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2
3 **Unpicking the tangled bank**

4
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11
12
13 It is now seven years since a conference was held at the University of Innsbruck, Austria, that for the
14 first time was dedicated exclusively to the emerging field of the Molecular Detection of Trophic
15 Interactions. Since then the technology, and as a consequence the ecological questions that can now
16 be addressed, has moved on considerably and the field continues to grow exponentially. In this
17 Special Issue we have published 27 papers prepared by attendees at the second such meeting held
18 in May 2013, at the University of Kentucky, organised locally by James Harwood. These papers cover
19 a diverse array of ecological studies and diagnostic techniques, and include predation, parasitism
20 and herbivory, by and/or on vertebrates and invertebrates, in both terrestrial and aquatic systems.
21 Together they provide, in a single issue of the journal, an excellent opportunity to assess the current
22 state of play in this rapidly developing field.

23
24 Probably the biggest change since the last meeting has been the application of Next Generation
25 Sequencing (NGS) to trophic analyses. What a predator eats depends upon what else is available,
26 and for the first time it is possible to rapidly detect the whole spectrum of prey or plant remains that
27 a predator or herbivore may have eaten. Nine of the 27 papers use this technology, which has
28 dropped enormously in price, bringing it within reach of cash-strapped ecologists. This has partly
29 been brought about by the introduction of bench-top sequencers, such as the Ion Torrent machines,
30 but these in turn are being replaced by even smaller and cheaper equipment as new developments
31 are made at an accelerating pace. In most recent studies, DNA from predator gut or faecal samples
32 has been tagged, by individual or sub-group/treatment, permitting the spectra of prey consumed to
33 be separately analysed across a population or between populations. Seven of these nine papers
34 analyse the diets of bats (Burgar *et al.* 2014; Clare *et al.* 2014b,b; Emrich *et al.* 2014; Krüger *et al.*
35 2014a,b; Sedlock *et al.* 2014), one the diets of shrews and skinks (Brown, Burger *et al.* 2014a), and
36 one prey consumption by seals (Thomas *et al.* 2014), reflecting the dominant (but not exclusive) use
37 of NGS to date for analysis of vertebrate (versus invertebrate) predator diets. In all cases, these were
38 predators that could have chosen, in the field, to eat from among tens to hundreds of different prey
39 items; no other approach could have analysed such samples as efficiently.

40
41 One of the problems with NGS, particularly in tropical rainforest and other biodiversity hot spots, is
42 that many prey species cannot be identified from their DNA sequences. Barcoding of species in such
43 locations is very patchy, requiring molecular operational taxonomic units (MOTUs) detected within
44 faecal samples to be classified (as far as possible) by family or even order. Where diets are compared
45 between different predator species, it is possible to analyse niche breadth and niche overlap entirely
46 by MOTUs, even when few or none of the sequences can be identified to species (e.g. Brown *et al.*
47 2014a; Burgar *et al.* 2014; Sedlock *et al.* 2014). Thus, even though it is not known precisely what they
48 were eating, it is possible, for example, to detect resource partitioning (e.g. Burgar *et al.* 2014;
49 Emrich *et al.* 2014; Krüger *et al.* 2014a; Sedlock *et al.* 2014) and direct competition (Brown *et al.*
50 2014). Nevertheless, this gap in our knowledge should provide a further incentive for extensive
51 barcoding of biota. Where possible, and where the range of items in the diet is limited, those using

52 NGS may in the meantime need to barcode biota within the habitats under study if they are to
53 improve the resolution of their analyses.

54

55 The range of ecological questions being addressed by the papers using NGS is broad but like almost
56 all molecular diagnostic work on diets there are biases that have to be considered. The hope
57 originally was that numbers of sequences generated by NGS would provide a good estimate of
58 biomass consumed. Unfortunately, this is not the case. Homology at the primer site, rates of
59 digestion of different tissues, density of mtDNA copies per cell, competitive PCR biases and other
60 factors all affect numbers of sequences amplified, even when the same predator species and the
61 same primers are used throughout. Here, Thomas *et al.* (2014) found that lipid content of different
62 fish fed to seals can also affect results and could be incorporated into models adjusting for bias. Such
63 adjustments may, as in this case, be specific to a particular study system. With NGS, therefore, many
64 authors now simply record numbers of predators testing positive for a target prey or plant species,
65 providing a pragmatic and useful surrogate for truly quantitative information.

66

67 Next Generation Sequencing is certainly not a universal panacea. For example, analysis of the role of
68 predators in crops or even natural ecosystems, where relatively few potentially influential prey
69 species are present, can be done cost-effectively using species-specific primers (Raso *et al.* 2014;
70 Schmidt *et al.* 2014; Šerić Jelaska *et al.* 2014). Some studies are concerned with analysing the range
71 of predators attacking a single target species (e.g. Lundgren & Fergen 2014). The great advantage of
72 species-specific primers is that large numbers of predators can be individually and rapidly screened,
73 providing the levels of replication often needed for analysis of invertebrate food webs.

74

75 The question of biases is greatest where different predator species are compared and/or where
76 results are obtained using different primers. The wider use of qPCR can increase sensitivity and help
77 quantify prey DNA in samples (Eitzinger *et al.* 2014; Leal *et al.* 2014a; Lundgren & Fergen 2014; Redd
78 *et al.* 2014), and is excellent for comparing relative predation rates of particular predator-prey
79 pairings over time or between treatments, but is still subject to these biases. Thus, the whole
80 question of biases remains problematic, but in this Special Issue there is a spectrum of studies where
81 biases are reduced or managed in a range of effective ways. Invertebrate food webs are the primary
82 challenge, where there is great interest in how interactions between and within several trophic
83 levels can lead to changes in populations. The opinion piece by Greenstone *et al.* (2014) highlights
84 the need for adjustments to be made based on feeding trials and digestion rates, allowing the sum
85 effects of these biases to be parameterised. Where few species are involved, this is relatively simple
86 (e.g. Welch *et al.* 2014). However, such feeding trials are not always practical in complex food webs,
87 where multiple predators are feeding on multiple prey (and each other). One option is to do feeding
88 trials on representative species within predator groups (defined by taxonomy, hunting mode or
89 feeding mode), but not all species (Schmidt *et al.* 2014). Alternatively, recognising that suppression,
90 for example, of crop pests can depend upon the concerted action of whole communities, it is
91 possible to avoid making comparisons between individual predator species and look instead
92 (separating predators only by feeding mode) at how predator abundance and diversity affect overall
93 predation on a target pest (Lundgren & Fergen 2014). The approach of Wallinger *et al.* (2014) was to
94 design sets of primer pairs that amplified DNA fragment of similar length, and they found (in earlier
95 trials) that this allowed detectability of plant DNA in rootworms to be similar across most plant
96 species. Eitzinger *et al.* (2014) looked at the interesting question of whether predator size, within
97 species, might affect digestion rates too, and found that it did not (at least in centipedes).

98

99 It was very good to see aquatic systems well represented within the Special Issue, one freshwater
100 and six marine. Krüger *et al.* (2014a) compared the diets of two sympatric 'aquatic' bats that trawl
101 for insects (using feet and tail membranes) across the surface of rivers. Small differences in bat
102 morphology were thought to be related to prey choice, one species feeding more on terrestrial prey

103 than the other. Alonso *et al.* (2014) analysed the diets of Cory's shearwaters feeding on fish and
104 invertebrates, comparing factors such as sex, breeding status and breeding phase. Both of these
105 papers report comparisons of morphological analysis of samples with molecular analysis, finding
106 many more trophic links and greater taxonomic precision with the latter. The Alonso *et al.* (2014)
107 paper in particular shows how the two approaches can be used in a complimentary way. Thomas *et al.*
108 *al.* (2014) performed captive feeding trials with seals, to try to calibrate quantitative variables
109 discussed above. Redd *et al.* (2014) highlight sources of error too, in their temporal and spatial
110 analysis of Rock Lobsters feeding on sea urchins, finding that juveniles in particular may be picking
111 up urchin DNA from the benthos, a problem that could potentially cause interpretive challenges with
112 other studies involving benthic food webs. Dietary work is also reported from coral reefs and deep
113 sea vents, by cnidarians (corals and sea anemones) and amphipods, respectively. It was shown in the
114 lab that both an anemone and a coral digested their prey far more slowly than had previously been
115 thought (Leal *et al.* 2014b), while some corals failed to capture and consume the microalgae offered
116 or showed evidence of choice (Leal *et al.* 2014a). Studying trophic interaction in the deep ocean is
117 even more of a challenge as the species will often only survive *in situ*. Olsen *et al.* (2014) found,
118 using denaturing high performance liquid chromatography of 18S rDNA, that amphipods feeding at
119 hydrothermal vents and cold seeps were far more omnivorous than previously suspected. The range
120 of these aquatic studies illustrates well the breadth of species, habitats and trophic associations that
121 can be studied using molecular diagnostics.

122
123 One group of predators that have rarely been studied before using molecular diagnostic are the
124 reptiles. Brown *et al.* (2014a), as mentioned above, examined the diet of the rare Telfair's Skink on a
125 Mauritian island, competing for invertebrate prey with the invasive Asian Musk Shrew. They also
126 analysed the diet of the locally rare Smooth Snake in the UK, using a panel of species-specific
127 primers, demonstrating that these snakes may be confined to small pockets of habitat where their
128 main prey, other reptiles, are abundant (Brown *et al.* 2014b). One little-recognised advantage of
129 analysing faecal DNA is that the same samples can be used to detect not only diet (and where
130 necessary the species and genotype of the animal producing the faeces) but also gut parasites. Thus
131 Brown & Symondson (2014) were able to examine a range of factors affecting prevalence of
132 nematodes in the Slow Worm, a legless lizard.

133
134 From its infancy, molecular diagnostics has been used to better understand trophic interactions
135 (both predation and parasitism) between invertebrates, in both natural and agricultural systems,
136 and such papers are well represented here. A novel application of the analysis of trophic pathways is
137 to use them to explain the movements of heavy metals through the environment. Šerić Jelaska *et al.*
138 (2014) were able to analyse the bioaccumulation of such metals in carabid beetles, by linking the
139 heavy metal burdens of the beetles to those of their main prey (in this case earthworms and slugs in
140 forest ecosystems). Equally interesting is work on pioneer communities on land exposed as glaciers
141 retreat. Few prey species are available to the spiders and carabids inhabiting such environments and
142 Raso *et al.* (2014) showed clearly (using a combination of molecular diagnostics and stable isotope
143 analysis) the intensity of competition between predator species, demonstrated by high rates of
144 intraguild predation.

145
146 Much has been published to date on the application of molecular diagnostics to the effective
147 detection of parasitoids within their hosts (often at an early stage of development), mostly in
148 agricultural systems. Here we have three papers that take this approach in new directions, both
149 technically and ecologically. Derocles *et al.* (2014) analyzed parasitoids attacking aphids in
150 agricultural fields vs. field margins, and showed that there was strong compartmentalization, with
151 few parasitoids attacking aphids in both environments. This suggests that, with parasitoids at least,
152 field margins may be a poor source of useful natural enemies, contrary to much evidence already
153 published. Two papers report the ability of molecular diagnostics to identify host and parasitoid

154 from empty pentatomid eggs (Garipey *et al.* 2014) and empty aphid mummies (Varenes *et al.*
155 2014). Garipey *et al.* (2014) were interested in determining the range of native parasitoids attacking
156 an alien pentatomid bug, and were only limited in this endeavor by lack of a comprehensive
157 parasitoid barcode database. Varenes *et al.* (2014), using general primers followed by single-
158 stranded conformation polymorphism (SSCP), experimented in the laboratory with detection of
159 parasitoid and hyperparasitoid DNA from empty aphid hosts. Such work is truly forensic and further
160 extends the reach of molecular diagnostics.

161

162 Useful work continues to be conducted on biocontrol of crop pests by invertebrate generalist
163 predators (Lundgren & Fergen 2014; Schmidt *et al.* 2014), although far fewer papers are represented
164 here than were reported at the last meeting in Austria. The opinion piece by Greenstone *et al.*
165 (2014) is aimed primarily at ways in which the accuracy of such work can be improved, using
166 calibratory feeding trials and modelling to estimate a rank order of predators in terms of their
167 effectiveness as biocontrol agents at a study site. Such efforts allow us to approach, if not finally
168 answer, the question of precisely how many prey individuals are being consumed. Conventional food
169 webs (such as host-parasitoid webs) require such numerical estimates in order to evaluate web
170 dynamics. However, much can be done based simply on number of predators testing positive, as
171 shown in the study of Lundgren and Fergen (2014), in which it was shown that both numbers and
172 diversity of natural enemies had significant effects on a focal prey, corn root worm. Similarly,
173 Schmidt *et al.* (2014) examined the wide range of predators attacking squash bugs, major pests of
174 cucurbits particularly in organic farming systems. They were able to compare the value of different
175 natural enemies as biocontrol agents of this pest under various organic management practices.

176

177 To conclude, we have hardly begun to exploit the full potential of molecular diagnostics as a tool for
178 improving our understanding of trophic interactions. There seems to be no limit to the interactions
179 that can be studied in this way, or to the habitats in which trophic interactions can be explored. A
180 major strength of this field had been the willingness of participants to embrace new technologies
181 which have taken us from early precipitin tests, using antibodies, through to NGS. This process will
182 continue, as will the use of data obtained by such means to test ecological theory. Current work
183 embraces everything from applied work, on ecosystem services, through analysis of behaviour to
184 conservation applications. It is safe to predict that all these areas, and more, will expand rapidly over
185 the next few years.

186

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192

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