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Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood

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Blood-feeding blackflies (Diptera: Simuliidae) transmit pathogens, harass vertebrate hosts and may cause lethal injuries in attacked victims, but with traditional methods it has proved difficult to identify their hosts. By matching mitochondrial DNA (mtDNA) sequences in blood collected from engorged blackflies with stored sequences in the GenBank database, relationships between 17 blackfly species and 25 species of vertebrate hosts were revealed. Our results demonstrate a predominance of large hosts and marked discrimination between blackflies using either avian or mammalian hosts. Such information is of vital interest in studies of disease transmission, coevolutionary relationships, population ecology and wildlife management.

Keywords: blood feeding; parasite–host webs; land–water interactions; claw morphology

1. INTRODUCTION

Blackflies (Diptera: Simuliidae) are found from arctic to tropical ecosystems, where they have a significant economic impact on humans and animal production, and may reduce the fitness of wildlife (Crosskey 1990; Adler *et al.* 2004). About 1800 species of blackflies are recognized worldwide (Crosskey 2002), and they often reach very high abundances (Wotton 1988) suggesting that their impact on wild animals in terrestrial landscapes is significant. Blackfly larvae develop exclusively in running waters and the annual number of blood-sucking blackflies that emerge from large, unregulated boreal rivers is huge, possibly in the range of billions of individuals per km of river. Therefore, investigating their impact on birds and mammals, including blackfly species composition and host choice, is important to describe the landscape-level interactions between an aquatic ecosystem and its terrestrial surroundings (Polis *et al.* 1997; Malmqvist 2002). Ecological processes operating across aquatic–terrestrial boundaries could be disrupted, for example, by large-scale disturbances such as river regulation and pest control.

Blackflies and hosts have traditionally been linked by using various serological methods (Simmons *et al.* 1989; Hunter & Bayly 1991), or by exposing potential hosts in cages *in situ* (Hunter *et al.* 1993); these methods, however, are not suitable for wholesale investigation of host choice for any blood-feeding insect in the wild. With the advent of novel molecular techniques, better methods of studying blackfly host interactions have become available (Boakye *et al.* 1999; Mukabana *et al.* 2002). We identified vertebrate hosts of blackflies collected in the field by extracting DNA from the blood in the midgut of engorged females, and sequencing a portion of the cytochrome *b* gene obtained using standard vertebrate universal primers.

2. MATERIAL AND METHODS

Sampling of blackflies in the landscape was performed using a trap (0.9 m × 0.5 m, mesh 1 mm) mounted on the roof of a car driven on roads along the following major rivers in northern Sweden: the Ume, Vindel, Skellefte, Pite, Lule, Kalix and Torne Rivers (latitude: 64–66 °N). Data derived from sampling performed in June through August 1999–2002. Blackflies are somewhat reluctant fliers after a blood meal; however, about 3 per 1000 females caught contained blood (a study in Ontario, Canada, yielded fewer than 1 per 1000 flies in 60 days of truck trapping (Hunter & Bayly 1991), but another from Alberta reported frequencies much higher than ours (Shemanchuk 1987)). Host body weights (averages of reported maximum and minimum values) were taken from the literature (Siivonen 1968; Cramp 1994). A regional account of bird densities is available (Olsson & Wiklund 1999), but corresponding data for mammal populations in this region do not exist. Hence, the analysis in relation to host abundance was restricted to birds. Collected blackflies were fixed immediately in 70% ethanol. Identifications were performed in the laboratory, using a key modified from a book on North American blackflies (Adler *et al.* 2004). In our material, females of five species combinations were morphologically inseparable (table 1). Additional species were collected but no engorged females were among these.

Total DNA was extracted from the abdomen of blackflies, using standard proteinase k digestion and phenol chloroform purification. We used the primers L14841 and H15149 (Kocher *et al.* 1989) to amplify a 305 bp segment of the cytochrome *b* gene (excluding primers) from the host DNA (these vertebrate-universal primers do not amplify blackfly DNA present in abdominal tissue). The PCR reactions were performed in 25 µl total volumes, including 25 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM $MgCl_2$, 1× PCR buffer (Perkin Elmer), 0.6 µM of each primer and 0.5 units of Platinum Taq DNA polymerase (Invitrogen). We tested a total of 230 samples of which almost 90% yielded a PCR product that we could sequence. Amplified fragments were sequenced directly with the primer H15149 (Big Dye) and loaded on an ABI PRISM 310 (Applied Biosystem). To identify the host species, the obtained sequences were compared with deposited sequences in the GenBank database using standard nucleotide BLAST searches. We found matching sequences for all samples except six that gave the closest fit to *Turdus* species with distribution ranges confined to East Asia and North America. To solve the identity of these host species, we sequenced the partial cytochrome *b* gene from the six species of *Turdus* that occur in the area of sampling.

The primers for the cytochrome *b* gene used here easily amplify from human material. We therefore took the following precaution to exclude the possibility that the obtained human sequences resulted from contamination of human DNA, either from persons collecting the insects or working with the material in the laboratory. We typed all of the samples for which we obtained human cytochrome *b* sequences along with DNA samples from experimenters BM, DS, OH and SB for two polymorphic microsatellite loci, GATA44 (GenBank G08546) and Mfd23 (Weber *et al.* 1990). We could exclude two samples that had matching alleles with one of the authors. For 12 samples we could not amplify any of the microsatellites, and we therefore tested them again for the cytochrome *b* gene. The re-amplification failed in seven of these cases, and these were excluded in the analyses because the first positive reactions might have resulted from temporary contamination.

Host specificity was assessed for each blackfly species (minimum $n = 9$) using the Shannon diversity index (Magurran 1988): $H' = -\Sigma\, p_i \ln p_i$, where p_i is the proportional abundance of the ith species. This index decreases with increasing host specialization.

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Table 1. Blackfly species with identified hosts, claw morphology and host specificity.
(Values in brackets denote the number of observations.)

blackfly species[a]	*n*	mammal hosts	bird hosts[c]	claw morphology[b]	host specificity (Shannon index)[c]
Metacnephia lyra (Lundstr.)	21	*Homo sapiens* (1), human	*Bonasia bonasia* (2), hazel hen *Fringilla montifringilla* (1), brambling *Grus grus* (1), common crane *Lagopus lagopus* (1), willow grouse *Numenius arquata* (1), curlew *Phylloscopus trochilus* (2), willow warbler *Tetrao tetrix* (4), black grouse *Tetrao urogallus* (6), capercaille *Turdus pilaris* (2), fieldfare	B	2.07
M. saileri (Stone)	1		*P. trochilus* (1), willow warbler	B	
Simulium annulus (Lundstr.)	5	*H. sapiens* (1), human	*Grus grus* (3), common crane *P. trochilus* (1), willow warbler	B	
S. curvans (Rubts. & Carlss.)	3		*P. trochilus* (1), willow warbler *Tetrao tetrix* (1), black grouse *T. urogallus* (1), capercaille	B	
S. curvistylus Rubts.	32	*Alces alces* (25), moose *Bos taurus* (4), domestic cattle *Equus caballus* (1), horse *H. sapiens* (1), human *Rangifer tarandus* (1), reindeer		S	0.78
S. dogieli (Rubts.)	3	*H. sapiens* (1), human	*Anas crecca* (1), teal duck *Bucephala clangula* (1), goldeneye duck	B	
S. intermedium Roubaud	1	*A. alces* (1), moose		S	
S. murmanum Enderl.	9	*A. alces* (9), moose		S	0.00
S. noelleri Frieder.	1	*A. alces* (1), moose		S	
S. ornatum Meig.	3	*A. alces* (1), moose *R. tarandus* (2), reindeer		S	
S. reptans (L.)	28	*A. alces* (22), moose *B. taurus* (1), domestic cattle *Capra hirtus* (1), common goat *E. caballus* (4), horse		S	0.71
S. rostratum (Lundstr.)	9	*A. alces* (7), moose *H. sapiens* (1), human *Sus scrofa* (1), domestic pig		S	0.68
S. subpusillum Rubts.	10	*A. alces* (8), moose *Microtus agrestis* (1), field vole	*T. urogallus* (1), capercaille	S	0.64
S. silvestre (Rubts.)	6 B	*H. sapiens* (2), human	*Turdus philomelus* (2), song thrush *Turdus viscivorus* (2), mistle thrush		
S. transiens Rubts.	29	*B. taurus* (1), domestic cattle *H. sapiens* (1), human	*Bo. bonasia* (2), hazel hen *P. trochilus* (5), willow warbler *T. tetrix* (6), black grouse *T. urogallus* (14), capercaille	B	1.40
S. truncatum (Lundstr.)	13	*A. alces* (12), moose *R. tarandus* (1), reindeer		S	0.27
S. tuberosum (Lundstr.)	26	*A. alces* (1), moose *Arvicola terrestris* (4), water vole *Clethrionomys glareolus* (3), bank vole *Clethrionomys rufocanus* (3), grey red-backed vole *Microtus agrestis* (9), field vole *Sciurus vulgaris* (6), red squirrel		S	1.62

[a] Females of the following five species combinations were morphologically inseparable: *S. dogieli*/*rendalense*, *S. pusillum*/*subpusillum*, *S. annulitarsis*/*tuberosum*/*vulgare*, *S. paramorsitans*/*posticatum*/*truncatum* and *S. curvistylus*/*morsitans*/*rubtzovi*. For readability, only the name of the species most commonly found is given, based on known distribution data of larvae, pupae and males (Adler *et al.* 1999).
[b] Claw morphology is indicated as B representing bifid or S representing simple.
[c] Host specialization is indicated in terms of Shannon's index; only species with nine or more tested individuals are included.

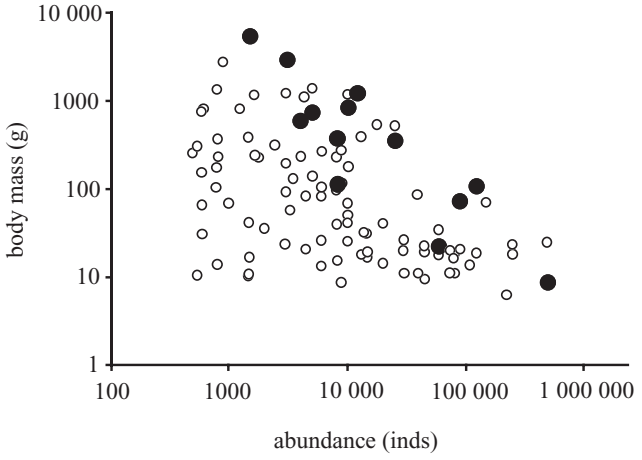

Figure 1. Plot of body weights versus abundances of bird species (number of individuals, 'inds') in the region of investigation. Species identified from blood in engorged blackflies are indicated by filled circles, others with open circles. Marine species and species with 250 or less breeding pairs are excluded.

3. RESULTS

Our material consisted of 200 engorged female blackflies of 17 species, and these flies had blood meals from 25 vertebrate species (table 1). Clear patterns emerged. First, blackflies could be separated into either mammalophilic or ornithophilic species. This observation also served as a test of the hypothesis that the design of the female claw reflects host type, with simple claws in mammalophilic species and bifid claws in ornithophilic species, the latter design presumably facilitating movement among feathers (Crosskey 1990). Our observations were perfectly consistent with the hypothesis (table 1). Further, the degree of specialization varied, with some species being highly specialized. Mammalophilic species, on average, were more host-specific than ornithophilic species. Moose (*Alces alces*) was the most common host for eight blackfly species, underpinning its importance as a source of blood. By contrast, *Simulium tuberosum* clearly is a rodent specialist, with squirrels and four species of voles as hosts in 25 out of 26 specimens. In four ornithophilic blackfly species, several kinds of grouse (black grouse (*Tetrao tetrix*) and willow grouse (*Lagopus lagopus*), capercaille (*Tetrao urogallus*) and hazel hen (*Bonasia bonasia*)) played a dominant role, similar to that of moose for mammalophilic species. *Simulium annulus* fed principally on cranes, *Simulium dogieli* on ducks (goldeneye (*Bucephala clangula*) and teal (*Anas crecca*)), and *Simulium silvestre* on true thrushes (*Turdus* spp.).

A second pattern was that most blackflies showed a marked preference for large hosts. If a host species was common this also increased the probability of attacks. Thus, considering bird hosts, large and widespread species were selected (figure 1). In mammals, for which abundance data were not available, the use of large hosts was apparent (except by *S. tuberosum*), with host median weight being 95 kg versus 0.245 kg for the entire range of mammals in the area.

4. DISCUSSION

Host choice is likely to be based on visual, olfactory and thermal cues, providing the blackflies with information about host location and type (Sutcliffe 1986), but may not always lead to specialization in a strict sense. For example, blackflies may feed in a particular habitat such as the forest canopy or lakeshore, with habitat taking precedence over the species of host (Bennett 1960). Both the pattern presented in figure 1 and the specificity in terms of Shannon diversity index (table 1) suggest that the choice of large or common bird hosts depends on the probability of encounters. Recent studies on host specialization in insects suggest that ecological host attributes, such as microhabitat, phenology and host-finding constraints, may be decisive for host preference (Janz & Nylin 1997; Tompkins & Clayton 1999; Stireman & Singer 2003). Since the most profitable hosts may be rare, acceptance of those of lower rank may take place, which would also lead to the inclusion of a broader range of hosts (Jaenike 1990).

Although the importance of host size has previously been observed (Anderson & DeFoliart 1961), the pattern of size and abundance that we observed makes it possible to predict other potential host species. Thus, expected but not yet documented hosts include chaffinch (*Fringilla coelebs*), European robin (*Erithacus rubecula*), tree pipit (*Anthus trivialis*), wood pigeon (*Columba palumbus*), crow (*Corvus corone*), raven (*Corvus corax*), mallard (*Anas platyrhynchos*), goosander (*Mergus merganser*), red-breasted goosander (*Mergus serrator*) and common gull (*Larus canus*). Alternatively, these species may be attacked more rarely owing to less overlap with blackflies or more effective defence mechanisms.

Our results demonstrate that host identification has been improved not only by using mtDNA in blood, but also by the availability and striking completeness of cytochrome *b* sequences in public gene databases.

By identifying the hosts of large-river blackflies we can predict that river regulation, through the destruction of larval habitats, decreases blackfly impact on birds in general and on grouse specifically by greatly reducing the populations of *Metacnephia lyra*. This species is characteristic of free-flowing large rivers in northern Scandinavia (Malmqvist 1999) and the major pest species in northern Finland on penned black grouse (Ojanen *et al.* 2002). Other mass-occurring blackflies of large, free-flowing rivers are *Simulium transiens*, which attacks grouse species, especially capercaillie, and *Simulium murmanum*, *Simulium reptans* and *Simulium subpusillum*, which are major blood suckers of moose in this study. Thus, the impact of river regulation might have repercussions for host populations by reducing large-river blackfly populations, a hypothesis that could be tested in comparative studies along free-flowing and regulated rivers. Effects on birds might not be entirely negative because insectivores should be favoured by mass emergence of blackflies.

The use of molecular techniques to identify host use of blackflies and other biting flies offers tremendous potential for revealing not only hosts but also vector potential. Future studies might focus on the spread of blood parasites for which blackflies are vectors, including species of the haemosporidian *Leucocytozoon* that are exclusively transmitted by blackflies (Valkiunas 1997), and linking

searching behaviour with host choice, using the molecular technique outlined here.

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