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INVITED TECHNICAL REVIEW

PCR enrichment techniques to identify the diet of predators

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Abstract

The increasing sensitivity of PCR has meant that in the last two decades PCR has emerged as a major tool in diet studies, enabling us to refine our understanding of trophic links and to elucidate the diets of predators whose prey is as yet uncharacterized. The achievements and methods of PCR-based diet studies have been reviewed several times, but here we review an important development in the field: the use of PCR enrichment techniques to promote the amplification of prey DNA over that of the predator. We first discuss the success of using group-specific primers either in parallel single reactions or in multiplex reactions. We then concentrate on the more recent use of PCR enrichment techniques such as restriction enzyme digests, peptide nucleic acid clamping, DNA blocking and laser capture microdissection. We also survey the vast literature on enrichment techniques in clinical biology, to ascertain the pitfalls of enrichment techniques and what refinements have yielded some highly sensitive methods. We find that while there are several new approaches to enrichment, peptide nucleic acid clamping and DNA blocking are generally sufficient techniques for the characterization of diets of predators and highlight the most important considerations of the approach.

Keywords: blocking primers, laser capture microdissection, locked nucleic acid, PCR enrichment, peptide nucleic acid clamp, predator, prey

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Introduction

A long-standing and critical goal of ecology is to understand the trophic links between organisms, that is, what eats what? When applied to this goal, the polymerase chain reaction can be of tremendous value. It enables the ecologist to amplify DNA remnants of prey from the regurgitate, dissected gut or faeces of a predator. The amplified DNA is sequenced, and the sequence is then matched to a database to identify its source. The amount of usable prey DNA from these sources is expected to be low because of degradation by digestive processes, but because PCR is a sensitive method, capable of amplifying trace quantities of DNA, it is an ideal technique for diet studies. Consequently, the technique is emerging as a popular method with ecologists to establish trophic links among organisms (Carreon-Martinez & Heath 2010). However, when PCR is applied to a heterogeneous sample that contains multiple DNA templates, then it is typically the case that the dominant ortholog will be preferentially amplified. This is problematic for diet studies where the target prey DNA may be overwhelmed by the DNA of the predator.

The problem of predator DNA swamping the PCR can be avoided using species- or group-specific primers that are designed to exclusively target a set of predetermined prey DNA sequences (King *et al.* 2008). This approach is successful and highly sensitive and predominates in the literature. It most frequently enables us to refine what we already understand about the diet of a predator. However, if our goal is to characterize the limits of a predator's diet, then by anticipating prey items to design prey-specific primers we run the risk of presupposing the results we are testing. This review aims to discuss the PCR tools that enable ecologists to discover new trophic interactions or reveal the full extent of the diet of generalist predators. There are many studies that have addressed these broader questions, either by using a carefully selected set of group-specific primers (Jarman *et al.* 2004; Corse *et al.* 2010) or by screening the amplicons from universal primers with methods such as PCR denaturing gradient gel electrophoresis (PCR-DGGE: Martin *et al.* 2006; Tollit *et al.* 2009). These approaches are very successful and their use has been reviewed (Symondson 2002; Sheppard & Harwood 2005; Gariepy *et al.* 2007; King *et al.* 2008; Beja-Pereira *et al.* 2009). However, there is another strategy employed by a handful of DNA diet studies that has so far eluded review. This strategy is to use PCR enrichment techniques such as combined amplification and restriction digestion (Blankenship & Yayanos 2005; Blankenship & Levin 2007; Dunshea 2009), DNA

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blockers (Vestheim & Jarman 2008; Deagle *et al.* 2009, 2010) and peptide nucleic acid (PNA) clamps (Chow *et al.* 2010). Enrichment techniques constitute a very small part of published DNA diet studies, but they are a very promising means to uncovering unknown trophic links.

While ecology has neglected PCR enrichment techniques, cancer research has been at the forefront of research into these methods. This is because genetic mutations can be used as markers to screen for genetic diseases such as cancer, but many mutant cancer alleles typically occur at low frequency in tissues that can be routinely (i.e. non-surgically) biopsied. Enrichment techniques have therefore been perceived as a way to dramatically improve the early detection of cancers and enable clinicians to monitor the efficacy of cancer treatments. This is evidenced by the rich literature documenting highly sensitive assays where activating mutations are detected in peripheral fluids such as blood or sputum (e.g. Lietman *et al.* 2005; Miyazawa *et al.* 2008). The clinical literature is vast and there are many good reviews of enrichment techniques (Parsons & Heflich 1997; Gocke *et al.* 2000; Milbury *et al.* 2009), although these tend to focus on emerging technologies, rather than the optimization of established techniques. No published review of enrichment techniques surveys the existing PCR enrichment literature to synthesize those results and infer which enrichment methods work best in different experimental frameworks for dietary studies. What motivates this current review is the sheer number of clinical studies that incrementally modify techniques to improve the yield and sensitivities of reactions. We believe that these studies are a resource that can be used to rapidly develop PCR enrichment studies in the field of ecology.

The PCR enrichment techniques discussed in this article apply to a broad range of biological problems, including other fields of ecology. For instance, enrichment with competitive primers has been applied to study microbial ecology (von Wintzingerode *et al.* 2000) and parasite detection (Troedsson *et al.* 2008). The use of this competitive primer technique (one-tube enrichment) is discussed below, and its application to ecology in general is the subject of an excellent review (Vestheim *et al.* 2011). However, diet studies are distinct from many other fields of ecology because the DNA target is degraded and fragmented by digestive processes (Deagle *et al.* 2006; Simonelli *et al.* 2009). This is not a trivial point; for instance, to detect a parasite on a host, a researcher is afforded the advantage of targeting long sequences of DNA that span ideal priming and enrichment sites. Conversely, to detect the host DNA digested inside the parasite, the researcher is constrained to dealing with short DNA fragments where the coincidence of priming, enrichment and taxonomically informative sites can become unlikely. Ancient DNA studies, if they wish to use enrichment techniques, must contend with this problem too (e.g. Gigli *et al.* 2009). The purpose of this article is to supplement the vast and excellent literature on DNA techniques for diet studies with a review of methods to enrich the DNA of prey in the gut, which greatly ease the task of identification. It is very likely that many of the findings reported here would also be useful to fields other than diet studies.

In the following, we review PCR-based methods that enable prey discovery in any predator, particularly predators that are generalist or whose trophic roles are uncharacterized. We deal with the mainstream approaches to generalist prey discovery, including the use of multiple group-specific primers in parallel single reactions or multiplexed reactions. We then discuss PCR involving universal primers and the techniques that have been used to selectively remove predator amplicons to enrich prey amplicons. These methods include electrophoretic methods that screen for unwanted amplicons (e.g. DGGE), restriction digestion, PNA clamps and DNA blockers. We also survey the clinical research literature to see how they report enhanced enrichment for these techniques. We finally discuss laser capture microdissection (LCM) as a technique that may mitigate the need for highly sensitive enrichment techniques because it enables the physical dissection of minute biological samples that contain target nucleic acids. LCM has recently been used to study diet in larval fish (Maloy *et al.* 2010). Altogether, this review is intended to supplement the methodological programmes for DNA diet studies that are prescribed in existing reviews (Symondson 2002; Sheppard & Harwood 2005; King *et al.* 2008; Beja-Pereira *et al.* 2009) by directly addressing methods suited to the discovery of prey species of generalist and uncharacterized predators.

Group-specific primers

Species-specific and group-specific primers amplify prey to the exclusion of the predator by targeting prey DNA regions where the prey and predator sequences have diverged. Group-specific primers are usually efficient and sensitive, which is not surprising, because designing primers to anneal to one sequence and not another is the essence of PCR. The publication that initiated modern PCR with the thermostable DNA polymerase of *Thermus aquaticus* (Taq) reported PCR sensitivities between $1{:}10^5$ and $1{:}10^6$ when amplifying mixed template (Saiki *et al.* 1988), and such sensitivities are sufficient for diet studies.

Using PCR to characterize the diet of herbivores has been facilitated by the plastid genome of plants, because as Jarman *et al.* (2004) comment, designing primers to amplify a plastid gene is basically designing group-specific primers to target an entire kingdom. Short fragments of the rbcL gene (~157 bp: Höss *et al.* 1992) and the P6 loop of the trnL (UAA) intron (10–143 bp: Taberlet *et al.* 2007)

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are sensitive enough to generate amplicons from not only faeces but also ancient coprolites that are as much as 28 500 years old (e.g. Poinar *et al.* 1998; Hofreiter *et al.* 2000). The P6 loop has been used in conjunction with a massively parallel sequencing approach to yield 97 737 amplicons (Valentini *et al.* 2009). Despite being a short fragment (20–85 bp long), the amplicons had sufficient phenetic information to resolve 50% of amplicons to species, 45% to genus and the remainder to family. This use of the P6 loop with massively parallel sequencing has continued with similar success (Soininen *et al.* 2009; Kowalczyk *et al.* 2011), indicating that DNA diet studies of terrestrial herbivores have transitioned from pioneering to fully developed methodologies. It is more problematic when a predator is more closely related to the prey they consume.

With a region of DNA that is hypervariable across taxa, it is relatively straightforward to find a DNA sequence that uniquely identifies a predator. It is more difficult to find a unique predator sequence in a region that is highly conserved across taxa (i.e. an autapomorphy). However, if a predator and several of their prey belong to the same taxonomic group, then the only way to exclusively amplify all prey with a *single primer set* and not amplify the predator would be to locate an autapomorphy. The design of group-specific primers and PCR enrichment methods looks for unique predator sequences in hypervariable regions, whereas a single primer approach, that sets out to discover autapomorphies, is potentially a significant bioinformatic task. Also, the analysis would also need to be performed anew for every predator studied. In one study, such a pseudo-universal primer set has been used to amplify amphipods, krill, bony fishes and squid, while excluding the predator, which was penguin (Deagle *et al.* 2007). Their primer set did not amplify penguins because of a 3′ T that was not present in birds but was present across the targeted prey taxa (Deagle *et al.* 2007). Although the primer set did not amplify all taxa with equal efficiency, it did establish this approach as a plausible option. Another option is to design multiple group-specific primers to amplify taxonomic groups excluding the predator (Jarman *et al.* 2004). These primers can be used in parallel single reactions (Jarman *et al.* 2006; Deagle *et al.* 2007; Marshall *et al.* 2010) or in multiplex reactions (e.g. Greenstone *et al.* 2005; Harper *et al.* 2005). By multiplexing, it is possible to have comprehensive taxon coverage, and as many as 34 primer pairs have been used to represent 53 prey families (Corse *et al.* 2010).

Universal primer approaches

Fingerprinting

Universal primers have an advantage over group-specific primers for studying uncharacterized and generalist predators because the target does not need to be known *a priori* for them to be effective. The problem with using universal primers is that predator amplicons will normally dominate, so that for every prey amplicon there will be potentially thousands of predator amplicons generated. Some diet studies have solved this by following PCR or cloning with an amplicon fingerprinting step that assorts target and non-target sequences by denaturing or temperature gradient gel electrophoresis (DGGE, TGGE) and restriction fragment length polymorphisms (RFLP: Symondson 2002; King *et al.* 2008). A range of gene targets, mostly 18S, have been screened by a finger printing method (refer Table 1). In addition to RFLP, DGGE and TGGE, a novel method of screening has been used to ascertain the animal portion of an ancient human diet using universal primers to amplify DNA extracted from human coprolites, followed by cloning into a vector for sequencing (Poinar *et al.* 2001). A second PCR with a human-specific primer informed the researcher whether clones contained host amplicons so they were not sequenced as potential prey.

Screening amplicons after PCR with RFLP, DGGE or TGGE enables universal primers to be used in diet studies, but it is a labour-intensive approach; therefore, it ultimately narrows the sample size examined. Screening methods also carry the risk that target amplicons might share the same genotypic profile as the predator, for example, the same RFLP band size. This means that target amplicons might be excluded erroneously from the diet study. These shortcomings have led to recent trials of PCR enrichment methods in diet studies.

Restriction as an enrichment strategy

The method of using restriction enzymes discussed in this section differs from the use described in the previous section. It involves physically *removing* the non-target amplicons from the workflow rather than visualizing amplicons so that the non-target can be *avoided*. There are a few DNA diet studies that cite restriction as a prey enrichment method to be used with universal primers (Blankenship & Yayanos 2005; Blankenship & Levin 2007; Dunshea *et al.* 2008; Dunshea 2009). Restriction enzymes can be used to cut non-target amplicons near their centre, reducing them into two short fragments that are removable by electrophoresis or another size selection method (Blankenship & Yayanos 2005; Blankenship & Levin 2007). In contrast, genomic DNA can be digested prior to PCR, thereby removing the non-target template (Dunshea 2009). Additional post-PCR restriction can further reduce the number of non-target amplicons (Dunshea 2009). While the success of these approaches has been attributed to the restricted fragments being removed by electrophoresis or incapacitated so they do

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Table 1 PCR studies that have used universal PCR primers with a method to visualize amplicon heterogeneity. Columns refer to the gene targeted, the predator, the universality of the genes and the general approach. Some primers amplify a subset of eukaryote targets, and in this case, the groups that were used to design the primers are designated by brackets.

Gene	Predator	Universality	Approach	Reference
18S*	Bivalve	Eukaryote	RFLP	Duplessis *et al.* (2004)
18S	Lobster larvae	Eukaryote	RFLP	Suzuki *et al.* (2006)
18S	Lobster larvae	Eukaryote	RFLP	Suzuki *et al.* (2008)
18S	Krill	Eukaryote	DGGE	Martin *et al.* (2006)
18S	Eel larvae	Eukaryote	DGGE	Riemann *et al.* (2010)
18S	Bivalves	Eukaryote	DGGE	Maloy *et al.* (2009)
16S	Sea lion	Cephalopoda and teleostii	DGGE	Deagle *et al.* (2005a)†
16S	Giant squid	Metazoan (Cephalopoda, teleostii and crustacea)	DGGE	Deagle *et al.* (2005b)
16S	Sea lion	Cephalopoda, teleostii and crustacea	DGGE	Tollit *et al.* (2009)†
Cytochrome oxidase I‡	Beetle	Invertebrate (insect)	TGGE	Harper *et al.* (2006)

RFLP, restriction fragment length polymorphisms; TGGE, temperature gradient gel electrophoresis.
*Duplessis *et al.* (2004) also use 16S universal primers to amplify bacterial DNA.
†These studies used a nested PCR approach, first amplifying with a universal primer set and then a group-specific one.
‡Harper *et al.* (2006) also use TGGE with 12S primers to earthworms.

not contribute to PCR, it is important to note that a TA cloning system was used in all studies. This is presumably significant because the restricted predator fragments cannot be incorporated into the clone vector because the restriction enzymes that were used do not provide an A overhang and cannot be utilized in TA cloning (Blankenship & Yayanos 2005; Blankenship & Levin 2007; Dunshea 2009).

Despite its successful application to diet studies, there are potential problems with restriction. Commercially available restriction enzymes have varying efficiencies (Parsons & Heflich 1997), so there is the danger that a critical amount of predator DNA will not be cut. This problem could be overcome by careful enzyme selection, and to this end, less efficient or poorly targeted enzymes should be avoided. Another possible solution comes from clinical studies that have observed that using thermostable restriction enzymes can improve efficiency (Sandy *et al.* 1992). The rationale is that thermostable enzymes survive the melting step of the PCR and therefore continue to remove predator template throughout the cycles of a PCR.

More troubling for the use of restriction enzymes in PCR enrichment is that enzyme recognition sequences are generally short (<6 bp) and therefore occur frequently throughout genomes. This means there is the danger that the nuclease restriction site might occur in polymorphic regions of the targeted prey as well as their unwanted orthologs and that the restriction enzyme might accidentally remove target sequences. To minimize the chances of removing a target, a 'rare cutter' can be used, which is an enzyme with a recognition sequence of 8 bp or more (Dunshea 2009). However, there are relatively few commercially available rare cutters, so it is a matter of luck if a rare cutter is available that can be used to discriminate sequences of interest. An alternative is to use at least two of the shorter, more readily available enzymes in separate reactions and combine the results (Blankenship & Yayanos 2005). However, performing digests in parallel creates additional work and increases the risk of contamination with extraneous DNA. Alternatively, artificial restriction cutters could be designed such as zinc finger nucleases (Porteus & Carroll 2005) or, more economically, artificial restriction cutters (ARCUT: Yamamoto *et al.* 2007).

Restriction-based enrichment differs from the 'one-tube' methods (discussed below) because enrichment and amplification are independent reactions. The advantage of this independence is that both PCR and enrichment can be independently optimized to maximize the efficiency of both (Dunshea 2009).

'One-tube' enrichment

'One-tube' enrichment involves a non-priming oligonucleotide being added to the PCR to specifically prevent the amplification of unwanted DNA sequences. This non-priming oligonucleotide is homologous to the predator so that when it binds it prevents polymerization of the predator DNA but allows polymerization of target DNA. Blocking primers are DNA oligonucleotides with the 3' end modified so that they will not prime amplification. They have been used successfully on both invertebrate and vertebrate predators such as krill (Vestheim & Jarman 2008), fur seals (Deagle *et al.* 2009) and penguins (Deagle *et al.* 2010). Another alternative has been to use a PNA clamp (Ørum *et al.* 1993). PNA does not prime polymerization, which in combination with its high affinity

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for DNA makes it an ideal candidate for specific PCR suppression. In diet studies, PNA has been used to ascertain the diet of lobster larvae (Chow *et al.* 2010).

All published diet studies that have used 'one-tube' enrichments successfully have designed the blocker/clamp to partially overlap with a primer-binding site, an approach called primer exclusion (refer Box 1: Chow *et al.* 2010; Vestheim & Jarman 2008; Deagle *et al.* 2009, 2010). Other blocking strategies were trialled in one diet study (Vestheim & Jarman 2008), but these were unsuccessful (Box 1 contains a discussion as to why these approaches may not have worked and alternative strategies). There are several limitations in applying primer exclusion enrichments to diet studies. They arise from the constraint

Box 1 The optimization of 'one-tube reactions'

'One-tube' enrichments have become a routine molecular tool for some diagnostic applications, and at least in the short term, we would recommend the use of 'one-tube' technologies for enriching diet studies. Because of the significance of this technique and because of initial difficulties with optimizing 'one-tube' techniques, we give special consideration to how this technology has been improved through gradual trial and error

DNA blockers and PNA clamps

Blocking primers are DNA oligonucleotides with the 3′ end modified so that they will not prime amplification. These oligonucleotides can be made from conventional nucleic acids but have a 3′ modification to prevent polymerization. Such modification has included a dideoxy (ddNTP) terminal base as is used in Sanger sequencing (Parsons *et al.* 2005). More commonly used terminals have been phosphorylation (Senescau *et al.* 2005; Thiede *et al.*, 2006; Hu *et al.* 2009; Ren *et al.* 2009) or the ligation of a C3 linker (Laughlin *et al.* 2008; Vestheim & Jarman 2008). These DNA blockers very often incorporate a few locked nucleic acid (LNA) nucleotides to increase sensitivity and increase the melting temperature (Tm) of the blocker above the primer Tm. The LNA molecule imparts these properties because it is an RNA, but with the sugar cross-linked between the 2′ oxygen and the 4′ carbon, which gives the oligonucleotide an 'open' conformation that favours hybridization

The majority of studies using 'one-tube' enrichments use a clamp constructed of peptide nucleic acid (PNA). PNA is an RNA analogue, but with the phosphodiester backbone replaced by an *N*-(2-amino-ethyl)-glycine backbone (Egholm *et al.* 1992). Because PNA is neutrally charged, the PNA–DNA base pairs have high thermostability. PNA clamps are ideal for suppressing PCR because they do not prime polymerization, which in combination with its high affinity for DNA makes it an ideal candidate for specific PCR suppression (Ørum *et al.* 1993). Mismatched base pairing between PNA and DNA is thermodynamically disfavoured (Igloi 1998), much more so than DNA duplex mismatches. This makes PNA clamping highly specific, which is generally an advantage for 'one-tube' enrichment, although if there is high intra-specific variation in the enriched gene it might be advisable to use DNA blockers to allow some blocker mismatches so that the entirety of the predator amplicons can be suppressed

There are two common approaches to designing PNA clamping or blocking primer reactions: 'primer exclusion' and 'elongation arrest' (refer Fig. 1: Ørum *et al.* 1993; Vestheim & Jarman 2008). In primer exclusion, the clamp/blocker prevents the annealing of either the forward or reverse primer of the unwanted (wild-type/predator) strand because it anneals to a region that overlaps with the 3′ end of the primer-binding site. The result is that polymerization cannot be initiated in the direction of the excluded primer. In elongation arrest, the clamp/blocker anneals to any region between the two primer sites on the unwanted DNA strand. Therefore, the function of the clamp is to impede the polymerization of the non-target DNA in one direction, preventing the synthesis of the complementary primer-binding site so that the incomplete strand cannot act as a template in the reverse direction

Primer exclusion

Primer exclusion was first used in a 'one-tube' reaction to enrich codon-12 mutations in K-ras (Thiede *et al.* 1996). This was achieved with a PNA clamp and yielded sensitivities of $1{:}2 \times 10^2$. Later, the same PCR primers and PNA clamp achieved a sensitivity of detection $>1{:}1 \times 10^3$ by being used in conjunction with a restriction digest (Behn *et al.* 2000). The restriction step was possibly an unnecessary complication, as sensitivities of $1{:}2 \times 10^3$ were later achieved using a PNA clamp alone (Taback *et al.* 2004). It was also found that while conventional Taq polymerase achieved sensitivities of $1{:}2 \times 10^3$, that this could be improved to $1{:}2 \times 10^4$ by instead using a polymerase with 3′ → 5′ exonuclease activity (Gilje *et al.* 2008). The lengthening of the PNA with two N-terminal C residues was probably another factor in this improved sensitivity, because it would have increased the Tm of the clamp (Gilje *et al.* 2008). With PNA clamps, it has been found that it is useful to have two annealing steps, one at a higher temperature that the clamp binds at and another step at a lower for primers to bind to the target sequence (Ørum *et al.* 1993).

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Box 1 Continued

Elongation arrest

Although it has been used frequently over the past 3–4 years, elongation arrest was at one point considered an unreliable method for suppressing non-target amplicons. There is even an early study that designed a system to *enhance* the synthesis of a strand by designing PNA clamps to bind down its length (Demers *et al.* 1995); that is, it used the methodology of elongation arrest to achieve the opposite goal. This study emphasizes that when using elongation arrest it is important to optimize the antecedent conditions; otherwise, the enrichment not only will fail to suppress PCR of unwanted amplicons but will fail spectacularly

Through trial and error, it was found that there are three important PCR parameters for optimizing elongation arrest with PNA clamping. The first of these is the relationship between the clamp/blocker Tm and primer Tm. For example, with K-ras enrichment, the main difference between the early poor attempts at exclusion and contemporary successful ones is that the latter use a longer PNA clamp that has a higher Tm. To enable PNA clamping of EFGR, it was found that dropping the PCR extension temperature from 72 to 60 °C made elongation arrest of the wild type possible (Luo *et al.* 2006). At 72 and 65 °C, the wild type entirely dominated the amplicons, which indicates the importance of Tm in affecting arrest. The only published diet study to use DNA blockers for arrest found it unsuccessful (Vestheim & Jarman 2008). Substituting LNA monomers into a blocker, which increases the blocker Tm, does enable enrichment (Laughlin *et al.* 2008). The blocker used in this study had the 5′ end modified on the assumption that this would prevent the 5′ → 3′ proofreading from digesting away the blocker (Laughlin *et al.* 2008). However, it was later found that an LNA blocker with no 5′ modification can affect elongation arrest with sensitivities of $1{:}3 \times 10^3$, so the success is almost certainly due to the benefits of LNA increasing Tm (Ren *et al.* 2009). The utility of increasing Tm of PNA–DNA binding can be enhanced by adding positively charged molecules to the N or C termini of the PNA oligonucleotide (e.g. Lysine: Ørum *et al.* 1993) or by introducing cationic PNA monomers into the oligonucleotide (Abibi *et al.* 2004; Dragulescu-Andrasi *et al.* 2006; Rapireddy *et al.* 2007). The other parameter that governs the sensitivity of elongation arrest PCR enrichments is the run-off distance from the PNA to the complementary reverse primer-binding site (Luo *et al.* 2006; Chiou *et al.* 2007). A minimum of 50 nt have been suggested as the minimum run-off length for successful elongation arrest (Chiou *et al.* 2007), and another found that extending run-off from 28 nt to 97 nt dramatically increased enrichment sensitivity (Luo *et al.* 2006). Fast ramping between PCR cycles has also been found to improve the sensitivity of enrichments (Luo *et al.* 2006; Chiou *et al.* 2007)

that the blocker or clamp can sit in only one of the two locations that overlap with the primer-binding sites. This means there is a fairly good chance that these two small regions will be unsuitable for the design of blocking/clamping oligonucleotides. For example, these regions might contain nucleotide sequences that complement the primers, are self-complementary or have extremely low or high GC content. With PNA, there is also a restriction on the number of purine repeats (Thiede *et al.* 1996), which make it an even more difficult molecule to accommodate. For example, a highly self-complementary PNA clamp with purine repeats had to be used when isolating prey DNA from oceanic larvae of spiny lobster (Chow *et al.* 2010), which potentially limited its success. Several strategies have been employed to minimize the difficulties associated with the design of primer exclusion assays, and these are discussed in the later section.

Another problem for using primer exclusion in PCR diet studies is that it requires that the sequence immediately adjacent to the primer-binding site be sufficiently variable to uniquely define the predator. This is a problem because massive alignments of diverse taxa indicate that there are few regions where highly conserved domains are immediately adjacent to regions with high entropy, rather there is gradual erosion from the conserved to the variable. This is most apparent in ribosomal gene alignments, which are the genes used most routinely in diet studies (Ali *et al.* 1999). Therefore, a conserved primer set designed to amplify all metazoans or all eukaryotes could end up located far from variable regions suited to exclusion blocking/clamping.

Despite the difficulty of applying primer exclusion to the PCR of a gene fragment, the technique has been used for diet studies. Adapting it can be achieved by conceding complete universality, and this might not be a problem for some biological questions. For example, the diet of fur seals is known to consist largely of chordates and cephalopods, so to discover whether the prey of seals were benthic or pelagic animals, a study restricted their primer set to target mostly chordates and cephalopods (Deagle *et al.* 2009). Given that the seals are also chordates, the study used a blocking primer, but by limiting their target prey to two phyla, it was possible to find a conserved region adjacent to a variable region that could be exploited for primer exclusion. The same study investigated the possibility of other metazoans being included

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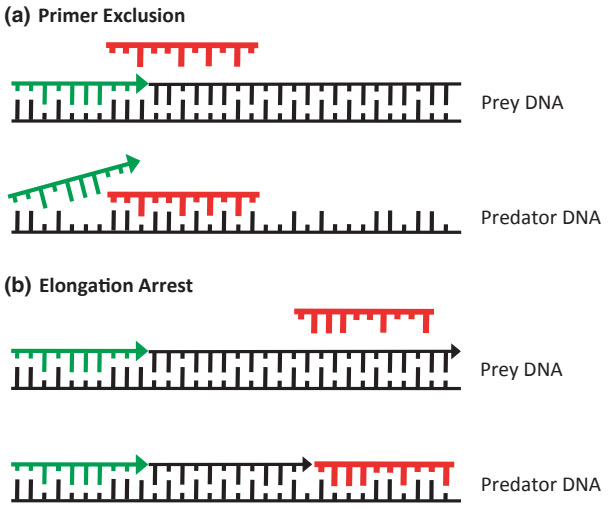

Fig. 1 Representation of two different approaches to 'one-tube' enrichments. In primer exclusion (a), the clamp/blocker (red) does not anneal to the prey DNA so the prey is amplified. The clamp/blocker does bind to the predator, which prevents primer-binding and stops the polymerization of predator amplicons. In elongation arrest (1), prey DNA is amplified. The primer binds to the predator DNA template but because the clamp/blocker binds downstream polymerization is stopped.

in the prey, but the investigation was constrained to using a short portion of the highly conserved 18S rRNA gene, which offers no possibility for primer exclusion. Instead of moving primer-binding sites nearer to variable regions that suit blocking, the blocker can be moved nearer the universal primer-binding site. The advantage of this approach is that the universality of the primers is not compromised, but the negative consequence is that the blocker can non-specifically inhibit the PCR of species other than the predator. For example, a blocker trialled for a krill diet study was found to match some species of decapod, but this consequence was not important to the study because those species of decapod were not listed as of interest to the study, which otherwise targeted a diverse range of taxa (Vestheim & Jarman 2008). An alternative to targeting fewer taxa is to target intergenic regions instead of intragenic regions. In this way, a study can place universal primers on conserved genes that are immediately adjacent to a hypervariable region that is suitable for clamp/blocker annealing. For example, a diet study has targeted the intergenic region between the 18S rDNA and the 5.8S rDNA (ITS1) for PNA clamping (Chow *et al.* 2010). The disadvantage with intergenic regions is that they can have massive variation in length and nucleotide composition, which can affect both PCR efficiency and the possibility of species identification (Chow *et al.* 2009, 2010). Another solution is to make a DNA blocker long enough to stretch from the conserved to the variable region. A dual priming oligonucleotide (DPO; Chun *et al.* 2007) with a 3′ C3 blocker was used to successfully enrich prey DNA extracted from Antarctic krill stomachs (Vestheim & Jarman 2008). The DPO is a long primer punctuated by an inosine bridge that forces the primer to have low affinity to mismatched DNA orthologues (Chun *et al.* 2007). There are therefore ways in which primer exclusion can be made to work for diet studies, but generally it does not appear to be the optimal strategy for designing a universal prey detection system for generalist or uncharacterized predators. For this, elongation arrest might be a better strategy.

For elongation arrest, the clamp/blocker anneals between the two PCR primers and prevents amplification by impeding polymerization (see Fig. 1). The method was not initially favoured as a PCR clamping technique because it did not perform as well as primer exclusion in both single nucleotide polymorphism studies (Ørum *et al.* 1993) and in ecological studies (von Wintzingerode *et al.* 1997). However, recently, elongation arrest has been further developed, has been reported to have very good sensitivity and has become a favoured method in clinical diagnostics (Miyazawa *et al.* 2008). Elongation arrest of PCR failed to work with DNA blockers in the context of diet studies (Vestheim & Jarman 2008), but it has been used with PNA clamps to detect parasites of crustaceans (Troedsson *et al.* 2008).

Purchasing 100 nmol of a high-pressure liquid chromatography (HPLC)-purified 25 nucleotide (25 mer) C3 blocker will cost a little over $100US with one C3 blocker or $145US if there is a blocker at either end (American price, November 2010). If a shorter blocker is required (to avoid PCR artefacts or to target a short sequence similar to that targeted by PNA clamps), then they can be synthesized with strategically substituted locked nucleic acid (LNA) bases. Each additional LNA base adds approximately $40US to the cost. An HPLC-purified PNA 14 mer will cost $520US, and the addition of lysine increases the cost to $550. DNA blockers can be synthesized and shipped within a few working days (our orders have occasionally taken more than 14 days to dispatch, but this is infrequent). PNA will generally take 3 weeks to ship. Because there is only one company that holds the international patent for synthesizing LNA, it means that demand can substantially increase wait times. However, on enquiry, the customer is supplied with a realistic estimate of synthesis time, which is reassuring when planning experiments involving LNA. These costs and time frames are for oligonucleotides purchased in late 2010 and early 2011, and global competition and market values will likely see these changes.

Before being purchased, blockers and clamps should be tested *in silico* to ensure that they have optimal characteristics for hybridization and that they do not form

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secondary structures or heteroduplex with primers. Conventional primer design software can be used to design DNA blockers (Vestheim *et al.* 2011, offer advice for this), but modified nucleotides have additional stringencies; useful websites for testing PNA and LNA oligonucleotides are http://www6.appliedbiosystems.com/support/pnadesigner.cfm and http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx. The companies that synthesize the latter oligonucleotides are also very helpful.

Dissection

Universal primers have been used without PCR enrichment methods to study the contents of shark regurgitate (Barnett *et al.* 2010; Dunn *et al.* 2010) and pieces of insects in red bat faeces (Clare *et al.* 2009). These predators are megafauna whose stomachs contain large pieces of prey and not just a homogenous digestive chyme, and therefore, enrichment techniques are unnecessary because DNA for PCR can be simply extracted from an isolated piece of prey tissue. This approach seems confined to large predators, because at some point the pieces of prey tissue become too small to be isolated with certainty. However, a recent publication has used LCM to excise prey DNA from folds in the gut of cod larvae (Maloy *et al.* 2010). LCM was designed to specifically remove cells (or one cell) from the bulk phase of very small tumours, therefore avoiding the problems associated with the co-dissection of wild-type cells (Emmert-Buck *et al.* 1996). A brief account of LCM is that it excises tissue from thin histology sections by firing a low-power laser at the target region. The excited tissue is captured onto a thin piece of film that is placed over the tissue (Emmert-Buck *et al.* 1996). The technique is highly accurate and relatively cheap and has been applied to numerous biological problems (e.g. Bonner 1997; Simone 1998). With cod larvae, the application of LCM to isolate cells of prey tissue from the gut produced excellent results using only universal 18S primers on gDNA (Maloy *et al.* 2010). The percentage of amplicons derived from cod and not prey ranged from 18% for the shortest amplicon (264 bp), to 48% and 52% for the larger amplicons (378 bp, 626 bp). It is possible to purchase purpose-built LCM platforms from commercial vendors (Applied Biosystems, Zeiss). After this initial outlay, the ongoing operating costs are the replacement of the laser burner (most core facilities charge around $30US/h), common histological consumables and the adhesive caps used to capture DNA ($6US). An Internet browser search will also reveal that there are several commercial LCM facilities that will perform LCM for upwards of $20US per sample. The recent introduction of this technology to molecular diet studies has great potential for the field.

Technological limitations and solutions

The public DNA databases are still largely incomplete for many metazoans, even for popular 'barcoding' genes (e.g. Bucklin *et al.* 2010). The incompleteness of databases is problematic for the study of uncharacterized predators because an impoverished database limits the ability to identify unknown prey. More importantly for experimental design, the database is required to test the universality of the primers. Enrichment PCR is even more affected by poor databases because a representative database is required to ensure the specificity of the enrichment. However, as the price of sequencing drops and through concerted sequencing efforts, this problem will gradually dissipate.

Perhaps the greatest problem for studying generalist and uncharacterized predators is finding a suitable 'barcoding' gene. Most genes are either too conserved or too variable across the metazoa. Having to work in concert with an enrichment method adds to the complication. Mitochondrial genes, such as cytochrome oxidase I, cytochrome b (Cyt B) and the 16S rDNA, have tended to be favoured in PCR-based diet analyses because they are abundant in cells and relatively variable, permitting a high level of species discrimination (King *et al.* 2008). However, this variability is problematic for designing universal PCR primers and also results in difficulties in taxonomic identification for taxa poorly represented in the databases. Of studies that are truly universal, the majority use a small portion of the 18S rDNA gene. The 18S gene is reasonably conserved across eukaryotes, and most eukaryotic phyla are represented by 18S in public databases. This makes it possible to identify 18S gene fragments to some level of taxonomic resolution. The disadvantage of 18S, which is the flip side of its being so conserved, is that it generally does not resolve to the level of species. However, when dealing with generalist and uncharacterized predators, the discovery of prey items at any level of taxonomic resolution is a valuable insight Further resolution can be obtained by other subsequent PCRs. Fragment length is also very important, especially in 'one-tube' enrichments where there is a tension between the requirements that a fragment that is sufficiently long to perform enrichments (Luo *et al.* 2006) and provide taxonomic information, and the requirements that fragments be short enough to be detectable after the digestive processes of the predator (Deagle *et al.* 2009). The problem of finding an appropriate barcoding gene can also be addressed by concerted sequencing efforts and intensive bioinformatic analyses.

Pseudogenes are non-functional copies of genes integrated into the genome. They mutate rapidly and create confusing signals if they are inadvertently sequenced. It is a problem for any diet study if prey pseudogenes are

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present (Dunshea *et al.* 2008), but it is a particular problem for PCR enrichment if there are predator pseudogenes because the high mutation rate of pseudogenes means that the enrichment site might not be present and therefore predator pseudogenes might feature in the sample (e.g. Dunshea *et al.* 2008; Chow *et al.* 2010). The best way around this is careful gene selection. Another method to avoid pseudogenes is to sequence reverse-transcribed RNA (e.g. Machida *et al.* 2009), but RNA is a molecule with a short half-life and its validity for diet studies needs to be assessed.

The increased sensitivity that enrichment enables increases the possibility of false positives owing to contamination. This contamination need not be an artefact of the laboratory or sampling. The environment is filled with liberated DNA, which can be co-sampled with specimens. A false positive may also arise through secondary predation, where the food of prey has been mistaken for prey species. It might be valuable to assess RNA as an alternative target to DNA because RNA is less stable so it is less likely to be present in the gut of the prey or as free RNA in the environment. Another strategy is to use the relative quantities of different amplicons/clones as an approach to sort out potential prey from contaminants—assuming that the amount of prey DNA in the gut exceeds contaminating DNA. However, although it is tempting to treat the distribution of amplicons as quantitative, this is potentially misleading (Acinas *et al.* 2005). The best way to minimize any misleading signal from exogenous DNA is to pair the enriched PCR with other quantitative molecular techniques. It is also likely that universal primers will amplify gut symbionts and parasites. This can create confusion, and it is also best dealt with by supplementing the DNA technique with a non-molecular technique (e.g. stable isotope analyses).

False positives can occur in PCR enrichments through the use of low gDNA concentration, high PCR cycle numbers, polymerase error rate and the size of the recognition sequence used by the enrichment method (Jacobs *et al.* 1999). Therefore, although the sensitivities of $1{:}10^5$ achieved by the enrichment methods are remarkable, these results may be inflated. It has been estimated that Taq will incorporate an erroneous base for every 9×10^3 bases it polymerizes (Tindall & Kunkel 1988) and Taq error during enrichment has been estimated as being one base in every 1.76×10^5 (Ohishi *et al.* 2004). With enrichments, there is a danger that Taq polymerase will induce errors in the predator amplicons, which will then be unrecognized by the enrichment method, and therefore, the unwanted DNA will proliferate. A PCR negative control containing only predator DNA can determine Taq error. Such negative controls suggest that Taq error can lead to false positives even when the target is outnumbered by only $1{:}1 \times 10^2$ (Luo *et al.* 2006). A DNA polymerase with $3' \rightarrow 5'$ exonuclease activity can substantially reduce the replication error rate compared to Taq polymerase (Gilje *et al.* 2008). This is serendipitous, because many diet studies already use proofreading enzymes because they are more sensitive to the nucleotide damage induced by digestion (Jarman *et al.* 2002; Deagle *et al.* 2005b) and because choice of enzyme affects the efficiency of PCR from low copy template (Beja-Pereira *et al.* 2009). To prevent the accumulation of errors in 'one-tube' enrichments, clamps could be made to complement both strands of the PCR template. This has already been practised, though for different purposes than to evade Taq error (Hancock *et al.* 2002; Gigli *et al.* 2009). However, the bidirectional use of a clamp/blocker seems necessary only when a high degree of sensitivity is required.

One solution to PCR-induced error is to eliminate PCR. Hybridization capture systems consist of numerous immobilized probes that are highly specific single-stranded oligonucleotides. These capture systems can be used in concert with next-generation sequencing technology to eliminate PCR from a sequencing workflow (See: Bau *et al.* 2008; Lee *et al.* 2009). This results in tens or hundreds of thousands of sequences of not only the probed DNA region but also the regions of the captured DNA strand that flank the probed region. For an ecology study, we imagine that an array of probes could target various metazoan groups and could be designed with the software used to design group-specific primers. The regions of DNA that flank the probed region would then provide sufficient genetic information to identify the isolated DNA. Capture arrays have been used to enrich ancient DNA (Burbano *et al.* 2010), and this is a good indicator that the method will be applied to DNA diet studies.

Hybridization capture systems can be commercially synthesized so are simple to implement, but despite the fact that their cost is dropping, they are still an expensive option. If PCR is not eliminated, then it might be beneficial to reduce PCR cycle number through technologies that increase the relative quantity of target (e.g. LCM). Also, enrichment techniques such as clamping, blocking and restriction give good results so long as a sufficient 'predator-only' negative control is used.

PCR enrichment in the context of diet studies

There are numerous methods that have been applied to studies of trophic interactions. Observing the behaviour of predators *in situ* and in laboratory scale, feeding trials are fairly fundamental approaches, but not always practical. One approach to situate a predator in a broad trophic context is to match the chemical composition of the predator (e.g. ratio of stable isotopes or lipids) to the chemical

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composition of various prey and environmental sources. However, there are three methods that are unique because they can identify dietary items with greater taxonomic precision; these are DNA techniques, serological techniques and hard-part analysis. Each of these methods has particular advantages and limitations. The main advantage of using universal PCR primers for DNA amplification is that they can identify a very wide range of prey but also give highly specific information about the prey species. Of course, this advantage lacks power without an effective enrichment strategy to prevent the predator homolog swamping the amplicon assemblage. Alternatively, a species/group-specific PCR approach can be used, but at the risk of overlooking unanticipated diet items. It is generally true that serological techniques have now been superseded by PCR techniques because they take considerably longer to optimize than PCR techniques (Symondson 2002; Carreon-Martinez & Heath 2010). However, the high specificity of serological techniques can also make them highly informative, especially when used in conjunction with a prey-enriched universal PCR approach. For example, a PCR approach might establish that bony fish are a part of a marine predator's diet. However, DNA cannot determine whether this predator consumes the adult fish, its larvae or eggs. Monoclonal antibodies can be raised against antigens present at a particular life stage, or tissue type, and can therefore be particularly informative as to the life stage of the prey (refer: Symondson 2002). Hard-part analysis has been used to identify prey remnants for many years and has been successfully applied to samples as small as copepod faeces (e.g. Turner 1987). One drawback with hard-part analyses is that it can over-represent dietary components owing to differences in gut residence times and digestibility for hard parts (Bowen 2000; Tollit *et al.* 2003, 2006). Hard-part analysis is also time-consuming and a highly specialized skill. However, hard-part analysis can be used to help validate that a PCR approach is not missing any prey species or to calibrate quantitative DNA methods. For example, a study on the diet of the arrow squid, a generalist predator, found that using DNA techniques and hard-part analysis in combination was more effective than using either method alone (Braley *et al.* 2010). They detected nineteen unique prey taxa, but only six of these were identified by both methods. Ten taxa were only detected using DNA techniques, and the other three were only detected by a morphological approach (Braley *et al.* 2010).

Integrating the data derived from prey-enriched PCR methods with other diet-study methods can enable highly supported hypotheses for trophic interactions involving generalist and uncharacterized predators. What techniques can be applied to any study is naturally constrained by the nature of the predator being studied. For example, the habitat of an organism will determine whether observing feeding behaviour is feasible or the digestive structures of an organism (e.g. if it has a specialized gut) will determine how easily samples can be dissected out for molecular analysis. Determining which techniques are suited to studying different clades of predators can involve synthesizing a massive number of studies, but reviews of the applicability of techniques to specific predators are available, for example, for seabirds (Barrett *et al.* 2007) and lobster larvae (Jeffs 2007).

Applying enrichment to diet studies: recommendations and conclusions

Of the enrichment methods discussed, the reagents of for restriction digests, DNA blocking and PNA clamping are readily available, at reasonable cost, and do not require any special development to be incorporated into even a rudimentary molecular ecology laboratory. While LCM is not standard laboratory apparatus, its utility means it is typically a part of the core facilities of larger research institutes. These techniques have been established in the clinical research for at least 15 years. Because of this long period of clinical application, there is a wealth of informative literature on how to optimize these techniques.

Next-generation sequencing technologies have the potential to enhance PCR-based studies' ability to represent the dietary component of gut/faecal contents. These technologies have enabled deep sequencing coverage at a drastically reduced cost. Diet studies have already benefitted from the application of second-generation sequencing technologies (e.g. Valentini *et al.* 2009; Deagle *et al.* 2009). More recently, there have been two developments in the next-generation technologies that will enable molecular ecology laboratories to access this technology, even if constrained by modest budgets. Small, low-volume platforms have been developed such as the Roche GS Junior (http://www.gsjunior.com) and Illumina's MiSeq Personal Sequencing System (http://www.illumina.com/systems/miseq.ilmn), and third-generation sequencing technologies such as the Ion Torrent (http://www.iontorrent.com; Rothberg *et al.* 2011) have come onto the marketplace. These third-generation technologies are characterized by non-optical detection systems that greatly reduce reagent costs. These developments will enable molecular ecology laboratories with modest budgets to obtain a depth of coverage that will enable them to perform crucial meta-analysis of the efficiency of PCR enrichment and assess the contribution of contaminants to data sets. The massive numbers of sequences produced by these sequencers reduce the negative impact of non-target amplicons that are caused by 'leaky' enrichment methods, resulting in gut symbionts

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and DNA contaminants being incidentally co-amplified with genuine diet items.

We anticipate that the very recent application of enrichment technologies to study trophic ecology will enable the rapid resolution of questions about what constitutes the diet of any predator. Enrichment techniques will therefore enable molecular studies of organisms that prey on closely related organisms to be conducted with an efficiency and sensitivity similar to that attained by molecular studies of trophy between unrelated organisms, such as herbivory. The study of herbivory still has some advantages over the study of animals that predate upon animals. For example, a study can simultaneously determine the diet of several herbivores using the same universal primer set (e.g. Valentini *et al*. 2009), but a study of several predators would require developing several corresponding enrichment techniques, which is potentially expensive. Future research needs to be performed on creating enrichment techniques that are readily *transferable* between predators. These could come from new technologies, for example capture arrays, but could also come from reinvestigating enrichment methods that have been proposed for clinical screening but have not been adopted. For example 'headloop' PCR is a primer-mediated technique for enriching target amplicons by inducing hairpins in non-target amplicons (Rand *et al*. 2006). This technique could be a cheap and quick way to enrich amplicons and therefore would be suited to designing enrichments for various predators. This is not an endorsement of 'headloop' PCR, as more research needs to be performed on the technique, but it is an example of the value of investigating other proposed enrichment techniques for ecological applications.

The least tractable biological questions are ones about the relative proportions of prey that constitute the diet of predators. This is a general problem faced by molecular ecology, largely because PCR does not necessarily preserve the composition of environmental samples (e.g. Acinas *et al*. 2005), and future research is required to demonstrate whether quantitative data from environmental samples are reliable. Sequencing directly from hybridization capture arrays is another potential solution to this problem, so long as the array is not saturated. This is because it removes the potential biases introduced through PCR. However, if the enrichment techniques discussed in the present review are only able to establish the presence/absence of potential prey in the diets of uncharacterized predators, it is a significant step forward in understanding the ecology of predation.

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