Ticks need not bite their red grouse hosts to infect them with louping ill virus

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For pathogens transmitted by biting vectors, one of the fundamental assumptions is often that vector bites are the sole or main route of host infection. Here, we demonstrate experimentally a transmission route whereby hosts (red grouse, *Lagopus lagopus scoticus*) became infected with a member of the tick-borne encephalitis virus complex, louping ill virus, after eating the infected tick vector. Furthermore, we estimated from field observations that this mode of infection could account for 73–98% of all virus infections in wild red grouse in their first season. This has potential implications for the understanding of other biting vector-borne pathogens where hosts may ingest vectors through foraging or grooming.

1. INTRODUCTION

Vector-borne diseases are of major health and economic importance and can have key roles in regulating biodiversity (Daszak *et al.* 2000; Malakoff 2002). They include many of the virulent zoonotic diseases that are currently emerging or re-emerging such as West Nile virus, tick-borne encephalitis (TBE) and Lyme borreliosis (Randolph 2001; Malakoff 2002). For such diseases, where vectors bite hosts, a common assumption is that vector bites are the sole or main route of host infection (Gilbert *et al.* 2000). To develop methods for the control of these diseases it is essential to fully understand the modes of infection of the host and vector, and if the assumptions are inaccurate, this could have important implications.

Louping ill virus (LIV) is part of the TBE virus complex and occurs mostly in the UK and Ireland where its main impact is in causing mortality and morbidity in livestock and wildlife. For example, LIV can cause 78% mortality in experimentally infected red grouse (*Lagopus lagopus scoticus*), an economically important game bird (Reid 1975). The sheep tick, *Ixodes ricinus*, vector has three life stages (larvae, nymphs and adults) that each takes one 4–7 day blood meal from each host. Unfed larvae are always uninfected since transovarial transmission is not known in LIV (Gaunt 1997), and adult ticks prefer mammalian hosts, such as hares, deer or sheep, and rarely bite birds. This has led to the assumption that only biting nymphs can infect red grouse with LIV (Gilbert *et al.* 2000).

While adult red grouse feed predominantly on heather (*Calluna vulgaris* L.), the chicks feed on slow-moving invertebrates including ticks during their first three weeks of life (Hudson 1986; Park *et al.* 2001). Therefore, the aim of this study was to use laboratory experiments to test whether hosts can become infected with virus by eating the vector. We then estimated the importance of this novel infection route to wild hosts using two different methods based on field data from two wild grouse populations. First, we compared the frequency that grouse chicks ingest ticks with the number of ticks biting them. Second, we estimated the discrepancy between the actual LIV seroprevalence observed in grouse with that expected if infection arose solely from tick bites.

2. MATERIAL AND METHODS

(a) *Feeding experiment*

Under licence from the UK Home Office, we hand fed eight grouse chicks with 100 μl LIV-infected suckling mouse brain (SMB; mean titre 7.0 log pfu (plaque-forming units) ml^{-1}), eight chicks with six infected, unfed, adult female sheep ticks (mean titre 2.3 log pfu per tick) and four chicks with six uninfected ticks as a control. All chicks were aged 18–20 days and force fed the material in solution with a syringe to ensure complete ingestion. Blood samples were taken on days 1 and 4 post-treatment and virus neutralization assays were undertaken using pig stable (PS) cells as previously described (Davies *et al.* 1986; Jones *et al.* 1997). To ascertain that chicks infected in this way can transmit LIV back to biting ticks, we infested four chicks from each treatment group with 20 uninfected nymphs and assayed those nymphs that fed (see Jones *et al.* 1997).

Ticks and virus were propagated as described previously (Jones *et al.* 1988, 1997). Briefly, the virus preparation was originally isolated from a fed female sheep tick using PS kidney cell monolayers, and subsequent virus stocks were developed by passage in SMB. Infected adult ticks came from nymph instars that had been inoculated intra-coelomically with 1.0 μl of LIV (estimated 5.0 log pfu per nymph).

(b) *Method 1: a comparison of ticks in droppings with ticks biting chicks*

To assess the relative importance of the oral infection route in wild hosts, we first compared the frequency that wild grouse chicks ingest ticks with the frequency of ticks biting them.

During June 1994–1997 in a population in Inverness-shire, Scotland, grouse chicks aged 3–16 days were caught after locating broods with pointer dogs. Chicks were meticulously examined for biting ticks, which were readily visible and always found on the head, especially around the eyes, ears and gape. Droppings, excreted during handling the chicks, were examined under a microscope for nymphs or adult ticks. We omitted larvae because unfed larvae are not infected, and they were too small for accurate counting in faeces. Hence, our estimate of the infection probability from the oral route may be conservative.

The probability, X, that a grouse will become infected during its first season can be estimated from the equation $X = TVPE$, where T is the number of potentially infective ticks (nymphs and adults) biting or being ingested per day; V is the virus prevalence in nymphs; P is the probability that an infected tick (biting or ingested) will cause infection in the host; and E is the period the grouse is exposed to potential infection. The parameter values were estimated as follows.

For the oral infection route, $T = ND$, where N is the number of potentially infective ticks (nymphs and adults) per dropping (see table 1) and D is the number of droppings per grouse per day. To estimate D, eight 24–28 day old grouse chicks were kept in a bare-floored

Table 1. *Estimating the probability, X, of a chick becoming infected with LIV in its first season from the oral versus tick-bite infection routes, by comparing ticks in faeces with ticks biting grouse (see § 2b).*

($X = NVPED$ for the oral route (grouse ingesting ticks) and $X = NVPE/A$ for the tick-bite route (ticks biting grouse). Values are means ($\pm$s.e.).)

route of host infection	grouse ingesting ticks	ticks biting grouse
sample size (number of grouse)	22	306
nymphs + adult ticks	0.182 ($\pm$0.142)	0.255 ($\pm$0.044)
log (nymphs + adults + 1), N	0.041 ($\pm$0.030)	0.059 ($\pm$0.009)
droppings per day per chick, D	29.92 ($\pm$2.95)	—
attachment period (days), A	—	5
nymph virus prevalence, V	0.003	0.003
probability of infection, P	0.109	1
period of exposure (days), E	21	90
$X = NVPED$ or $NVPE/A$	0.0084 ($\pm$0.0062)	0.0032 ($\pm$0.0005)
X (back-transformed)	0.0196 ($\pm$0.0143)	0.0074 ($\pm$0.0012)

enclosure for exactly 24 h. They produced 91.25 pellets per chick. Grouse droppings comprise discrete pellets, so to estimate the number of pellets per dropping, we collected additional droppings while handling 38 chicks between 6 and 20 days of age in 2001. These produced 3.25 ($\pm$0.32 s.e.) pellets per dropping. Assuming that 24–28-day-old chicks excrete at similar rates to 6–20-day-old chicks, we estimated that $D = 29.92$ ($\pm$2.95 s.e.) droppings per day. For the conventional tick-biting route, $T = N/A$, where N is the number of attached nymphs counted per chick (table 1) and the period of nymph attachment, A, is 5 days.

V, the virus prevalence in nymphs was estimated as 0.003 (Gaunt 1997).

P is the probability that one tick infects a host. Therefore, $(1 - P)$ is the probability of a chick not being infected by one tick. In our feeding experiment, each of eight chicks was fed six infected ticks, so the probability of not being infected by six ticks is $(1 - P)^6$. The proportion of chicks that became infected was 0.5. If $(1 - P)^6 = 0.5$, then $P = 0.109$.

The exposure period to potential infection, E, is different for the two modes of host infection. As grouse chicks feed on invertebrates mainly during their first three weeks (Hudson 1986), we estimate $E = 21$ days for the ingestion route. For the conventional tick-biting infection route, the exposure time is from early June when chicks hatch, to August/September when grouse are shot and blood sampled and questing nymph numbers decline (Lees & Milne 1951; Steele & Randolph 1985). Therefore we estimate $E = 90$ days for the tick-bite infection route.

(c) *Method 2: a comparison of the observed seroprevalence with that expected from ticks biting grouse*

Using a grouse population in Morayshire, Scotland, we estimated the discrepancy between the actual LIV seroprevalence observed in young grouse with that expected if infection arose solely from tick bites.

Biting ticks were counted on 6–20-day-old grouse caught during June 1993–1995 (table 2). From the same population we collected blood samples from three-month-old red grouse shot in August/September 1993–1995 and carried out standard haemagglutination inhibition antibody tests on the sera (Clark & Casals 1958). The seroprevalence was calculated as the proportion of all samples that tested positive to LIV antibody.

Taking into account virus-induced grouse mortality, we calculated the predicted seroprevalence, X_e, expected if host infections arose solely from nymphs biting grouse, from $X_e = PR/(PR + (1 - P))$. R is the probability of recovering from infection, which in experimental infections is 0.22 (Reid 1975). P is the probability of infection per chick = VNE/T. V, the virus prevalence in nymphs was 0.003 in 1993, 1994 and 1995 at this site (Gaunt 1997). N is the number of potentially infective ticks (i.e. nymphs) counted per chick, which varied each year (table 2). E is the period of exposure to infection, which is estimated to be 90 days for the tick-bite infection route (see § 2b). T is the period of attachment of a nymph, which is 5 days.

We assumed that the frequency of biting ticks counted in June is constant throughout the estimated period of exposure. More realistically, questing nymph numbers are probably higher in June than in July and August (Steele & Randolph 1985), so our estimates of the expected probability of infection from the tick-bite route (X_e) may be conservatively high.

For both methods, calculations were carried out using log (tick numbers + 1). The final estimates in tables 1 and 2 are back-transformed values. The values presented are means ($\pm$s.e.).

3. RESULTS

(a) *Feeding experiment*

All (eight out of eight) chicks fed with infected SMB became infected and 50% (four out of eight) of those fed with infected ticks became infected. No virus was detected in the control chicks.

Out of the eight chicks infested with uninfected nymphs, only four could be used to test for transmission back to ticks because the nymphs failed to bite one, and three did not acquire the virus. Overall, 75% (three out of four) of infected chicks transmitted the virus back to nymphs, comprising two out of three chicks fed with infected SMB and one out of one fed with infected adult ticks.

(b) *A comparison of ticks in droppings with ticks biting chicks*

Parameter values, sample sizes and calculation of the probabilities of infection, X, are shown in table 1. We estimated that the probability of a chick becoming infected during its first season as a result of eating ticks was 0.0196 ± 0.0143 (mean $\pm$ s.e.) and as a result of being bitten by ticks was 0.0074 ± 0.0011. From this, we estimated, unexpectedly, that 72.6% of all LIV infections in young grouse were derived from the oral infection route rather than from the conventional tick-bite route in this population.

(c) *A comparison of the observed seroprevalence with that expected from ticks biting grouse*

Calculations and comparison of the observed and expected LIV prevalences are shown in table 2. The observed LIV seroprevalences in grouse were 0.82 ($\pm$0.135), 0.71 ($\pm$0.098) and 0.78 ($\pm$0.077) for 1993, 1994 and 1995, respectively. The seroprevalences expected if infection arose solely from the tick bites (taking into account virus-induced grouse mortality) were 0.021, 0.017 and 0.012 for 1993, 1994 and 1995, respectively. Hence, overall, we estimated that a surprisingly high 97.8% of all LIV infections arose from birds eating ticks in this population.

Table 2. A comparison of the observed LIV seroprevalence (X_o) with that expected (X_e) if infections arose from tick bites alone (see § 2c).

($X_e = PR/(PR + (1 - P))$), where $P = VNE/T$. We then estimated the proportion of all infections that arose from tick ingestion as $(X_o - X_e)/X_o$. Values are means (±s.e.).)

year	1993	1994	1995
sample size (number of chicks)	132	155	177
biting nymphs/chick	6.96 (±0.69)	5.39 (±0.47)	2.51 (±0.21)
log (nymphs + 1), N	0.721 (±0.227)	0.614 (±0.167)	0.422 (±0.047)
period of attachment (days), T	5	5	5
period of exposure (days), E	90	90	90
virus prevalence in nymphs, V	0.003	0.003	0.003
probability of chick infection, P	0.0389	0.0332	0.0228
chick recovery rate, R	0.22	0.22	0.22
expected LIV prevalence, X_e	0.0088	0.0075	0.0051
back-transformed X_e	0.0205	0.0174	0.0118
number of grouse tested for LIV	22	47	81
observed LIV prevalence, X_o	0.818 (±0.135)	0.710 (±0.098)	0.780 (±0.077)
percentage of infections from oral route, $(X_o - X_e)/X_o$	97.5	97.5	98.5

4. DISCUSSION

The feeding experiment demonstrated that grouse can become infected with LIV from ingesting virus-infected material, and that grouse infected in this way can transmit virus back to feeding ticks. There are several reports of arbovirus infection without involving the vectors, for example, orally or intranasally (Kuno 2001), but this study, importantly, demonstrates arbovirus infection from ingesting the vector. Furthermore, this study suggests that this novel infection route may cause 73–98% of host infections of this tick-borne virus during the host's first summer. Hitherto, it has been assumed that red grouse acquire LIV only from being bitten by nymphs (Hudson *et al.* 1995), but we can now suggest that adult ticks and possibly other tick stages and states can infect grouse with LIV. As virus titres and prevalence rates are greater in fed and adult ticks than in biting nymphs (Gaunt 1997), ingesting ticks would increase the probability of host infection. Moreover, if siblings within a brood allopreen ticks, this could theoretically lead to faster transmission rates with a single chick infecting siblings indirectly. This is in contrast to the general assumption that a single tick can cause infection in only one chick per season.

Non-viraemic transmission, a route first described between co-feeding ticks for Thogoto virus (Jones *et al.* 1987), is now known to occur for other pathogens and vector types (Randolph *et al.* 1996; Ogden *et al.* 1997; Mead *et al.* 2000). Likewise, our finding that vector ingestion is a major infection route for LIV may have implications for other arboviruses where hosts ingest vectors through foraging or grooming. Potential examples include TBE virus (Randolph 2001) and West Nile virus (Malakoff 2002) that can be acquired orally by hosts (see Kuno 2001). Perhaps mammalian herbivores may also gain infection of pathogens if they ingest infected vectors, such as questing ticks, while grazing. Hitherto, it has been assumed that vector bites are the sole or main route of host infection for these zoonoses. Thus, to fully understand the persistence and spread of these zoonoses and to impose control measures, there is clearly a need to re-examine the assumed key infection routes for vector-borne pathogens.

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