prey interactions (analogous research has focused on parasitoid–host, pathogen–host, blood-meal identification and the movement of entotoxins through complex food-webs), this review will concentrate on the application of, and difficulties associated with, two molecular techniques for predator–prey studies: monoclonal antibodies and DNA-based detection.

Monoclonal antibodies

INTRODUCTION TO MONOCLONAL ANTIBODIES

Antibodies have been used for 60 years to study predation; Brooke & Proske (1946) first described a polyclonal antibody to study predation on the mosquito *Anopheles quadrimaculatus* (Say) (Diptera: Culicidae). However, owing to the inherent lack of specificity associated with polyclonal antisera (Miller 1979), monoclonals have become the optimal system for studying predator–prey interactions in the field. Their development was first described by Köhler & Milstein (1979), documenting the fusion of lymphocytes with myelomas to create antibody-producing hybridomas, making it possible to develop family, species-, stage- or even instar-specific monoclonal antibodies (Greenstone 1996; Symondson 2002). For example, the high degree of specificity achieved in an egg-specific antibody unravelled the behaviour of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae cannibalizing eggs (Sigsgaard, Greenstone & Duffield 2002).

USE OF MONOCLONAL ANTIBODIES TO STUDY PREDATION

Despite the number of monoclonal antibodies reported as being developed against invertebrate pests, very few have made the transition from laboratory evaluation to the quantification of predation in the field (Table 1). In addition to the disproportionately high number of monoclonals reported, relative to those used in the field, all studies have focused on terrestrial invertebrate predator–prey interactions even though polyclonal antisera have been used for studying invertebrate (e.g. Venter *et al*. 1999) and vertebrate (e.g. Walter, O'Neill & Kirby 1986) predation in marine systems. The most frequently employed assay for the detection of these insect-specific proteins is the enzyme-linked immunosorbent assay (ELISA). This enables the rapid screening of predators to obtain accurate data on gut content (reviewed by Sunderland 1988; Greenstone 1996). It is the simplicity and sensitivity of ELISA screening protocols that explain why they have, until recently, been the principal method for measuring trophic connections through food-webs. However, a number of prerequisites need to be fulfilled to ensure accurate reporting and documentation of predation events in the field (e.g. determining detection limits and factors affecting antigen decay rates, measuring potential error due to scavenging and recording the likelihood of secondary predation). Without this optimization, incorrect trophic links could be implied, predation events overestimated, and the differential rates of feeding between species, genders and/or stages could incorrectly be assumed to exist.

The most important factor to consider in the development of an antibody-based assay is the level of sensitivity and specificity achieved. Although highly specific monoclones can be developed, they must be specific to the target prey. Once specificity is achieved, through the development of a suitable monoclonal cell line and the utilization of appropriate immunoassay which can vary in efficacy (Hagler 1998), further laboratory optimization is necessary to quantify rates of antigen decay, the effects of temperature on decay rates, the consequence of alternative prey consumption on detection periods, and differences in detection limits between predators. All these factors can influence detection of prey material (Sunderland 1996). As important as these preliminary characterization trials, but frequently ignored, are 'errors of predation' caused by secondary predation and scavenging. All methods of gut-content analysis enable prey material to be recorded but, crucially, none accounts for the way in which it was consumed by the predator – direct predation, scavenging or predation on a primary predator which itself consumed the target prey (reviewed by

Table 1. Studies qualifying trophic connections and interaction pathways in the field using monoclonal antibodies

Target	Predator	Reference
Lygus hesperus (Hemiptera: Miridae)	Hemipteran predators	Hagler *et al*. (1992)
Bemisia tabaci (Homoptera: Aleyrodidae)	Multiple predators	Hagler & Naranjo (1994a)
Pectinophora gossypiella (Lepidoptera: Gelechiidae)	Multiple predators	Hagler & Naranjo (1994a)
Bemisia tabaci (Homoptera: Aleyrodidae)	*Collops vittatus* (Coleoptera: Melyridae) *Hippodamia convergens* (Coleoptera: Coccinellidae)	Hagler & Naranjo (1994b)
Pectinophora gossypiella (Lepidoptera: Gelechiidae)	*Collops vittatus* (Coleoptera: Melyridae) *Hippodamia convergens* (Coleoptera: Coccinellidae)	Hagler & Naranjo (1994b)
Bemisia tabaci (Homoptera: Aleyrodidae)	Multiple predators	Hagler & Naranjo (1996)
Pectinophora gossypiella (Lepidoptera: Gelechiidae)	Multiple predators	Hagler & Naranjo (1996)
Helicoverpa armigera (Lepidoptera: Noctuidae)	Multiple predators	Sigsgaard (1996)
Gastropoda: Pulmonata	*Pterostichus melanarius* (Coleoptera: Carabidae)	Symondson *et al*. (1996)
Otiorhynchus sulcatus (Coleoptera: Curculionidae)	Coleoptera: Carabidae, Staphylinidae	Crook & Solomon (1997)
Heliothis virescens, *Helicoverpa zea* (Lepidoptera: Noctuidae)	Multiple predators	Ruberson & Greenstone (1998)
Nilaparvata lugens (Homoptera: Delphacidae)	*Pirata subpiraticus* (Araneae: Lycosidae)	Lim & Lee (1999)
Gastropoda: Pulmonata	*Pterostichus melanarius* (Coleoptera: Carabidae)	Bohan *et al*. (2000)
Annelida	*Pterostichus melanarius* (Coleoptera: Carabidae)	Symondson *et al*. (2000)
Homoptera: Aphididae	Araneae: Linyphiidae	Harwood *et al*. (2001b)
Helicoverpa zea (Hemiptera: Anthocoridae)	*Orius* sp. (Heteroptera: Anthocoridae)	Sansone & Smith (2001)
Helicoverpa armigera eggs (Lepidoptera: Noctuidae)	*Helicoverpa armigera* larvae (Lepidoptera: Noctuidae)	Sigsgaard *et al*. (2002)
Homoptera: Aphididae	Araneae: Linyphiidae	Harwood *et al*. (2004)
Bemisia tabaci (Homoptera: Aleyrodidae)	*Hippodamia convergens* (Coleoptera: Coccinellidae)	Hagler & Naranjo (2004)
Nilaparvata lugens (Homoptera: Delphacidae)	Araneae	Zhao *et al*. (2004)
Homoptera: Aphididae	*Pachygnatha degeeri* (Araneae: Tetragnathidae)	Harwood *et al*. (2005a)
Homoptera: Aphididae	Coleoptera: Carabidae	Winder *et al*. (2005)
Bemisia tabaci (Homoptera: Aleyrodidae)	Multiple predators	Hagler & Naranjo (2005)

Sunderland 1988, 1996). Furthermore, the mass collection of arthropods for gut-content analysis can yield 'false-positive' data due to surface-level contamination with target prey or increased interactions between predators and prey due to inappropriate sampling protocols (Harwood & Obrycki 2005). Without factoring scavenging and secondary predation into analyses, incorrect trophic links could be implied. These considerations are discussed below, in relation to both antibody and DNA techniques.

The simplicity of screening protocols associated with monoclonal-based assays has allowed large-scale field analyses of predator–prey interactions (e.g. Harwood, Sunderland & Symondson 2004; Hagler & Naranjo 2005). This technique of using pest-specific monoclonal antibodies was pioneered in the early 1990s (e.g. Hagler *et al*. 1992; Hagler & Naranjo 1994a,b) and has subsequently contributed to our understanding of the role of invertebrate predators in biological control (Table 1). Long detection periods for prey antigens following their consumption (e.g. Harwood *et al*. 2001a; Schenk & Bacher 2004) compared with the relatively short ones for prey DNA (e.g. Agustí *et al*. 2003a; Sheppard *et al*. 2004) can sometimes make immunological techniques advantageous in the field-assessment of predation (discussed below). Using an aphid-specific monoclonal antibody, Harwood *et al*. (2004) screened over 1700 linyphiid spiders and correlated the relationship between pest consumption with the availability of pest and non-pest prey in the field. In the largest gut-content study to date, Hagler & Naranjo (2005) assayed 32 262 predators (from nine taxa) by indirect ELISA against a *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) monoclonal antibody. In addition to documenting whitefly predation rates, including those of species not previously reported as feeding on these pests, Hagler & Naranjo (2005) were able to conclude that insecticide regimes had little effect on feeding rates within the assayed predator complex. The scale of such studies, where thousands of predators are analysed, is possible only through the mass-screening protocols associated with monoclonal antibody-based techniques whereby

samples can be assayed cheaply and very rapidly. As such, where detailed ecological interactions between predators and prey (especially in relation to biological control of agricultural pests) are required, monoclonal antibodies offer the most viable methodology for measuring trophic interactions in the field.

While these and other (Table 1) studies tend to report specific trophic interaction pathways between a predator and a single prey item, multiple probes of predator guts have been used with two insect-specific monoclonal antibodies (Hagler & Naranjo 1994a,b). Therefore, if the ultimate goal is to document individual, or limited numbers, of trophic connections between predators and prey, monoclonal antibodies convey significant benefits over molecular techniques because they are inexpensive and easy to use when screening predators (especially if the antibody has already been developed). Their application to other predator–prey systems, including the study of vertebrate predation, is as viable as the DNA-based methodologies that have been used to study these interaction pathways (discussed below). In complex food-webs, however, where multiple monoclonal antibodies would be required, the high expense and time-consuming nature of their development (Chen *et al*. 2000) would make antibody-based techniques less useful than other molecular detection systems. Furthermore, given that monoclonal production cannot be assured (Chen *et al*. 2000), the development and characterization of multiple antibodies for studies of complex food-webs would be problematic. Despite these difficulties, antibody-based technology can, in many cases, provide valuable information on predator–prey interactions in the field. For example, life-stage specific monoclonal antibodies have been developed (e.g. Hagler *et al*. 1994; Greenstone 1995) which enable field analysis of predation events and cannibalism (e.g. Sigsgaard *et al*. 2002). Such levels of specificity have not been achieved using DNA-based techniques, and there is a significant advantage to the use of monoclonal antibodies when studying such trophic connections in the field. Risk assessments of genetically modified crops to the non-target food chain have also been measured using antibody and ELISA technology in the laboratory (e.g. Raps *et al*. 2001) and the field (Harwood, Wallin & Obrycki 2005b). With the development of new transgenic crop varieties, and concerns surrounding their incorporation into modern farming practices, there is great potential for monoclonal antibody-based ELISAs to continue to enhance our understanding of the complex interactions between transgenic crops and the non-target food chain.

DNA-based techniques

INTRODUCTION TO DNA TECHNOLOGY

The principal limitations of monoclonal antibodies are that they are expensive and time-consuming to develop. This is less of a problem when the aim is to discern the predator range of single prey species (commonly a pest), but when the diet of a generalist predator is to be characterized it may be impossible to develop a sufficient number of antibodies. DNA-based techniques are frequently used in biological research and the facilities and expertise required to conduct sophisticated analyses are widely available. Furthermore, there is an enormous bank of sequence information available for thousands of species which can be accessed to provide species-specific targets for DNA analysis.

Molecular experiments tracking trophic interactions in food-webs are based on the ability to differentiate between unique pieces of DNA from predator and prey species. The polymerase chain reaction (PCR) (Ehrlich 1989) provides the means for amplification, and thus visualization, of the DNA, but the key step in the process is the differentiation of DNA. The segregation of species-specific DNA sequences relies on the application of various strategies. Broadly speaking these follow two standard experimental patterns. The first technique involves the PCR amplification of total (predator and prey) DNA from tissue homogenates (e.g. gut sample). This DNA then undergoes secondary analysis to distinguish the different sequences (species) it represents. Such secondary DNA analyses (described later) may include sequence BLAST (basic local alignment search tool) searches, high-resolution gel/capillary separation, restriction digestion and DNA denaturation. The second generalized approach to identifying prey DNA from a mixture of fragments involves the amplification of prey DNA, to the exclusion of that of the predator, using species-specific probes. This has become popular because it is relatively simple and inexpensive to design PCR primer sets that target organisms at various taxonomic levels. These can then amplify extracted and purified target DNA from predator–prey homogenates. There are numerous variations to these methods and new techniques are continually being developed. The principal approaches to molecular detection of prey in predator diets are described in Fig. 1.

OVERVIEW OF DNA-BASED TECHNOLOGIES IN FOOD-WEB STUDIES

The first study to amplify prey DNA from the gut of a predator documented predation upon juvenile Stone Flounder, *Kareius bicoloratus* Basilewsky (Pleuronectiformes: Pleuronectidae) by sand shrimps, *Crangon affinis* (De Haan) (Decapoda: Crangonidae) (Asahida, Yamashita & Kabayashi 1997). A 2·5–2·8 kb of the D-loop region of mtDNA was amplified and, following restriction fragment length polymorphism (RFLP) analysis and cloning and sequencing of products, species-specific primers were designed that could amplify a 1·46 kb region of prey DNA from the guts of 50% of predators 5 h after feeding. This experiment demonstrated a new method for quantifying predation in the

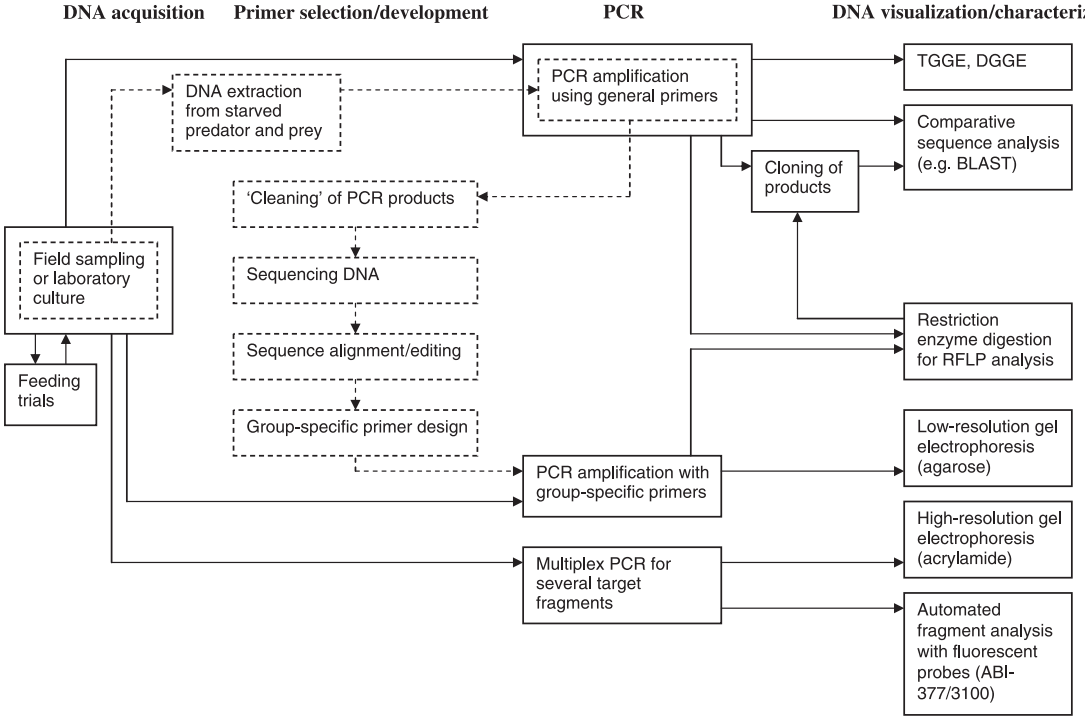

Fig. 1. Gut content analysis using DNA-based techniques. Broken lines describe processes required for species-specific primer design.

field and formed a model for later studies. Another laboratory study (Zaidi *et al*. 1999) used DNA techniques to detect interactions between arthropods. Small fragments (146 bp) of multiple-copy esterase genes from mosquitos (*Culex quinquefasciatus* Say (Diptera: Culicidae)) could be amplified from the gut of beetles (*Pterostichus cupreus* L. (Coleoptera: Carabidae)) up to 28 h after consumption. The conclusions of these early studies was that large DNA fragments break down more quickly in the digestive system and that it is better to target multiple copy DNA to maximize the chances of successful amplification. These conclusions are the same as in faecal analysis where degraded DNA is amplified (Kohn & Wayne 1997; Farrell, Roman & Sunquist 2000).

Multiple copy nuclear rRNA genes have been used as targets in invertebrate predator–prey studies (Hoogendoorn & Heimpel 2001) but mitochondrial genes (mtDNA) tend to dominate (Chen *et al*. 2000; Agustí *et al*. 2003a; Agustí, Unruh & Welter 2003b; Sheppard *et al*. 2004; Harper *et al*. 2005). Mitochondrial genes have been widely studied in insect phylogenetics (Caterino, Cho & Sperling 2000) providing a target for family, genus and species level discrimination of prey remains in predator guts (Symondson 2002). Several regions of the mitochondrial genome have been targeted in predator–prey studies and are discussed below. For example, the cytochrome oxidase subunit I (*COI*) gene contains conserved regions, ideal for rapid amplification and sequencing of DNA extracted from animal tissues (Folmer *et al*. 1994), and variable regions that are suitable target sites for group-specific primers. These characteristics have led to a 645 bp region of this gene being proposed as a suitable target for sequencing across species and entry into a database as the 'Barcode of Life' (Hebert, Ratnasingham & DeWaard 2003a; Hebert *et al*. 2003b). The *COI* gene has also been used for diagnostic identification of adult and juvenile arthropod predators in the field (Greenstone *et al*. 2005).

USE OF DNA-BASED TECHNIQUES IN VERTEBRATE FOOD-WEBS

Vertebrate food-webs often involve visible predator–prey interactions, making field observations relatively easy. However, this is rarely the case for invertebrates. The application of molecular methods to characterize cryptic trophic links in invertebrate systems often involves laboratory-based feeding trials and the killing of large numbers of specimens. For ethical reasons this is not acceptable for vertebrates and alternatives, such as faecal analysis, have been developed.

Much of the analysis of faecal material has been in population genetics to phylogenetically characterize the animal that produced the faeces. In these studies, epithelial cells sloughed off from the wall of the lower intestine are targeted for DNA extraction from the scats (e.g. Reed *et al*. 1997). Scats from predators, that have been identified in this way, have been physically analysed for solid prey remains to link prey and predator species (Reed *et al*. 1997; Farrell *et al*. 2000), but when prey remains are too degraded for morphological examination there is potential for amplifying prey DNA from faecal samples.

Jarman *et al*. (2002) used group-specific PCR primers to amplify regions of the small subunit (SSU) and the large subunit (LSU) rDNA molecule from Pygmy Blue Whale, *Balaenoptera musculus brevicauda* Ichihara (Cetacea: Calaenopteridae), and Adelie Penguin, *Pygoscelis adeliae* (Hombron & Jacquinot) (Sphenisciformes: Spheniscidae), faeces, respectively. An expansion segment of the krill (Crustacea: Malacostraca) mitochondrial genome was targeted using krill-specific primers. DNA from different species was subsequently separated on the basis of size (gel mobility) using high-resolution gel electrophoresis confirmed by sequencing. In a more recent study (Jarman, Deagle & Gales 2004), this method was taken further by designing primers that targeted group-specific regions of LSU and SSU mtDNA specific to Eukaryote, Bilateria, Chordata and Nototheniodei in the faeces of *Balaenoptera physalus* L. (Cetacea: Balaenidae) and Adelie Penguins. Following PCR amplification, clone libraries were created and cloned sequences were compared with sequences in the GenBank database using the BLAST search algorithm (Altschul *et al*. 1997) to produce prey DNA nearest-neighbour joining trees. Terrestrial ecosystems have also been characterized by molecular analysis of the dietary composition of scats. Group-specific PCR primers have been used to amplify parts of the 12S region of mtDNA from bird faeces (Sutherland 2000). Cloned PCR products (165 bp) from *Parus caeruleus* L. (Passerines: Paridae) and *P. major* L. (Passerines: Paridae) were analysed by RFLP analysis, sequencing and BLAST searches to determine the diet of these closely related species. Both species were shown to feed predominantly upon coleopteran and lepidopteran prey.

Analysis of scats is not the only non-invasive method for molecular determination of prey consumption. Regurgitated stomach contents from birds can also be targeted for prey DNA (Taberlet & Fumagalli 1996; Jarman *et al*. 2002, 2004). The bones of small mammals from regurgitated owl pellets have yielded amplifiable nuclear and mitochondrial DNA (Taberlet & Fumagalli 1996). Although it may be desirable to conduct non-invasive studies of vertebrate diets, large-scale invasive methods have, occasionally, been undertaken (Scribner & Bowman 1998). The Glaucous Gull, *Larus hyperboreus* Gunnerus (Charadriiformes: Laridae) is a predator of the chicks of various waterfowl. A subsample of a population was shot, and microsatellite loci were used to demonstrate that the rare Spectacled Eider *Somateria fischeri* (Brandt) (Anseriformes: Anatidae) was not an important prey item but Emperor Geese, *Chen canagica* (Sevastianov) (Anseriformes: Anatidae), were. This is a relatively unusual application of the study of microsatellites which are more commonly targeted to study population genetics within species (e.g. Vaughan & Russell 2004). Such applications are sometimes relevant to predator–prey studies, for example microsatellites have been used to demonstrate the non-selective nature of cannibalism upon eggs and juveniles taken from the gut of the Tessellated Darter, *Etheostoma olmstedi* Storer (Perciformes: Percidae) (DeWoody *et al*. 2001).

ADVANCES IN DNA-BASED TECHNIQUES FOR INVERTEBRATE PREDATION STUDIES

Techniques and primers have been developed to detect predation in numerous studies (Table 2). Although the majority describe predation by invertebrate predators, to date, only five have analysed invertebrate predation in the field (Hoogendoorn & Heimpel 2002; Agustí *et al*. 2003a; Dodd 2004; Kaspar *et al*. 2004; Harper *et al*. 2005).

The first step in most of this research was the development of prey-specific primer sets that were then used to amplify prey DNA from predator gut samples. As described for faecal analysis, secondary analysis of DNA from PCRs can further divide products and RFLP analysis has been used to separate *Rhopalosiphum insertum* (Walker) (Hemiptera: Aphididae) products from the gut of the predatory mite *Anystis baccarum* (L.) (Acarina: Anystidae) (Cuthbertson, Fleming & Murchie 2003). Rapid analysis of samples is possible using this approach to study the diversity of a specific target prey range in predator guts but it is of no use in isolation, when the aim is characterization of unknown prey for which no specific primers have been developed. The standard method for describing unknown DNA is cloning and sequencing following PCR using general or group-specific primers. As well as being relatively slow and expensive, these methods rely upon the BLAST search algorithm and the availability of matching sequences on databases such as GenBank. An alternative is the use of molecular profiling technology.

Invertebrate identification, phylogenetics and population ecology research have incorporated molecular profiling techniques involving the use of randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), minisatellites, microsatellites, temperature/denaturing gradient gel electrophoresis (T/DGGE) and RFLP analysis. In some cases quantification, not characterization, of species diversity is the principal objective. DNA profiling techniques, especially DGGE/TGGE, pioneered through studies of microbial diversity in environmental samples, have achieved this aim in examining bacterial diversity (e.g. Felske, Akkermans & De Vos 1998; Sheppard *et al*. 2005b). Generalist predators eat multiple prey species, and it is not always practical to analyse predator responses to prey diversity using the species-specific primer approach. It is possible to use TGGE-based approaches for directly profiling the diversity of invertebrate predator diets, as used in examining bacterial diversity (above). General primers can be used to amplify the DNA from the guts of predators. Fragments of the same length, but with small sequence differences, are then separated on vertical acrylamide gels over a temperature gradient to denature

Table 2. Studies of trophic connections and interaction pathways based principally upon DNA-PCR gut-content analysis techniques

Target	Predator	Target genes	Reference
Kareius bicoloratus (Pleuronectiformes: Pleuronectidae) larvae and juveniles	*Crangon affinis* (Crustacea: Malacostraca)	D-loop (mtDNA)	Asahida *et al*. (1997)
Ducks (*Anatidae*) and geese/swans (*Anserinae*)	*Larus hyperboreus* (Charadriiformes: Laridae)	Various microsatellite loci	Scribner & Bowman (1998)
Helicoverpa armigera (Lepidoptera: Noctuidae) eggs	*Dicyphus tamaninii* (Heteroptera: Miridae)	RAPD-PCR isolated sequences (genomic DNA)	Agustí *et al*. (1999)
Culex quinquefasciatus (Diptera: Culicidae)	*Pterostichus cupreus* (Coleoptera: Carabidae)	Amplified esterase genes (nuclear DNA)	Zaidi *et al*. (1999)
Trialeurodes vaporariorum (Homoptera: Aleyrodidae)	*Dicyphus tamaninii* (Heteroptera: Miridae)	RAPD-PCR isolated sequences (genomic DNA)	Agustí *et al*. (2000)
Homoptera: Aphididae	*Hippodamia convergens* and *Coccinella septempunctata* (Coleoptera: Coccinellidae), *Chrysoperla plorabunda* (Neuroptera: Chrysopidae)	COII (mtDNA)	Chen *et al*. (2000)
Cacopsylla pyricola (Hemiptera: Psyllidae)	Multiple invertebrate predators	COI (mtDNA)	Agustí & Symondson (2001)
Ostrinia nubilalis (Lepidoptera: Crambidae)	*Coleomegilla maculata* (Coleoptera: Coccinellidae)	rDNA (18S/ITS-I/5·8S) (nuclear)	Hoogendoorn & Heimpel (2001)
Ostrinia nubilalis (Lepidoptera: Crambidae)	*Coleomegilla maculata* and *Harmonia axyridis* (Coleoptera: Coccinellidae)	rDNA (18S/ITS-I/5·8S) (nuclear)	Hoogendoorn & Heimpel (2002)[2]
Krill (Crustacea: Malacostraca)	*Pygoscelis adeliae* (Sphenisciformes: Spheniscidae)	LSU rDNA (mtDNA)	Jarman *et al*. (2002)[2]
Collembolla: Isotomidae	Araneae: Linyphiidae	COI (mtDNA)	Agustí *et al*. (2003a)[2]
Cacopsylla pyricola (Hemiptera: Psyllidae)	Multiple invertebrate predators	COI (mtDNA)	Agustí *et al*. (2003b)
Rhopalosiphum insertum (Homoptera: Aphididae)	*Anystis baccarum* (Acari: Anystidae)	ND1, LSU rDNA (mtDNA)	Cuthbertson *et al*. (2003)
Emiliania huxleyi (haptophyte alga, Isochrysidales)	*Calanus finmarchicus* (Crustacea: Maxillopoda)	18SrDNA (nuclear)	Nejstgaard *et al*. (2003)
Paralchthys olivaceus (Pleuronectiformes: Paralichthyidae) larvae and juveniles	Multiple predators	Mitochondrial control region (mtDNA)	Saitoh *et al*. (2003)[2]
Deroceras reticulatum (Mollusca: Pulmonata)	*Pterostichus melanarius* (Coleoptera: Carabidae)	12S rDNA gene (nuclear)	Dodd (2004)[2]
Multiple prey items	*Architeuthis dux* (Teuthoida: Architeuthidae)	SSU rDNA (nuclear), LSU rDNA (mtDNA) and valine tRNA (mtDNA)	Jarman *et al*. (2004)[1,2]
Multiple prey items	*Polistes humilis* and *Vespula germanica* (Hymenoptera: Vespidae)	16S rDNA (mtDNA)	Kaspar *et al*. (2004)[2,3]
Eupithecia sp. and *Scotorythra* sp. (Lepidoptera: Geometridae)	*Halmus chalybeus* (Coleoptera: Coccinellidae)	COI (mtDNA)	Sheppard *et al*. (2004)
Eupithecia sp. and *Scotorythra* sp. (Lepidoptera: Geometridae)	*Halmus chalybeus* (Coleoptera: Coccinellidae)	COI (mtDNA)	Sheppard *et al*. (2005a)[2]
Earthworms, aphids, weevils and molluscs	*Pterostichus melanarius* (Coleoptera: Carabidae)	COI and 12S rDNA (mtDNA)	Harper *et al*. (2005)[2]
Melolontha melolontha (Coleoptera: Scarabaeidae) larvae	*Poecilus versicolor* (Coleoptera: Carabidae) larvae	COI (mtDNA)	Juen & Traugott (2005)

[1]Faecal analysis also formed part of these studies.
[2]Analysis of field-caught predators was carried out.
[3]Prey items collected from mouth parts.

DNA in melting domains defined by the proportion of G:C and A:T bonds in the sequence. Where necessary, GC clamps can be incorporated into the forward primer (Sheffield *et al*. 1989) to alter the fragment melting behaviour and enhance band separation. The strength of this technique, however, lies in its ability to separate species where unique primer sites cannot be found or prey have not been identified to species.

Molecular profiling provides a rapid means of quantifying prey diversity within predators but when there are specific prey DNA targets, PCR with group-specific primers is the principal method of choice (Symondson 2002). This is fine for simple laboratory studies, but when there are multiple potential target prey species (Sheppard *et al*. 2004) or fragments (Hoogendoorn & Heimpel 2001), the time required to assay each predator for each potential target becomes limiting. In field studies the mean number of prey items in a generalist predator gut may be as few as 1 or 2 but as many as 40, requiring 40 separate PCR assays

(Harper *et al*. 2005). This effectively precludes many useful field-based ecological studies.

Rapid PCR-based screening systems for the study of the prey diversity of generalist predators, have been developed to expand the potential of molecular detection into various areas of food-web research. The techniques described by Harper *et al*. (2005) use a single multiplex-PCR to simultaneously amplify DNA from a range of prey species. Fluorescently labelled PCR primers enable the highly sensitive simultaneous detection of multiple amplicons using an automated sequencer-based detection system. Multiplexing with numerous primer sets is not new, but it has been made easier with the development of multiplex kits. Multiple DNA fragment analysis has been used in the field of population biology, to score size variation in Variable Number Tandem Repeats (VNTR) markers (e.g. Vaughan & Russell 2004) and to separate species (e.g. Hinomoto *et al*. 2004). Harper *et al*. (2005) used a multiplex-PCR approach, incorporating fluorescent markers, to amplifying the mitochondrial DNA (12S rDNA and COI) of semidigested prey items from predators' guts simultaneously. PCR products were separated on polyacrylamide gels using an ABI377 sequencer. The carabid beetle *P. melanarius* was shown to consume aphids, earthworms, weevils and molluscs using group-specific PCR primers and, while the main prey of these beetles was found to be earthworms and molluscs, some field-caught beetles contained DNA from four different prey. Although this field study was conducted on a very small subset of predators, it demonstrated the viability of this technique for screening gut contents using multiple prey-specific probes. However, the likelihood of using such techniques in large-scale ecological studies, on the scale of those with antibody-based techniques (e.g. Hagler & Naranjo 2005), is small owing to the higher costs and time associated with PCR screening protocols.

The retention time for DNA within the gut of a predator during digestion is influenced by factors including the size of the target DNA molecule. Predation by the coccinellid beetle *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) upon the eggs of the European Corn Borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) has been characterized by single-plex PCR amplification of four fragments of prey genomic DNA of different sizes (492, 369, 256 and 150 bp) from predator guts (Hoogendoorn & Heimpel 2001, 2002). Predator weight, size, developmental stage and meal size had no effect on detection period but in all cases the shortest fragment (150 bp) was detected for the longest time after feeding (up to 12 h). A similar study of a coccinellid–Lepidoptera, predator–prey system in Hawaii, produced analogous results with short (151 and 140 bp) prey COI mtDNA fragments being detectable in a greater proportion of beetle guts than larger (170 bp) ones (Sheppard *et al*. 2004). It may therefore be possible, given that prey DNA retention/detection time is inversely proportional to fragment length, to calibrate a system to determine, through post-mortem analysis, when individual prey items were consumed. However, such applications have not, to date, been tested.

Discussion and conclusions for the molecular detection of predation

It is clear that multiple fragment analysis provides significant advantages, and improved technology has enhanced the detection of prey DNA. Molecular analyses, however, are not without limitations that begin at a molecular level and extend to interpretation of ecological data. The research is fundamentally dependent upon the ability to distinguish species-specific sequences for primer design or direct sequence analysis. This can be difficult under circumstances where gene sequences vary intraspecifically. Such variation has been shown in internal transcribed spacer (ITS-1) regions in several arthropods (e.g. Wesson, Porter & Collins 1992; Tang *et al*. 1996) and there is even potential for variation in copies of this region within an individual. Another obstacle to selectively targeting species-specific sequences is the potential presence of pseudogenes. These occur when a coding region of DNA, for example the cytochrome oxidase codon, becomes transposed into the nuclear genome. The rate of evolution of non-functional pseudogenes is slower than the coding genes in the mtDNA so sequence divergence occurs over time. This has been observed in insects (e.g. Zang & Hewitt 1996) and could explain the presence of double bands on gels that can be difficult to interpret (Symondson 2002). The problem of intraspecific variation and pseudogenes can usually be overcome by judicious selection of target DNA or cloning to isolate and eliminate sequence ambiguities. There is an unescapable paradox, however, that the small DNA fragments that are necessarily targeted when looking at degraded DNA contain less information than longer fragments that would be easier to separate by sequence (e.g. TGGE, RFLP analysis).

It is evident that the impediments to selecting appropriate target sequences have largely been overcome but the interpretation of data is difficult as many studies remain limited to laboratory-based microcosms. An example of the difficulty in extrapolating from laboratory studies to the field is with temperature variation. A temperature increase from 20 to 27 °C significantly increased the digestion rate and reduced the prey DNA detection period in coccinellid beetles (Hoogendoorn & Heimpel 2001). A similar result was observed in tests using antibodies (Hagler & Naranjo 1997). Central to the problem of qualifying predation in the field is the time when the predation event occurred. Detection times of prey material in predator guts vary widely from a few hours to over a week. Although long detection times maximize the likelihood of achieving a positive screen against target prey, they can be counterproductive in that relatively old predation events still

screen positive. This increases the likelihood of obtaining a historic perspective of feeding behaviour, rather than describing predation events that have occurred recently. Conversely, very short retention and detection times are likely to yield few, if any, positive results in the field (especially agroecosystems) given than many generalist predators are frequently in a state of starvation (Bilde & Toft 1998) and are rarely captured with full guts. Consequently, optimizing an assay is a compromise between maximizing the likelihood of prey detection and minimizing the probability of overestimating predation events in the field. Obtaining a workable assay, with suitable detection periods, is therefore likely to be affected by the particular organisms being studied and the environment in which the field study is being undertaken.

The main problems with all post-mortem gut-content analyses are associated with interpretation of field data. It is extremely useful to get data from undisturbed systems (Sunderland 1988; Harwood & Obrycki 2005) but one cannot be sure that the prey material detected within the guts of predators got there as a result of predation. When trying to model predation from molecular data this assumption is often made (Mills 1997). Scavenging is the most obvious direct cause of potential error. Calder *et al*. (2005) showed that prey corpses (the Field Slug, *Deroceras reticulatum* (Müller) (Mollusca: Pulmonata)) decay slowly on soils for several days and antibody detection is possible in the gut contents of carabid predators (*Pterostichus melanarius*) that scavenge. Although detectability following the consumption of decayed prey was relatively low, thus minimizing the likelihood for false-positive estimates of direct predation, determination of carrion availability and scavenging potential of predators in the field is clearly important. Similar work has been carried out using PCR amplification of mtDNA (*COI*) to detect scavenging by the carabid beetle *Poecilus versicolor* (Sturm) (Coleoptera: Carabidae) upon *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) (Juen & Traugott 2005). Consumption of prey was found to be negatively correlated with cadaver age, but all carrion prey (1–9 days old) was detected as efficiently as fresh prey using PCR.

Prey material may also get into the gut of a predator through secondary predation. Generalist predators may feed on resources at more than one trophic level in natural systems (Pimm & Lawton 1978). One example of this trophic omnivory is when a predator consumes other predators with which it shares common herbivore prey (Polis, Myers & Holt 1989; Polis & Holt 1992; Sunderland 1996). This type of intraguild predation could potentially restrict the interpretation of data from gut-content analyses. This potential 'error' is regularly cited in reviews (Sunderland 1988, 1996; Symondson 2002; Harwood & Obrycki 2005), but rarely considered in field studies of predation. Although this error was documented as negligible in antibody-based systems (Harwood *et al*. 2001a) using an aphid-specific monoclonal antibody resistant to digestion, the sensitivity of PCRs to detect DNA fragments need further investigation to test for potential interference from secondary predation, yielding 'false-positive' detection of primary predation in the field.

Molecular techniques provide valuable opportunities to study complex trophic interactions in the field. A prerequisite is the extensive characterization and optimization of the system. With careful consideration of potential sources of error and difficulties of interpretation, valuable data can be gathered that would otherwise be impossible to obtain. DNA-based techniques have some advantages over antibody-based technologies, particularly for the study of complex generalist predator–prey food-webs. However, in many instances, especially in relatively simple predator–prey communities (where biocontrol may be evaluated), monoclonal antibodies continue to provide valuable information on interaction pathways in large-scale studies in the field.

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