Beyond genes-for-behaviour: the potential for genomics to resolve questions in avian brood parasitism

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Abstract

Brood parasite-host interactions are among the most easily observable and amenable natural laboratories of antagonistic coevolution, and as such have intrigued evolutionary biologists for decades. It is therefore surprising they have not been at the forefront of genomic studies on evolutionary adaptation. Here we review state-of-the-art molecular methods in studying avian brood parasitism, a model system in behavioural ecology. We highlight outstanding questions to bring examples of how genomic tools are not merely about 'finding a gene for behaviour', but can be used to study the causes and mechanisms of (co)evolutionary adaptation. In doing so, we promote behavioural and molecular ecologists to integrate Tinbergen's questions into a collaborative, coherent science aiming to solve the mysteries of nature and apply current methodology into other model systems in behavioural ecology.

Behavioural ecology and genomics

The need to close the gap in knowledge between phenotype and genotype has long been discussed [1]. However, reading genome sequences has been insufficient to explain phenotypic variation [2–4] and has instead revealed that closing the gap requires understanding links between the genotype, phenotype, environment and species interactions across biological levels (e.g. [5,6]). Consequently, recognising when and how genomic data may be useful to study the ecology and evolution of behaviour is yet to progress beyond human genome pioneer

Eric Lander's famous quote: "Genome: Bought the book; hard to read". Bridging behavioural and molecular ecology could however bring a major advance. Behavioural ecology provides a rich understanding of why behaviour and associated traits (e.g. morphology and physiology) evolve at the phenotypic level and a tool-kit of quantitative and experimental methods [7] to address how the actions of individuals influence population-level processes in the wild [8]. However, behavioural ecology continues to work mostly at the level of the phenotype (figure 1a), and is sometimes viewed as the 'soft underbelly' of evolutionary biology [8]. In contrast, the declining costs of next-generation sequencing (figure 1a) have helped molecular ecologists use genomic data to identify genes and traits relevant for fitness and adaptation [9,10] including in non-model organisms [11]. Behaviour, however, is rarely considered in molecular ecology, despite it influencing heritability (i.e. non-genetic inheritance and indirect genetic effects [12]) and being one of the major drivers determining how genes interact in time and space [13] and experience selection.

While we are not the first to call for the use of genomic tools to study behaviour (e.g.[14–18], they are still rarely adopted in behavioural ecology (figure 1a) despite earlier genetic approaches leading to major advances (e.g. microsatellites to determine parentage [19]). This might be because 'finding the gene/s for a behaviour' is often the focus (e.g.[14,15]) and this is rarely feasible or cost effective in the wild [18] (See box 1). However, genomic tools can be used to do more than uncover the mechanistic basis of traits; they can help us find answers to questions about trait function, evolutionary history and development (i.e. Tinbergen's Four questions [20]). For example, (i) whole-genome sequencing data can help improve the robustness of phylogenetic trees and comparative analyses, by resolving discrepancies between different loci [21]; (ii) by comparing neutral and selected markers across genomes, we can now infer population demographics and local adaptation in addition to population structure and gene flow [22]; and (iii) genome sequencing provides increased resolution to determine parentage [23] and facilitates inferring offspring (or hybrid) fitness (through pedigree reconstruction, e.g.[24]). Perhaps most importantly, (iv) genomics allows us to go further than just improving on previous genetic methods: we can acquire information about species' evolutionary history and adaptive potential from the genome [25,26] to predict impacts of future change [27]. This is now a critical question for everyone working in ecology and evolution.

One of the largest stumbling blocks to integration is a cultural divide: while both behavioural and molecular ecologists study adaptation and address both ultimate "why" and proximate

"how" questions, the two fields are separated by different approaches, jargon (see table 1 for examples) and working styles. Behavioural ecology was founded on the 'phenotypic gambit', where knowing heritable genetic mechanisms is considered unnecessary as long as the trait correlates with fitness proxies [28]. Meanwhile, to resolve technological and methodological issues inherent to studying the heritability of polygenic traits (like behaviour), molecular ecologists have largely focused on those thought easier to measure (e.g. morphology [29]) and assumed to be determined by few, large-effect loci [26]. However, it is becoming evident that this approach has biased conclusions, and a single gene or supergene is unlikely to be a common explanation for variation across phenotypic traits [18], including in model systems (e.g. chicken plumage and egg coloration [30]). Furthermore, while heritability estimates for behavioural traits (when investigated) are often within range of estimates for physiological and life-history traits [29], recent genomic studies suggest that mechanisms of heritability are more complex than previously thought (see e.g.[3] for epigenomics and regulatory mechanisms) and the genome is now considered an environmentally responsive entity [6] somewhat analogous to behaviour [14].

Massive genomic datasets for species where we already have (or have the potential to collect) rich behavioural data are becoming available (e.g. Avianbase [31]), so how can we overcome this cultural divide? Here we bring together behavioural and molecular ecologists (box 2) to work through a case study example and demonstrate how we can combine information from both the genome and behaviour to study adaptation. We use one of the most classic textbook examples in behavioural ecology (avian brood parasitism [32]) and assess what is (and what is not) possible with the rapidly expanding, and sometimes overwhelming [33–35], range of genomic tools and analytical methods available [1,36] to address broad questions of interest.

Avian brood parasitism as a case study

Obligatory brood parasites (about 1% of birds, some insects and the cuckoo catfish [32]) trick hosts into rearing foreign offspring as their own. This selects for the evolution of host defences, with counter-adaptations in parasites [37] (figure 2) and has provided behavioural ecologists with a particularly tractable system for studying coevolution in the wild [38]. Experimental methods to test hypotheses about behavioural defences and cuckoo trickery [39] are well defined, and techniques to measure phenotypes are taking advantage of technological

developments (e.g. egg pattern and colour [40] to identify individual cuckoos [41]. Compared to the rich body of knowledge for behavioural adaptations, almost nothing is known about the molecular mechanisms underpinning behavioural defences and offences and the heritability of traits remains mostly assumed. The field of genetics has, however, already provided some insight into the evolutionary history of brood parasitism [42] and host-race specialization [43–45]. For example, microsatellites have been used to estimate levels of gene flow between parasitised and unparasitised magpie populations [46,47], leading to a candidate marker for its egg rejection behaviour [46]. Nevertheless, the same marker was not associated with egg rejection in another host species (the great reed warbler, [48]) and this line of inquiry has not continued.

Despite repeated predictions that genomics would re-revolutionize the study of brood parasitism (e.g. [17,49,50]), there has been relatively little work making use of data or insights from high-throughput sequencing (figure 1). The few examples are recent and limited to a small number of species (discussed in more detail below): improved phylogenies [51,52], population structure of brood-parasitic sister-species [53], adult parasite diet [53,54] and nestlings' microbiota [55], the heritability of parasite and host egg coloration [55,56], and the loss of parental care in brood parasites [57]. This is surprising, since biologists working on birds were instrumental in developing the field of quantitative genetics with an animal model approach [58], and after entering the genomic era [59] they quickly generated a detailed understanding of avian genome structure [60] and conducted some of the first studies of genomic adaptation in wild populations (e.g. [27]; [26]). Furthermore, high-quality reference genomes are now available for brood parasite hosts with rich behavioural data (e.g. superb fairy wren: [61], great reed warbler: [62,63], reed warbler: [64]). Therefore, here we discuss how applying genomic methods to avian brood parasitic systems could be used to better predict (i) where and when brood parasitism should evolve, (ii) when and how hosts defend, or (iii) how coevolutionary trajectories depend on ecological change, three major questions in a field aiming for a deeper understanding of coevolutionary dynamics [32]. Even though we focus on long-standing questions in avian brood parasitism, these three key concepts (evolutionary origins, plasticity, geographic selection mosaics) are of broad relevance across ecology and evolution.

1) Where and when should brood parasitism evolve?

Darwin [65] proposed that brood parasitism evolved from an ancestor with parental care. Since then, three key pathways have been suggested: by evolving directly from parental care [66], via conspecific brood parasitism as a 'stepping stone' [67,68], or via cooperative breeding as a precursor [69]. Ecological conditions and changes in life-history traits are also likely to have influenced the transition [69,70]. However, these hypotheses remain surprisingly difficult to test and understanding the origins of avian brood parasitism remains one of the most fundamental open questions [42]. Molecular methods in the early 2000s produced a more robust phylogeny for brood parasitic birds than had been available previously [71], and facilitated more accurate estimates of when, and in which families, brood parasitism evolved (e.g. [17,71–73]. Adopting this new phylogeny [71], however, changed conclusions of analyses into the life-history conditions that surrounded the evolutionary transitions to brood parasitism: for example, first comparative analyses using Aragón's [74] and Johnson's [75] molecular phylogenies suggested that brood parasites evolved to reduce the costs of reproduction associated with migration and a change in diet [76], whereas a similar analysis using the new phylogeny with a different topology and branch lengths suggested migration evolved after parasitism [75,77]. With only seven independent evolutionary origins of brood parasitism, can we expect to ever know the conditions that facilitate it?

To understand the challenges of using the phylogenetic comparative method (PCM) to infer the ecological drivers of evolution, it is useful to consider recent development in the field more broadly. Genomic data and the methods used to build phylogenies and conduct comparative analyses have become increasingly more accessible [78,79], but also more complex [80–83]. Essentially, increasing data (in terms of both loci sequenced and taxa sampled) has led to more cases of discordance between gene trees and the species tree (e.g. because of introgression and incomplete lineage sorting, [52,84,85]. This in turn has led to a conceptual change ([86] where the initial assumption that the "true" species tree could be reconstructed from the average signal of multiple genes has gradually been replaced by evolutionary models that incorporate various mechanisms now known to cause and sort genetic variation differently across the genome (e.g. varying mutation rates, recurrent hybridisation, gene flow, drift). The revolution of phylogenetics to phylogenomics has also meant that applying PCMs to answer eco-

evolutionary questions is now more vulnerable to inferential errors as model assumptions are not always considered [81,87]. Multiple evolutionary pathways can lead to the same outcome (e.g. [88] and although correlation from PCM can reject or give support to certain hypotheses, it cannot prove causality. For example, a PCM study by [89] suggested that brood parasitism promotes cooperative breeding in hosts, whereas later work concludes the opposite - brood parasites are attracted by cooperatively breeding hosts [90]. Therefore, it is necessary to formulate accurate hypotheses that enable robust tests [91], and preferably use methods that can compare multiple different hypotheses simultaneously (e.g. [92,93]. Using such an approach, Griesser et al. [94] demonstrated that a 2-step process including both family living and subsequent variable environmental conditions best explains the evolutionary origins of cooperative breeding.

Even the most sophisticated comparative methods are, however, dependent on the quality of data in inferring the origins of brood parasitism (e.g. [42,78]. Existing brood parasite phylogenies could benefit from additional sampling of missing species and molecular markers. For example, [71] used only a small fraction of the mitogenome and no nuclear markers, leaving some of the older branching events and relationships among subfamilies unresolved. Among brood parasites and hosts, ecological data has traditionally been highly biased towards the temperate regions (e.g. [32,91], leaving replicated data on reproductive mode and other life history traits of brood parasite and host taxa in the tropics lagging behind (although this is improving, see [95]).

While we do not yet have data for a full comparative analysis using the PCM across all birds, determining the conditions that facilitated the origins of brood parasitism is within our grasp if we split the question into smaller steps (e.g. [96]. First, PCM can be used to infer trait evolution linked with parasitism within lineages where all extant taxa have been sampled [97], dense sampling). Second, sequencing and assembling whole genomes of non-model species opens up the possibility to use comparative genomics and transcriptomics to investigate the origins of avian brood parasitism and bypass the issue of only seven independent origins. For example, Jackson et al. [98] were able to pinpoint evolutionary innovations associated with becoming a parasite by comparing gene networks across genomes of three species: kinetoplastids, protozoan parasites and their free-living relatives. When more functional annotations of genes involved in parental care become available, it also becomes possible to track the evolution of brood parasitism back in time using extant species. Given this framework, genomic

comparisons of closely related brood parasites and their non-parasitic sister species or even within facultative brood parasite species (e.g. [99]) could also prove informative. While the specific functions and regulatory pathways of genes associated with behavioural traits in higher organisms remain poorly known (Box 1), the first steps towards understanding the molecular basis of behavioural traits related to avian brood parasitism have already been taken. Lynch et al. [100] used transcriptomics to compare gene expression between parasitic and non-parasitic blackbird (Icterid) species, focusing on the preoptic area in the brain previously known to affect maternal behaviour. In species without maternal care, gene expression patterns remained more juvenile-like (i.e. neotenic) in adults. More detailed studies of different functional networks of genes related to brood parasitism and ecological conditions within and between the evolutionary lineages of extant brood parasites are thus likely to illuminate not only the mechanisms, but also the ultimate causes behind becoming an obligate brood parasite.

2) When (and how) should hosts defend?

Given the fitness costs of raising a parasite, explaining why many host species lack defences (e.g. almost 40% of avian brood parasite hosts tested do not remove foreign eggs from their nest [38,101]) remains unresolved. In part, this is because it is challenging to tell from behaviour alone whether a defence trait is yet to evolve (i.e. evolutionary lag, [102]) or is present but there are insufficient cues to elicit its expression (i.e. cryptic plasticity, [103]). Many brood parasites make use of behavioural and phenotypic tactics (figure 2) that increase the costs of host recognition errors to achieve effective parasitism [38,101]. This then, in theory, has selected for plasticity, with some hosts becoming more likely to defend with repeated experience of parasitism (e.g. magpies become more likely to reject cuckoo eggs as they age [104], or in response to environmental cues about local risk (e.g. reed warblers adjust mobbing of adult cuckoos and rejection of eggs based on increased mobbing behaviour of neighbours [105–107]. However, an alternative explanation for a lack of defences is that, instead of preventing successful parasitism events (i.e. ‘resistance’), hosts may mitigate the fitness costs of raising a parasite (‘tolerance’, [108,109]). Tolerance could, for example, include hosts altering their behaviour or adjusting life history strategies by reducing investment in current clutch size to save resources for later broods [110,111]. In other words, hosts without apparent ‘defences’ may actually still be defending their fitness from a brood parasite [112]. Testing whether defences (or tolerance) have not yet evolved (or are cryptic) has, however,

proven challenging [110,111,113]; This is because of two major stumbling blocks: (i) it is difficult to measure lifetime reproductive success and fitness of hosts (but see [104], and (ii) field methods to test for plasticity in host defences are still rarely employed [114] and require careful efforts to rule out cryptic defence traits. Could genomic tools be used to resolve whether the lack of defences in hosts reflects a missing adaptation (or loss of trait due to relaxed selection) or an alternative strategy (i.e. tolerance), given the inherent plasticity of behaviour?

One potential way to overcome the limitations of tracking individual fitness through time in the field could be to use population genetic theory to ask if observed genomic patterns fit one of the evolutionary scenarios (i.e. missing adaptation vs. tolerance). For example, if we have both behavioural and genomic data from one host population from before and after invasion by cuckoos, we could use simulations to compare allele frequency changes to a neutral model without selection (i.e. using coalescent theory Glossary, [25]). Such changes can then be compared to the output of flexible evolutionary simulation frameworks (SLiM, [115]; Nemo, [116]), which would take into account key evolutionary parameters inferred from the populations of interest. In addition, analysing genomic data from behaviourally tested rejector and acceptor individuals from e.g. a recently parasitised population (where selection for rejection is strong) and contrasting results to those of evolutionary simulations could help better quantify the fitness effects of rejection behaviour. However, study systems sampled in at least two time points with a known population history can be difficult to find in the wild (but see [117] for some particularly amenable study systems under range shifts).

Given the difficulties of "finding a gene for behaviour" (Box 1), it is easier, and perhaps also more meaningful, to study the genomic architecture of the trait (number and genomic distribution of loci involved, along with their functional interactions) and its evolution in a phylogenetic context (as in [118]). For example, by focusing on host species with well-developed field methods to detect plastic trait expression (e.g. reed warblers), genomes could be compared between rejector and non-rejector individuals to identify candidate genomic regions associated with egg rejection defences. This could be done with association analyses, which measure the correlations between genotypes and either phenotypic traits (Genome-Wide Association Study, GWAS) or environmental variables (Gene-Environment Association, GEA, [119]). This could also be conducted using FST scans which identify genomic regions of high differentiation between populations from divergent environments or contrasting phenotypes

[120]. We could also generate high-quality reference genomes and compare chromosome-level synteny across host species to identify e.g. candidate inversions associated with behavioural traits [118]. This approach would work best with species that show little to no plasticity in their defences (e.g. Cowbird hosts [121,122], but, together with the intraspecific approach, results could be used in future to develop genomic markers to detect defences in species where either behavioural data is unknown or difficult to obtain, or where they may be cryptic. Identifying the genomic regions underlying defences would also improve our understanding of trait evolution [56]. For example, if egg rejection behaviour is associated with an inversion, then it may be difficult to evolve as it requires large changes in the genome. However, it could arise via introgression from other populations (see Question 3) or during speciation [123]. Discovery of inversions have revolutionised our understanding of the evolution and maintenance of polymorphisms in social behaviour (e.g. fire ant [124]) and ruff mating strategies (e.g. [125]). However, all these approaches would benefit from more phenotypic data, collected across a broader range of species, that takes plasticity into account.

3) Will coevolution persist across time and space with ecological change?

As discussed in Question 2, all potential host species should in theory evolve defences and render parasitism untenable given sufficient time (also see [38]). How is it possible then, that brood parasites have persisted for millions of years (Question 1)? Furthermore, and perhaps most importantly, can we predict what will happen next to hosts and brood parasites, given rapid environmental change? The Geographic mosaic of coevolution theory (GMT, [126,127] provides a compelling framework to answer these questions. First, it explains how antagonistic coevolution can continue for long periods: local environmental variation, population dynamics and demographics, gene flow, mutation and drift combine to produce mosaics of reciprocal selection ('hot spots') and non-reciprocal selection ('cold spots') in time and space (figure 3). The key cold spot that allows parasites to persist is where selection on hosts is sufficiently relaxed that they lose their defences. This then allows parasites to eventually reinvade (although some work suggests that it is variation in the parasite's virulence that determines long term success [128]. Second, insight into the component parts of GMT can better facilitate predictions about adaptive potential and interacting species' resilience to rapid environmental change [129–132]. However, there have been few attempts to test GMT with avian brood parasites and

their hosts [114], despite them being a putative example in the theory's seminal publications [126,127]. Behavioural experiments have revealed spatial correlations between host and parasite traits (e.g. reed warblers vs. common cuckoo: [133–136]; magpies vs. great spotted cuckoo: [137]; prinias and parrotbills vs. common cuckoo: [138,139], providing evidence for hot spots as well as cold spots where parasitism is absent and defences vary, but we lack quantitative estimates of the strength of selection and trait remixing at the genomic level. These estimates are essential [140,141] to explain how coevolutionary interactions persist in time [142] There have been some attempts to quantify gene flow and local allele frequencies of a putative candidate marker for egg rejection in magpies [47], but this is where studies on brood parasitism, and tests of GMT for behavioural coevolution more broadly, have hit a stumbling block.

How can we quantify selection mosaics and trait remixing in avian brood parasite-host systems, given that the heritable mechanisms of coevolved traits remain largely unknown (Introduction)? Here again, combining empirical data with new molecular methods could offer ways forward. Landscape genomics (table 1) uses genomic and environmental data collected across a species distribution, or along an environmental gradient of interest. Neutral markers are used to infer the underlying spatial and genetic population structure, and any remaining markers associated with the environmental variable (e.g. current parasitism rate) are evidence of selection. However, this method has rarely been used to test for associations with biotic variables (e.g. [143]). The space-for-time substitution inherent to landscape genomics [144] could also help solve a major issue: detecting selection usually requires long-term data [145]. However, behavioural studies are rarely replicated in time and space, although recent invasions of brood parasites to new areas and range edge populations provide unique opportunities to observe coevolution in action [117]. Studying spatial variation in egg polymorphism [146] and egg rejection [117] has been suggested to overcome the lack of long-term behavioural datasets, but there have thus far been no attempts to evaluate the genomic consequences of environmentally varying brood parasitism risk in avian hosts.

Complementarily, we could use comparative phylogenetics (Question 1) between different hosts and their parasites to look at host-parasite interactions at varying stages of coevolution [117]. For example, parasitic lineages of *Viduidae* (parasitic whydahs) and *Anomalospiza*

(cuckoo finches), common cuckoos, and cowbirds have been estimated to have existed for 13, 6-8 and 3-4 mya, respectively [147–150], providing considerable variation in the duration of potential coevolutionary interactions. As more molecular data and sophisticated analysis methods become available, this variation could be used to resolve whether brood parasites and hosts evolve at different rates, how brood parasites diverge, and the timing of host specialisation [149–151].

With these landscape and comparative genomic approaches, it becomes possible to directly measure levels of selection, gene flow and trait remixing. In systems where we have detailed knowledge of host and parasite behaviour, demography, and genomics, it could also be possible to move beyond the phenotypic gambit. For example, genome-wide markers have been associated with climate adaptation in yellow warblers, and used to predict future vulnerability to climate change [27]. Yellow warblers, however, are also a common host of the brown-headed cowbird and the expression of host defences and levels of parasitism vary geographically [152]. These data could be used to zoom in and look for the molecular mechanisms underlying behavioural coevolutionary adaptations (Box 1). Molecular resources are also becoming available for the reed warbler [64] to complement the wealth of existing knowledge on geographic variation in defence behaviours [136]. Studying such avian brood parasite and host systems would complement current molecular studies on biotic selection, as examples of behavioural coevolution are few (e.g. ants: [128]), and most studies of the genomic changes associated with defence traits in antagonistic coevolution come from systems in vivo (e.g. [153]). However, accurate predictions of future coevolutionary trajectories requires further development of models that can disentangle genomic patterns caused by processes other than selection (i.e. migration in the molecular sense, mutation, recombination and drift). This is a major goal in population genomics [22,154], and methods are rapidly developing (e.g. [155]. In summary, analysing genomic data along with behavioural data in a brood parasite-host system in the wild could not only test the theory of a geographic mosaic of coevolution using direct genetic evidence but also help study three avenues of inquiry: speciation of hosts and parasites, persistence of antagonistic coevolutionary interactions in time and maintaining resilience to rapid ecological change.

Concluding remarks

Here we have shown that investing in analysing genomic data along with behavioural data in a brood parasite-host system in the wild could lead to advances in understanding the evolutionary origins of behavioural strategies, the fitness outcomes of plastic trait expression, the persistence of antagonistic coevolutionary interactions in time, and how resilience to rapid ecological change may be maintained. These themes are of broad interest beyond avian brood parasitism, and show that genomic tools can be used to find answers to more than mechanistic questions. By applying genomic comparisons at different levels ranging from within individuals to between populations and species, we can also address how behaviour develops or changes through ontogeny, its adaptive value, and its evolutionary past. In other words, genomic tools can be integrated with behaviour to find answers to all of Tinbergen's 'four questions' (see Box 2/Table 1, Outstanding questions).

Although we focussed our review on how genomic tools could be used to answer questions about function, evolutionary history, and plasticity, having a mechanistic understanding of trait heritability (Box 1) would nevertheless be beneficial to further advance many of the approaches we've described here. For example, knowing the "genes for egg rejection" would allow us to track its evolutionary history across taxa (Question 1), make it possible to determine the 'rejector' status directly from the genotype without behavioural testing (i.e. 'reverse ecology', [156], disentangle the heritable and plastic components of trait expression (Question 2), collect direct evidence of selection (Questions 2 & 3), test the theory of a geographic mosaic maintaining coevolutionary trajectories in time (Question 3) and even predict future adaptations [128]. Ideally, the underlying genomic mechanisms of plastic traits would be studied by measuring levels of gene expression during a behavioural response. This popular method can reveal the genes and regulatory mechanisms underlying differences in egg rejection behaviour even if the responses are fully plastic and produced e.g. by post-translational mechanisms from the same genotype, but are currently limited by uncertainty over which tissue (and when) to sample (Box 1). However, field methods could be developed to experimentally induce plasticity to the maximum and rule out cryptic defence traits, and emerging studies on host hormone levels could guide which tissues to target, as hormone receptor expression is a crucial step in the molecular pathways of behavioural responses [157]. Gene expression experiments are, however, usually lethal which is not only problematic ethically but also means we are unable to continue behavioural measurements to probe individual variation and

plasticity. Developing and validating methods using blood samples may provide a useful alternative (e.g. [158]) although the crucial step of functional validation of (plastic) candidate genes (using e.g. CRISPR, see Table 1) remains unlikely for avian brood parasites and their hosts (see Box 1).

Throughout this article we have argued why integrating genomics into behavioural ecology could be beneficial, but behavioural ecologists could also help resolve several outstanding issues in genomics. For example, genomics has been criticised for being data-driven rather than led by hypotheses [159,160], and increasingly reliant on searching for correlations rather than experiments to test causation [161]. At the same time, adopting a Tinbergian approach from behavioural ecology could help move forwards as it integrates mechanisms with development, function and evolutionary history to test hypotheses (see [162] for discussion of how this could revolutionise many fields). Similarly, variation in behaviour is often perceived to be more difficult to measure than physical traits, but the underlying heritability of both is intertwined [163,164]. Behavioural ecologists could bring a deep understanding of how and what to measure, as well as the ecology underlying the trait in question [30], to frame hypotheses appropriately. We now have a plethora of sequencing techniques and massive datasets becoming available. Utilising these data will require appropriate analysis methods that are carefully designed to address questions about polygenic traits with inherent plasticity, something best achieved by forming collaborative teams (Box 2) that make use of our wide range of complementary skill sets.

Outstanding questions

- Examples of other questions regarding avian brood parasitism: Do rates of evolution vary between avian brood parasites and hosts? What are the molecular mechanisms that facilitate rapid changes in egg coloration during evolving host-parasite arms races? How is egg or plumage coloration polymorphism maintained?
- What platforms are needed to best bring behavioural ecologists and molecular ecologists together to better integrate Tinbergens' four questions?
- If we find candidate genes for behaviours, can field-friendly methods be developed to experimentally test gene function?

- When does behavioural plasticity facilitate or hinder adaptation? This will require deeper integration of behavioural experiments with epigenomics.
- Can a richer understanding of behavioural interactions improve heritability estimates in studies of genetic and non-genetic inheritance (or indirect genetic effects)?
- Do new behavioural adaptations occur from selection acting on standing genetic variation, introgression or new mutations?

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Glossary

Bioinformatics - Science of collecting and analysing biological big data (e.g., nucleic sequences, genomes, gene / protein expression, biological networks, single cell data).

Coalescent theory - A population genetics model that allows reconstructing genealogies of all alleles of a gene in a sampled population. Coalescent theory treats genealogies as random providing null models for testing causes of genetic variation.

DNA - The deoxyribonucleic acid (DNA) is a macromolecule consisting of two chains forming a double helix and including four nucleotides: adenine, thymine, cytosine and guanine. The nucleotide sequence contains heritable instructions for the development, function, growth and reproduction for all living organisms (and some viruses).

Epistasis – Phenomenon where the effect of one mutation on a phenotype is dependent on the presence (or absence) of additional mutations in at least one gene.

Evolutionary simulations - Simulations of realistic, individual-based, and genetically explicit evolutionary scenarios using dedicated computer programs (e.g., nemo, SLiM). These programs are highly flexible and allow simulating hundreds of generations of evolution in terms of population genetics and/or life history trait (e.g., host-parasite relationships).

Gene - A philosophical concept of the unit of inheritance, which may often correspond to e.g. a protein-coding DNA sequence. However, it is no longer clear where “a gene” begins or ends in the

genome as the mechanisms of reading DNA have turned out to be various. Analogous to a word describing an ingredient if the genome is the cook-book.

Genetics - Study of genes, genetic variation and their heritable effects on organisms.

Genome - All genetic material of an organism, consisting of DNA (or RNA), and found in the nuclei of (almost) every cell of a multicellular organism. In a sense, contains all heritable information needed to build and operate the organism, i.e. the cook-book for an organism and its functions.

Genomics - Field of biology focusing on the structure, function, and evolution of genomes.

"Next-generation genomics" - A term referring to methods developed since 2005 and which aim at reconstructing the nucleotide sequence (sequencing). "Next-generation genomics" (NGS) differ from traditional Sanger sequencing in that billions of small DNA fragments can be sequenced simultaneously (shotgun sequencing). More descriptive names include massively parallel sequencing or high-throughput sequencing.

"Third generation genomics" - This term refers to methods sequencing very long molecules of DNA (> 10,000 nucleotides) contiguously (such as PacBio [165]), which help assembling reference genomes and identify structural variations such as inversions.

Post-genomics - Era starting after the completion of the Human Genome Project, where life sciences progressed beyond a gene-centred view to better understand genome functions and its evolution.

'Omics' - An informal term for several branches of science in biology aiming to characterize and quantify pools of biological molecules that translate into organisms' structures, functions and dynamics. Examples of omics include genomics, metagenomics, transcriptomics, metabolomics and proteomics or their integration in multiomics.

Gene expression - Process by which the information contained in the gene sequence is used to synthesise a gene product (usually a protein). This can be measured for single genes or across the genome (with RNA-seq, see Table 1 for transcriptomics) from different tissues and/or conditions.

Introgression - Transfer of genetic material between genetically distinct populations (e.g., species) through hybridization.

Inversion - Reversal of the orientation of a section of chromosome. Within the inversion, recombination is suppressed between inverted and non-inverted alleles.

Microsatellite - Tract of DNA made of the repeat of a single motif, composed of few nucleotides. The number of repeats is highly variable (polymorphic) between individuals, and for this reason microsatellite loci have been used as genetic markers since the late eighties.

Plasticity - When the same genotype produces different phenotypes according to environmental conditions.

Pleiotropy – When variation at one gene influences two or more different, seemingly unrelated traits.

Phenotypic gambit - an assumption that the genetic mechanisms underlying behavioural traits can be ignored or modelled as simple biallelic alternative strategies. Instead, one can determine the expected evolutionary dynamics of a population based on fitness of the different phenotypes.

QTL - A quantitative trait locus (QTL) is a section of DNA correlating with variation in a complex trait (for which phenotypic variation is continuously distributed in natural populations, e.g. height).

QTLs are identified (or 'mapped') by measuring the statistical association between genetic markers (such as SNPs, microsatellites) and a trait of interest, usually using crosses between lineages differing in their average trait values.

SNP - A single nucleotide polymorphism (SNP) is a substitution of one base pair at a given position in the genome. Analogous to a letter in a word that is the gene.

Box 1. Behavioural genomics in the wild

Many of the previous calls for behavioural ecologists to adopt genomic tools have focused on using approaches from behavioural genomics [14–16,166]. Here, the goal is to uncover the molecular mechanisms underlying behavioural traits and ideally this requires measuring levels of gene expression during a behavioural response (within the whole organism, organs or single cells; see transcriptomics in Table 1), determining which genes are up- or downregulated compared to when the behaviour is not expressed, then experimentally testing causation by e.g. gene knock-out experiments or crosses (gene editing in Table 1). Most of the methods available, however, were originally developed using model organisms (e.g. humans, *Drosophila*, zebra fish, laboratory mice and rats), and much of the success thus far has come from studying organisms that lend themselves to highly controlled laboratory-based experiments (e.g. great tits, [167]) or where lethal sampling of tissues has fewer ethical concerns (e.g. Heliconius butterflies: [168]). This limits wide-scale adoption to study behavioural traits in wild populations. For example:

quantitative trait locus (QTL) analyses designed to account for polygenic heritability require large sample sizes of behaviourally-phenotyped and pedigreed individuals, coupled with long-term monitoring, which can be difficult to obtain and require significant resources, including long term funding, for organisms in the wild [18].

Top-down approaches such as **genome-wide association studies** (GWAS) are most effective when traits are determined by few genes or 'supergenes' and associations are not masked by environmentally induced plasticity (Glossary, see Table 1 for examples). However, large-effect loci and simple genetic architectures only occasionally explain phenotypic variation (e.g. [18]) and identifying small effect genes contributing to behavioural traits has proven challenging even in humans, despite hundreds of thousands of genomes sequenced [169]. In addition, regardless of the genetic architecture, environmental variation may only affect the presence of alleles in one setting (i.e. conditional neutrality, [170]), or produce an opposing gene expression

pattern in two different environments (i.e. plasticity), meaning that the different phenotypes cancel out the association. GWAS methods are however being developed to account for confounding variation (e.g. naturalGWAS, [171] and RepeatABLE [172].

Bottom-up **gene expression studies** depend on the researchers' ability to induce and measure relevant and consistent behavioural responses (including the control or reference behaviour), and determine the correct timing and location to sample tissue where the genes are expected to be expressed (which can be measured within the whole organism, organs or single cells). This is especially problematic when studying behaviour in the wild as there is substantial uncertainty regarding where and when to sample expression of genes and behaviour [173]) and most tissues require lethal sampling.

Furthermore, the lack of relevant **functional annotation** of genes and knowledge of **regulatory gene networks** is a major hindrance for any genome-wide or gene expression studies in the wild [15,30], although available annotations for markers expressed (or underexpressed) are increasing, e.g. social behaviour in quail [174].

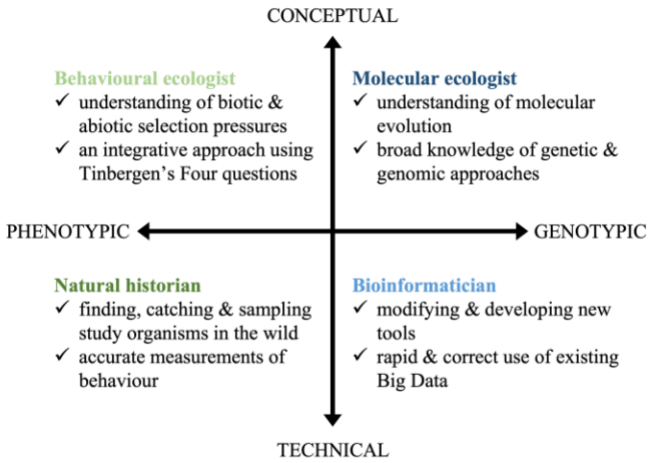
Finally, **gene editing approaches** using methods such as CRISPR-cas9 (Table 1) to experimentally test associations are not feasible (or ethical) with most wild vertebrate study organisms [175] and are still largely a blunt tool to probe complex traits.

More broadly, there is ongoing debate as to whether searching for a 'gene (or genes) for behaviour' is worthwhile (e.g. [33,169]). Detailed studies on the 3D-structures of (human) genomes and epigenomics are revealing that phenotypic traits are often determined by complex regulatory pathways, affecting the timing of expression in networks of tens to hundreds of interacting genes (e.g. epistasis and pleiotropy, Glossary), and even the formation of supergenes is regulated by several genes associated with hormones [176]. These developments have led to a paradigm shift in genomics from focusing on gene sequences, to understanding the regulatory and evolutionary mechanisms occurring at the molecular level [3,6], see e.g. [177] for insights from single-cell genomics). In this review, we therefore outline approaches and methods to be used with wild organisms without the need to 'find a gene for behaviour' to advance the field of behavioural ecology with genomic data and the most appropriate tools that are readily available.

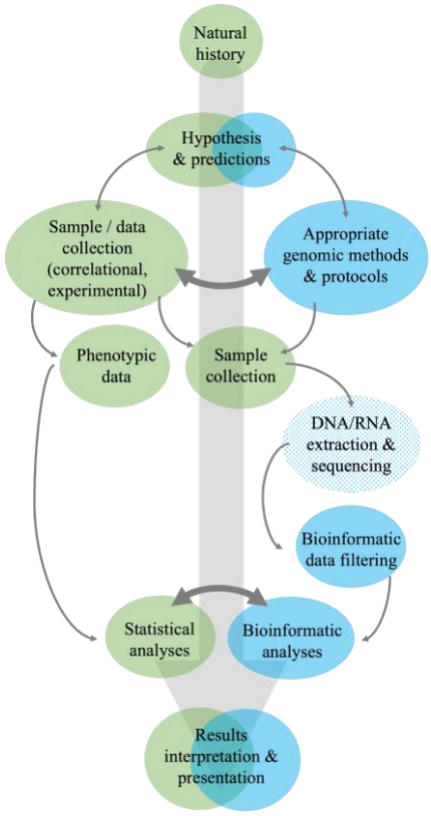
Box 2. Forming collaborative teams to take the field forward?

Behavioural ecology was built on the foundation of Tinbergen's four questions [20]: we can only understand a behavioural trait by investigating its underlying mechanisms, how the trait develops, its evolutionary history, and its function (i.e. effect on fitness). Tinbergen stressed that answers to each question were complementary rather than mutually exclusive, and behavioural ecology has since grown into one of the most integrative fields in biological sciences [178]. While genomic methods could become a useful part of our toolkit to answer aspects of each question, our goal is not to advocate for all behavioural ecologists to become experts in genomics. Neither should molecular ecologists necessarily all become experts in behaviour. Rather, we should form collaborative teams that make use of our wide range of complementary skill sets (Box 2 figure 1): the four specialists represent the different levels of inquiry from conceptual question framing to technical problem solving and move between phenotypic and genotypic approaches. Naturally the number of people does not need to be four - many scientists may sit closer to the centre on both axes and thus bridge the gap between solely phenotypic or genotypic approaches.

(*a*)



(*b*)



Box 2 figure 1. (*a*) Composition of an ideal collaborative team to bridge the gap between molecular and behavioural ecology. (*b*) A hypothetical workflow of behavioural ecologists and natural historians (green) working with molecular ecologists and bioinformaticians (blue), from project conception to completion (large grey arrow). Smaller arrows indicate each step with their width highlighting essential points in the collaboration. Note that DNA/RNA extraction and sequencing (shown as stippled) is likely to be outsourced.

To illustrate the potential benefits of interdisciplinary collaboration here we present a hypothetical workflow (Box2, figure 1b) from planning, data collection and analysis to reporting. Behavioural ecologists ask *hypothesis-driven* questions based on detailed knowledge of *the natural history of the study organism* to account for different selection pressures and confounding factors. However, jumping to the genomic era will require detailed knowledge of the *available methods and the theory* behind them to enable efficient communication between fields. Collecting *accurate behavioural data* and analysing it in a meaningful way, or even finding and sampling *enough individuals* in their natural habitats requires field skills. DNA extraction and sequencing can often be outsourced, yet the subsequent preprocessing of the massive genomic data and *choosing appropriate methods* to draw biologically meaningful inference from it requires knowledge of *bioinformatics*. Finally, collaborative discussions throughout the process will aid the team in reporting their results and conclusions in a precise but understandable way to the benefit of wider audiences than may be currently interested in behavioural evolution.

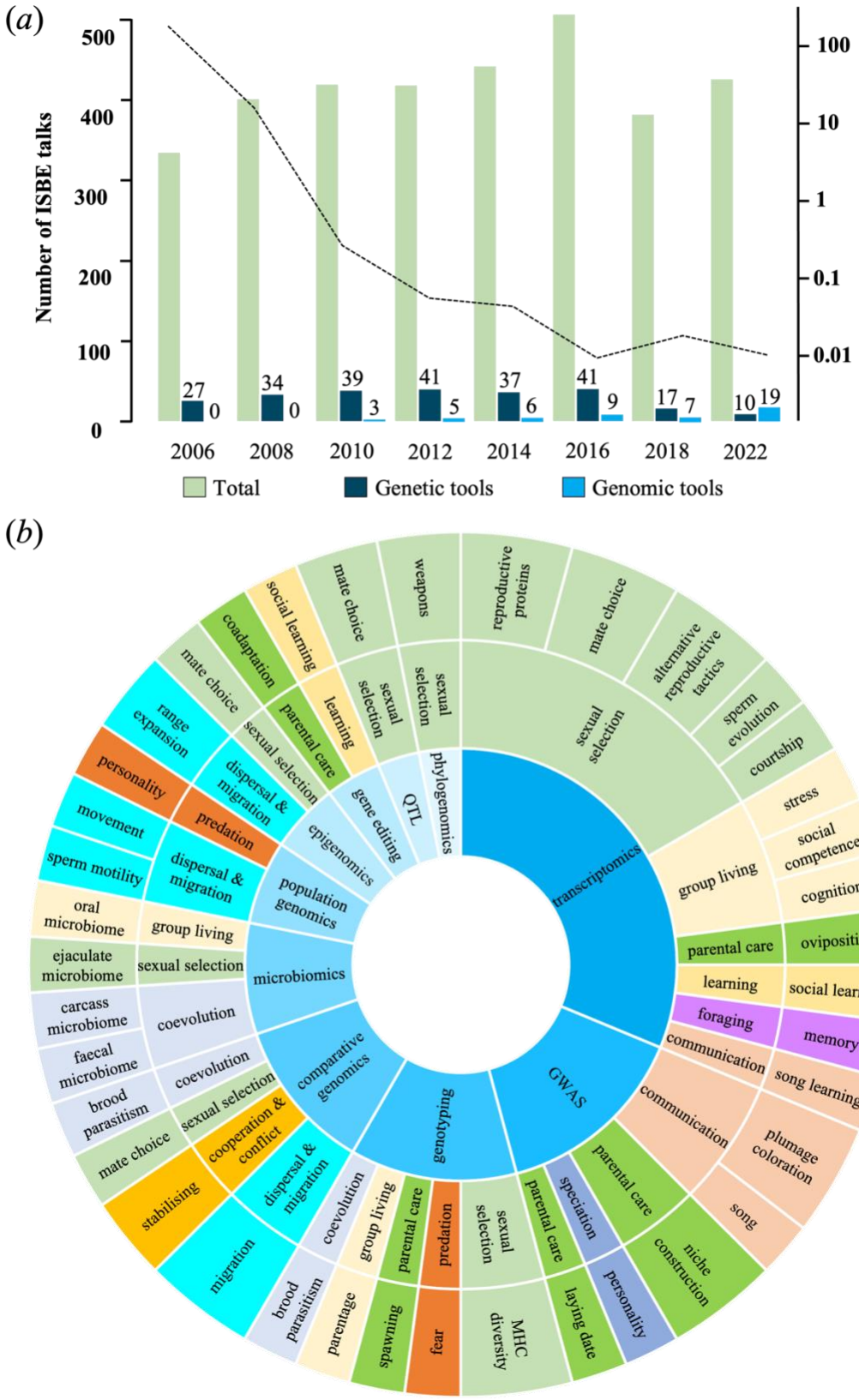
633 Table 1. Genomic tools to study behaviour.
634
635 These include common methods and -omics approaches already utilised in behavioural
636 ecology (see figure 1). Example studies include behavioural traits but are not necessarily
637 from behavioural ecology. Suggestions for further reading provide broad overviews.

638

Tool	Definition	Goal	Example studies	Further reading
Genotyping	Determining differences in individual genotypes	Identifying DNA markers for defining biological populations, parentage, breeding or tracking disease	[179] and [180] for great tit HapMap projects.	[181]
Quantitative genomics	Statistical analysis of quantitative trait loci (QTL)	Inferring the genetic basis and heritability of polygenic traits	[182] found a simple oligogenetic basis for mate recognition in *Heliconius* butterflies	[183]
Microbiomics	Study of the microbiome, which is the community of micro-organisms (e.g. bacteria, protozoa, fungi and viruses) inside a given habitat (e.g. body part, organism or environmental sample)	Understanding the factors causing variation in microbiomes and how the microbiome interacts with its environment	[184] for burying beetles regulating carcass microbiota for their offspring, [185] for factors affecting wild bird microbiota.	[186]
Phylogenomics	Study of evolutionary relationships using phylogenetic inference drawn from genomic data	Testing evolutionary hypotheses either by sequence-based methods or whole-genome features	[94] for evolution of cooperative breeding in birds. For other examples see Question 1	[187]

Comparative genomics	Comparisons of the genomic features of organisms	Understanding heritability and genomic evolution across organisms	[188] to investigate genes related to tool use in corvids	[189]
Transcriptomics	Study of all ribonucleic acid RNA in a cell	Detecting genes and defining regulatory pathways underpinning trait expression	[190] aggressive host responses to cowbirds	[177,191,192]
Epigenomics	Study of epigenetic processes in the genome, such as methylation and altered expression rates.	Understanding variation in behaviour that is not determined by genotype	[193] parent exposure to predation alters offspring mating behaviour in three-spined sticklebacks.	[4] for discussion on evolutionary implications and twin studies.
GWAS	Genome-wide studies of associations between genetic markers or functional gene networks and a phenotypic trait	Detecting relevant loci to study molecular mechanisms underlying phenotypes of interest	Supergene determining behavioural lekking strategies in the ruff [194] and [195] for resistance genes in Daphia against a bacterial pathogen	[196]
Gene editing	Manipulation of the genome by deleting, inserting, or replacing a gene sequence	Producing transgenic individuals to test gene functions	[197] used RNAi to knock-out genes in earwigs to study parental care. [198] used CRISPR/CAS9 to knock-out genes associated with social behaviour in raider ants.	[199–201]
Population genomics	Study of evolutionary processes at the	Understanding populations, microevolution,	[202] for population structure across	[203], [204]

	population level with genomic concepts and technologies	demography and phylogenetic history	elevations in Mountain chickadees	
Landscape genomics	Study of environmental variables associated with genetic adaptation to those variables	Demonstrating natural selection, studying spatial variation in adaptation	[205] used Gene-Environment Association analyses (GEA) to detect signs of selection during a range expansion in a damselfly, and Question 3	[144]


(a)
Number of ISBE talks
500
400
300
200
100
0
100
10
1
0.1
0.01
27 0
34 0
39 3
41 5
37 6
41 9
17 7
10 19
2006
2008
2010
2012
2014
2016
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Total
Genetic tools
Genomic tools
(b)
transcriptomics
GWAS
genotyping
comparative genomics
microbiomics
population genomics
epigenomics
gene editing
QTL
phylogenomics
sexual selection
reproductive proteins
mate choice
alternative reproductive tactics
sperm evolution
courtship
weapons
mate choice
sexual selection
sexual selection
social learning
learning
coadaptation
parental care
mate choice
sexual selection
range expansion
personality
movement
sperm motility
dispersal & migration
predation
dispersal & migration
group living
stress
social competence
cognition
parental care
oviposition
learning
social learning
foraging
memory
communication
song learning
communication
plumage coloration
song
parental care
niche construction
personality
laying date
MHC diversity
sexual selection
parental care
speciation
parental care
predation
fear
spawning
parentage
brood parasitism
migration
coevolution
group living
dispersal & migration
cooperation & conflict
sexual selection
coevolution
coevolution
brood parasitism
faecal microbiome
carcass microbiome
sexual selection
ejaculate microbiome
group living
oral microbiome
dispersal & migration
mate choice
stabilising

Figure 1.

The use of genomic (i.e. next-generation and third-generation methods using high-throughput sequencing) and genetic (any non-genomic method utilising DNA or RNA) tools in behavioural ecology using (*a, b*) abstracts from International Society for Behavioural Ecology (ISBE) conference talks. Despite a dramatic reduction in sequencing costs (dashed line), genomic tools are used rarely in behavioural ecology (*a*), although a range of methods and approaches are applied across a wide range of topics (*b*). The titles and abstracts of accepted talks in ISBE conferences between 2006 and 2022 were checked manually from abstract booklets for mentioning usage of "genomic methods" (i.e. next generation, high throughput, deep or RAD sequencing, *omics*, transcriptom* or RNA-sequencing). Talks mentioning usage of molecular methods (or DNA or RNA) that were not genomic were counted as using "genetic methods". Only regular talks with abstracts in the programs were included, leaving out plenaries, keynote speakers and cancelled talks from ISBE2022 from the total number of accepted talks. Only articles clearly stating the genomic methods used were included to make figure 1b. The included ISBE talks with abstracts are listed in the Supplementary Table 1.

Figure 2.

Examples of the arms races between avian brood parasites and their hosts. Row 1: virulence (*a*) low - bronzed cowbird with Bewick wren host chicks, (*b*) high - common cuckoo removing reed warbler's egg (*c*) mutualism - great spotted cuckoo and crow host; Row 2: defences (*d*) mobbing, (*e*) egg rejection, (*f*) chick rejection; Row 3: mimicry (*g*) hawk mimicry by adult *Cuculus* cuckoos, (*h*) *Prinia* egg mimicry by cuckoo finch, (*i*) host chick (left) mimicry by *Chalcites* cuckoo species (right).

Image credits:
(A) Rolf Nussbaumer / Alamy Stock Photo, (B) Richard Nicoll, (C) Vittorio Baglione
(D) Alan McFadyen / Scottishphotographyhides.com, (E) Oldrich Mikulica
(F) Alfredo Attisano, (G) left panel: Frans Lemmens/Alamy Stock Photo; right panel, Mike Lane/Alamy Stock Photo, (H) Claire Spottiswoode, (I) Naomi Langmore

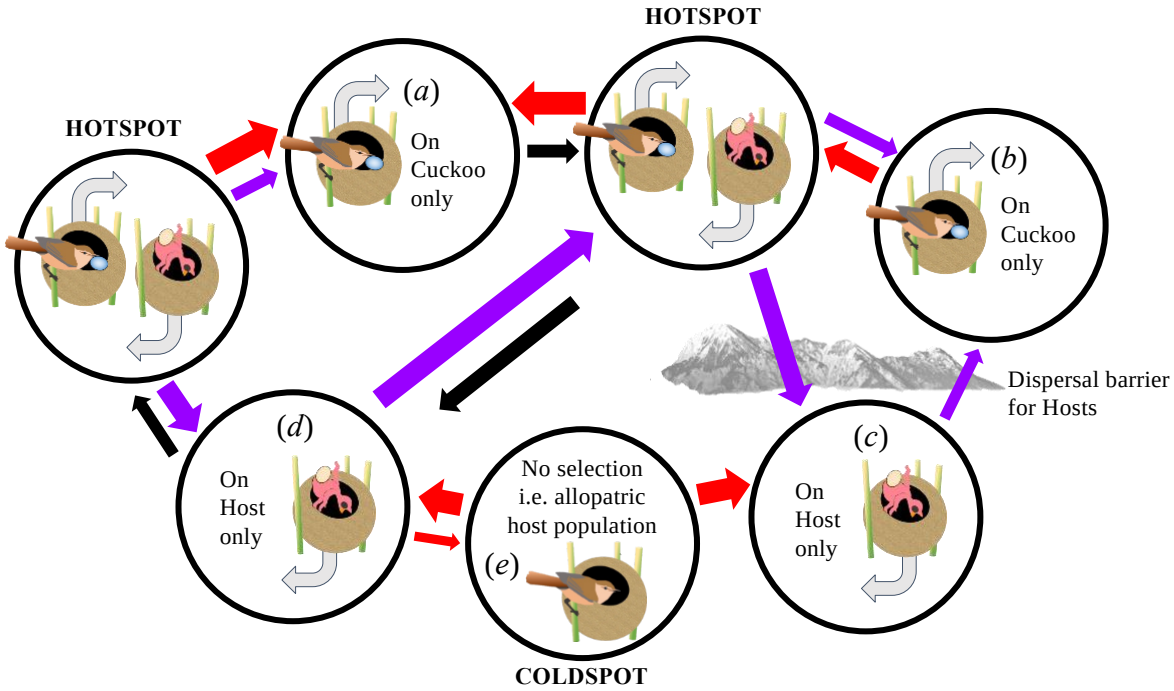

Figure 3.

A hypothetical geographic mosaic of coevolution between a brood parasitic cuckoo and a warbler host (key components in bold text, modified from [127]: Circles represent locations where **selection varies** and arrows between circles describe the direction of **gene flow** (red is host, purple is cuckoo, and black represents similar gene flow of both). Arrow thickness indicates **trait remixing** essential for maintaining genetic variation: if gene flow is absent then local fixation of alleles increases, whereas high levels of gene flow cancel out local adaptation. Reciprocal selection occurs in 'hotspots' and non-reciprocal selection occurs in 'coldspots'; these vary according to local population dynamics e.g. (*a*) strong gene flow of experienced hosts or (*b*) locally fixed host defences exert stronger selection on local cuckoos, or (*c*) a recently invaded host population lacks defences or (*d*) 'spill over' of cuckoos from another host species exerts stronger selection on hosts. Coldspots also arise when (*e*) hosts and cuckoos do not co-occur (i.e. relaxed selection). Behaviour could influence each component: local environmental conditions determine the relative fitness of expressing or retaining behavioural defences (especially when plastic) when parasitism is low (i.e. strength of selection), movement of behavioural phenotypes is unlikely to be homogenous (i.e. affecting direction and specific genotypes during gene flow), and both defences and population dynamics can depend on the behaviour of others' phenotypes/genotypes (i.e. indirect genetic effects).

Supplementary material

Table S1

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