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Original article

Sex ratios of the tick *Ixodes arboricola* are strongly female-biased, but there are no indications of sex-distorting bacteria

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ABSTRACT

Studies on sex ratio are of fundamental importance for understanding the biology of populations and biological control of pests and pathogens. In most *Ixodes* tick species, only females feed in the adult stage and, hence, contribute to pathogen transmission. The tree-hole tick *Ixodes arboricola* infests cavity-nesting birds and has limited dispersal possibilities. It plays an important role in the maintenance of zoonotic disease cycles. Here, we quantified the sex ratio of 718 adult *I. arboricola* ticks obtained from a laboratory stock at nine distinct periods (cohorts) from 2008 to 2015. In addition, we screened 93 specimens, collected from four study sites in 2011 and 2012, for the presence of six maternally inherited bacterial parasites known to manipulate arthropod sex ratios. We found significantly female-biased sex ratios in seven out of nine cohorts. There were no infections with members of the *Wolbachia*, *Arsenophonus* or *Cardinium* bacterial genera, whereas 96.8% of the screened ticks were infected with *Rickettsia vini*, 22.6% with *Rickettsiella* sp., and 14.0% with *Spiroplasma ixodetis*. Male and female *I. arboricola* were found equally infected. Our results suggest skewed sex ratios in *I. arboricola* are not caused by these bacterial infections, although there may be other, untested candidates driving sex ratios. Alternatively, female-biased sex ratios may be an adaptation in females to high local densities and low dispersal, where the production of daughters has a selective advantage because a few sons can fertilise all daughters.

1. Introduction

Sex ratio is an important parameter which characterises the state and dynamics of natural populations of animals. Studies on sex ratio and its distorters, the mechanism of sex determination and sex allocation are of fundamental importance for understanding the biology of populations and biological control of pests and pathogens (Fourie et al., 1996; Wrensch and Ebbert, 1993). In arthropod vectors, sexes often contribute differently to pathogen transmission (Sonenshine, 1991), hence the dynamics of sex ratio characteristics can ultimately influence the epidemiology of vector-borne diseases.

Skewed sex ratios are observed frequently under natural circumstances and may have different reasons. For instance, skewed sex ratios could result from unequal mortality between males and females (Kiszewski et al., 2001). As such, sex ratios calculated from field data can depend on the season and venue of collection, and may change with cohorts. In arthropods, sex ratios can be skewed towards females by reproductive parasites that depend on this gender for their (transovarial) transmission. At least five maternally inherited bacteria belonging to the *Arsenophonus*, *Cardinium*, *Rickettsia*, *Spiroplasma* and *Wolbachia* genera are known to manipulate the reproduction of their host species towards the production of daughters (the transmitting sex) through the induction of parthenogenesis, feminisation or male-killing (Engelstädter and Hurst, 2009; Stouthamer et al., 1999; Werren et al., 2008). These reproductive parasites are common in arthropods (Duron et al., 2008), including ticks (Duron et al., 2017). In addition, another maternally inherited bacterium belonging to the *Rickettsiella* genus has been observed in a parthenogenetic laboratory stock of the tick *Ixodes woodi* (Kurtti et al., 2002). This tick species is generally known to be bisexual, suggesting a possible role of *Rickettsiella* infection in the induction of asexuality (Kurtti et al., 2002). Sex ratio skewness may also be adaptive. For instance, under high local densities and low dispersal there may be considerable competition among closely related males for access to females (Clobert et al., 2001; Poulin, 2007). Females that produce female-biased offspring have a selective advantage, because few sons are required to fertilise many daughters, and, therefore, producing more sons does not increase a mother's fitness (Werren, 1980).

In the current study, we investigated the sex ratio of the nidicolous

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tick *Ixodes arboricola* Schulze and Schlottke 1929. This tick chiefly infests a variety of cavity-nesting bird species, with great and blue tits (*Parus major*, *Cyanistes caeruleus*) as its principal hosts (Hudde and Walter, 1988; Petney et al., 2011; Walter et al., 1979). Although great and blue tits are very mobile, ticks can only disperse by feeding on birds that move among cavities before the ticks detach. This does not occur frequently because nesting and roosting tits usually remain in the same cavity, and natal dispersal is followed by a period of shunning cavities (Gosler, 1993; Tyller et al., 2012). This may lead to inbreeding of ticks within cavities (Van Oosten et al., 2014). Dispersal of adult female ticks may be even more restricted than of the earlier instars, as adult females feed almost exclusively on nestling birds (Heylen et al., 2014b). Male ticks feed only in the larval and nymphal stages and remain in the host's cavity in the adult stage, where they copulate with both unfed and engorged female ticks (Heylen et al., 2012b; Van Oosten et al., 2016a). *Ixodes arboricola* is important for the disease ecology of humans, pets and livestock, as it carries and transmits tick-borne encephalitis (TBE) virus (Gresikova and Kaluzova, 1997; Hillyard, 1996; Lichard and Kozuch, 1967) and rickettsiae, which may cause spotted fever (Špitalská et al., 2011), and carries several avian *Borrelia* genospecies (Heylen et al., 2012a; Špitalská et al., 2011). By co-infestation with *I. ricinus*, not necessarily at the same time, these pathogens can be bridged from bird-tick cycles to humans, pets and livestock (Heylen et al., 2014a). Knowledge concerning sex ratios in ixodid ticks is very important, because in the adult stage − in contrast to males that do not feed − female ticks feed for several days, which strongly increases the chance for pathogen transmission.

Here, we report the sex ratio of our laboratory stock of *I. arboricola* and tested whether reproductive parasites that are known for sex-distorting bacteria (*Arsenophonus*, *Cardinium*, *Rickettsia*, *Spiroplasma*, *Wolbachia*, including also *Rickettsiella*) are correlated with sex ratio distortion. If bacterial infections of any of these genera are different between male and female ticks, this indicates sex ratio distortion is driven by sex-distorting bacteria.

2. Materials and methods

A laboratory stock of *I. arboricola* was established in 2008 with ticks (cohort 2008-V; 303 larvae, 111 nymphs and 19 adult females) from nest boxes in the study areas Peerdsbos, Brasschaat, Belgium (PB; 51°16′29”N, 4°29′03”W) and Boshoek, Lier, Belgium (BH; 51°07′43”N, 4°31′52”W) (Heylen and Matthysen, 2010). This stock has been maintained by allowing ticks to infest great tits, and occasionally other birds, in several studies (Heylen and Matthysen, 2011; Van Oosten et al., 2016b), and the occasional addition of ticks from PB, BH and three other nearby study areas: Park Middelheim, Antwerp, Belgium (MI; 51°18′33”N, 4°40′69”W), Park de Warande, Oostmalle, Belgium (WA; 51°17′53”N, 4°43′46”W) and Wortel Kolonie, Wortel, Belgium (WK; 51°24′10”N, 4°49′29”W). Nest boxes have been present in these areas for many years as part of long-term population studies on great and blue tits (Matthysen et al., 2011, 2001). Sex ratios in nest boxes are slightly female-biased (Heylen et al., 2014b), but the overall sex ratio in the field may be more female-biased because a significant portion of females may be attached to bird hosts. The laboratory stock was kept in the following conditions: engorged ticks were always kept at 25 °C, which ensures rapid moulting, and unfed ticks at 12 °C, which ensures increases longevity; relative humidity for all ticks was 85%, which is sufficiently humid for the ticks and at the same time restricts fungal growth; and a light cycle 12 h light:12 h dark, which is the average hours of light per day throughout the year. Before each breeding season, ticks were mated by placing them together in vials (diameter 1.5 cm, height 12 cm) for several days, either with all ticks together (until 2013) or in random pairs (2014 and 2015).

2.1. Sex ratio

Sex ratios were recorded between May 2008 and February 2015, when nymphal *I. arboricola* ticks fed on great tits under controlled conditions and moulted in the laboratory for previous experiments. In this way, 718 adult *I. arboricola* ticks from nine distinct periods (cohorts) were obtained. Because field-collected ticks have occasionally been added to the laboratory stock, the generation and origin of individual ticks cannot be traced back. However, all have been part of the laboratory stock at least since the larval stage (i.e. no field-collected nymphs were used). After detachment, ticks were placed in individual 1.5-ml Eppendorf tubes to moult under controlled climatic conditions (see above). Ticks were inspected every three days and gender was recorded once moulting was completed.

2.2. Screening of sex-distorting bacteria

A total of 93 *I. arboricola* ticks (45 males, 48 females) has been collected in the study areas MI, PB, WK and WA for a previous analysis of population genetic structure in 2011 and 2012 (Van Oosten et al., 2014). All ticks were screened for the presence of the five most common known reproductive parasites (*Arsenophonus, Cardinium, Rickettsia, Spiroplasma and Wolbachia*; Duron et al., 2008), and also of *Rickettsiella* (Kurtti et al., 2002).

Tick DNA was individually extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer instructions. Infections have been next investigated using amplifications through polymerase chain reaction (PCR) in each individual host for the six targeted bacteria. Independent and specific assays for infection by each of the six bacteria were performed using PCR amplification following existing protocols using specific primers (Duron et al., 2017). All bacterial and tick primers are listed in Table 1. Maternally-inherited bacteria were detected through either nested, semi-nested or non-nested specific PCR amplifications depending on the targeted genus. Nested and semi-nested PCR amplifications were performed as follows: the first PCR run with the external primers was performed in a 10 µl volume containing 20–50 ng of genomic DNA, 3 mM of each dNTP (Thermo Scientific), 8 mM of $MgCl_2$ (Roche Diagnostics), 3 µM of each primer, 1 µl of 10 × PCR buffer (Roche Diagnostics), and 0.5 U of Taq DNA polymerase (Roche Diagnostics). A 1-µl aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR was performed in a total volume of 25 µl and contained 8 mM of each dNTP (Thermo Scientific), 10 mM of $MgCl_2$ (ThermoScientific), 7.5 µM of each of the internal primers, 2.5 µl of 10 × PCR buffer (Thermo Scientific), and 1.25 U of Taq DNA polymerase (Thermo Scientific). All non-nested PCR amplifications were performed following conditions similar to the first PCR run used in the nested PCR assays. All PCR amplifications were performed under the following conditions: initial denaturation at 93 °C for 3 min, 35 cycles of denaturation (93 °C, 30 s), annealing (Tm = 50–56 °C, depending on primers, 30 s), extension (72 °C, 1–2 min), and a final extension at 72 °C for 5 min.

Infected-positive and infected-negative individuals were used as positive and negative controls, respectively, in each PCR assay. DNA template quality of all infected-negative specimens was systematically verified by PCR amplification of the eukaryotic 18S ribosomal RNA gene (18S rDNA) using universal primers (Table 1). All PCR products were visualized through electrophoresis in a 1.5% agarose gel. Positive PCR products of randomly sampled individuals were purified and sequenced in both directions (EUROFINS) to ensure that the record represented a true positive and not a PCR artefact or related bacterium. The chromatograms were manually inspected and cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html) and sequence alignments were done using CLUSTALW (Thompson et al., 2002), both implemented in MEGA (Kumar et al., 2004).

Table 1
Genes and primers used in this study.

Gene		Hypothetical product	Primers (5′-3′)		Tm	Fragment size	Reference
TICK							
	18S rDNA	Small ribosomal subunit (18S rRNA)	NSF4 –	CTGGTTGATYCTGCCAGT	50 °C	Non-nested PCR assay:	Hendriks et al. (1990), Hendriks et al. (1991)
			NSR399 –	TCTCAGGCTCCYTCTCCGG		NSF4/NSR399: 404bp	
BACTERIA							
Coxiella							
	rpoB	DNA-directed RNA polymerase beta chain	CoxrpoBF2 –	GGGCGNCAYGGWAAYAAAGGSGT	56 °C	Nested PCR assay:	Duron et al. (2015)
			CoxrpoBR1 –	CACCRAAHCGTTGACCRCCAAATTG		1 st round PCR: CoxrpoBF2/CoxrpoBR1: 607–610bp	
			CoxrpoBF3 –	TCGAAGAYATGCCYTATTTAGAAG		2nd round PCR: CoxrpoBF3/CoxrpoBR3: 539–542bp	
			CoxrpoBR3 –	AGCTTTMCCACCSARGGGTTGCTG			
Rickettsiella							
	GroEL	Chaperone protein GROEL	CoxGrF1 –	TTTGAAAAYATGGGCGCKCAAATGGT	56 °C	Nested PCR assay:	Duron et al. (2014)
			CoxGrR2 –	CGRTCRCCAAARCCAGGTGC		1 st round PCR: CoxGrF1/CoxGrR2: 655bp	
			CoxGrF2 –	GAAGTGGCTTCGCRTACWTCAGACG		2nd round PCR: CoxGrF2/CoxGrR1: 619bp	
			CoxGrR1 –	CCAAARCCAGGTGCTTTYAC			
Arsenophonus							
	yaeT	Outer membrane protein assembly factor	yaeTF-	GCATACGGTTCAGACGGGTTTG	50 °C	Non-nested PCR assay:	Duron et al. (2010)
			yaeT-	GCCGAAACGCCTTCAGAAAAG		yaeTF/yaeTR: 470bp	
Wolbachia							
	wsp	Surface protein	81F-	TGGTCCAATAAGTGATGAAGAAAC	50 °C	Non-nested PCR assay:	Braig et al. (1998)
			691R-	AAAAATTAAACGCTACTCCA		81F/691R: 610 bp	
Rickettsia							
	gltA		RICKF1-	CCTATGGCTATTATGCTTGCGGC	56 °C	Semi-nested PCR assay:	Duron et al. (2017)
			RICKR1-	CATCTTTAAGAGCGATAGCTTCAAG		1 st round PCR: RICKF1/RICKR1: 672bp	
			RICKF2-	GGTTCTCTTTCKGCATTTTATCC		2nd round PCR: RICKF2/RICKR1: 645bp	
*Spiroplasma (*ixodetis *group)*							
	16S rDNA	Small ribosomal subunit (16S rRNA)	SpixoF2-	TTAGGGGCTCAACCCCTAACCGC	56 °C	Semi-nested PCR assay:	Duron et al., (2008), Duron et al. (2017)
			SpixoR2-	CCCTGCCTTTGGCAGCTCCCTCC		1 st round PCR: SpixoF2/SpixoR2: 865bp	
			SpixoR1-	TCTGGCATTGCCAACTCTCGTGG		2nd round PCR: SpixoF2/SpixoR1: 822bp	
BACTEROIDETES							
Cardinium							
	16S rDNA	Small ribosomal subunit (16S rRNA)	ChF-	TACTGTAAGAATAAGCACCGGC	52 °C	Semi-nested PCR assay:	Zchori-Fein and Perlman, (2004), Gotoh et al. (2007)
			CloR1-	GTTGGTAGAGCTTGAAGACAGTGG		1 st round PCR: chF-CloR1: 957bp	
			ChR-	GTGGATCACTTAACGCTTTCG		2nd round PCR: ChF/ChR: 390bp	

2.3. Statistical analyses

Differences in sex ratio of adult *I. arboricola* ticks across cohorts (logit-link, binomially distributed residuals) were investigated with a generalised linear model, with cohort as a fixed factor. Post-hoc testing was conducted with goodness-of-fit chi-squared tests for each cohort, applying Bonferroni correction to correct for multiple testing.

The effect of reproductive parasite infection on sex ratio (logit-link, binomially distributed residuals) was analysed with a generalised linear model, including parasite genus, second- and third-order interactions, and sampling location as fixed factors. Only those parasite genera for which at least one infection was found were included in the model (i.e. *Rickettsia*, *Rickettsiella* and *Spiroplasma*).

All data analyses were done in R v 3.3.3 (R Core Team, 2017). In all models a stepwise selection procedure was used in which the model was iteratively refitted after exclusion of the least significant covariate. Terms were not removed if they were part of a higher order significant interaction.

3. Results

3.1. Sex ratio of I. arboricola

Of the 718 inspected individuals, 160 were males and 558 females (ratio 1:3.49 M:F). There were significant differences in sex ratio among cohorts ($\chi^2_8 = 1488.72$, $P < 0.001$), ranging from 1:1.27 to 1:10.43. Post-hoc testing revealed that, with the exception of cohorts 2008-XII and 2013-I, sex ratio was female-biased in all cohorts ($P \leq 0.002$; Table 2, Fig. 1).

3.2. Screening of sex-distorting bacteria

A summary of the infection proportions and results of the statistical tests can be found in Table 3. None of the 93 specimens (45 males and 48 females) were infected with *Arsenophonus*, *Cardinium* or *Wolbachia*. Ninety were positive for PCR detection of *Rickettsia*, 21 for *Rickettsiella* (of which 19 were also infected with *Rickettsia*), and 13 for *Spiroplasma* (all also infected with *Rickettsia*, of which 2 were also infected with *Rickettsiella*). For these three genera (i.e. *Rickettsia*, *Rickettsiella* and *Spiroplasma*), sex ratio was not significantly different between infected and uninfected tick individuals (*Rickettsia*: $\chi^2_1 = 0.406$, $P = 0.524$; *Rickettsiella*: $\chi^2_1 = 0.877$, $P = 0.349$; *Spiroplasma*: $\chi^2_1 = 0.010$, $P = 0.920$), nor were there any significant interactions, i.e. one parasite species driving the effect of another on tick sex ratio (*Rickettsia:Rickettsiella*: $\chi^2_1 = 0.905$, $P = 0.342$; *Rickettsia:Spiroplasma*: $\chi^2_1 = 0$, $P = 1$; *Rickettsia:Spiroplasma:Rickettsiella*: $\chi^2_1 = 0$, $P = 1$). There was no significant effect of sampling location ($\chi^2_4 = 1.444$, $P = 0.837$) (Fig. 2).

All *gltA Rickettsia* sequences were strictly identical and matched (100% identity at the nucleotide level) *Rickettsia vini* sequences available in GenBank (Accession numbers: KX159434 − KX159436), a recently described species commonly found in *I. arboricola* (Novakova et al., 2016; Palomar et al., 2012; Špitalská et al., 2011). Three different sequences of *GroEL Rickettsiella* were obtained and each (100% identity at the nucleotide level) with the strains of *Rickettsiella* sp. from *I. arboricola* available in GenBank (Accession numbers: KY677996-KY678000). All 16S rDNA *Spiroplasma* sequences were strictly identical and matched (100% identical at the nucleotide level) with the *S. ixodetis* from *I. arboricola* available in GenBank (Accession numbers: KY674400-KY674401). The three remaining *I. arboricola* specimens were devoid of any of the targeted bacteria but have satisfactory DNA template quality as shown by positive PCR amplification using the 18S rDNA arthropod universal primers; they were further considered as uninfected.

Table 2
Summary of the chi-squared goodness-of-fit tests conducted on sex ratio for each of seven experiments separately. Significant effects are given in bold.

Experiment	No. males	No. females	Sex ratio (M:F)	z	*P*
2008-V	31	61	1:1.97	3.069	**0.002**
2008-XII	23	34	1:1.48	1.448	0.148
2012-X	6	26	1:4.33	3238	**0.001**
2013-I	26	33	1:1.27	0.909	0.363
2013-IV	13	105	1:8.08	7.105	< **0.001**
2014-IV	7	73	1:10.43	5.925	< **0.001**
2014-X	17	48	1:2:82	3.678	< **0.001**
2014-XII	7	73	1:10.43	5.925	< **0.001**
2015-II	30	105	1:3.50	6.051	< **0.001**

4. Discussion

Sex ratio of *I. arboricola* ticks was strongly female-biased in seven out of nine cohorts. This includes the first cohort (2008-V), which was used to initiate the laboratory stock using field-collected nymphs. Sex ratio therefore does not seem to change over generations in the laboratory. There were no differences in the prevalence of any of the investigated reproductive parasites between male and female ticks. This suggests skewed sex ratios in *I. arboricola* are not caused by bacterial infection. However, it is likely that the three reproductive parasites we observed, i.e. *Rickettsia vini*, *Rickettsiella* sp. and *S. ixodetis*, have some effect on *I. arboricola* fitness. Recently, a diversity of endosymbionts has been emphasised as a source of evolutionary innovations in arthropods: they alter host phenotype and, because they are heritable, contribute to host adaptation (Ferrari and Vavre, 2011). Beyond manipulating host reproduction, diverse maternally inherited bacteria exert a significant influence on arthropod ecology and evolution, providing resistance to environmental stress or broadening host trophic interactions (Moran et al., 2008; Wernegreen et al., 2012). The nature of interactions of *Rickettsia vini* and *S. ixodetis* with *I. arboricola* remains to be investigated.

Our results are in stark contrast with a German study, where sex ratio of *I. arboricola* kept at 20 °C and 90% RH and fed on chickens for five generations was 1:1 (Liebisch, 1996). Lab conditions were comparable to ours, yet chickens are artificial hosts for this songbird-tick. Furthermore, nymphs that originated from engorged larvae were exposed to the hosts almost immediately after these larvae had moulted, whereas in our studies several months passed between feedings. The beforementioned study did not report the sex ratio of the first generation, and it is therefore unknown whether the sex ratio changed in the laboratory or is 1:1 in the field too. Which factors (e.g., contrasting rearing conditions, local adaptation or undefined reproductive parasite species) explain the contrasting outcomes between the two labs remains to be resolved.

Skewed sex ratios in *I. arboricola* may be adaptive. The dispersal capabilities of *I. arboricola* are limited because hosts show high site fidelity when using cavities (Gosler, 1993), and ticks within nests can be highly related (Van Oosten et al., 2014). Under high local densities and low dispersal, ticks in a nest are closely related. Closely related males compete for female access because they can mate repeatedly (Van Oosten et al., 2016a). As such, the production of female offspring has a selective advantage (Werren, 1980). It remains to be investigated experimentally whether the skewed sex ratio in *I. arboricola* is indeed an adaptation to counteract inbreeding.

Skewed sex ratios in *I. arboricola* could also be caused by differential survival between sexes. Since males do not feed in the adult stage, they do not face the risk of being killed while feeding (Van Oosten et al., 2016a). Females may be more abundant to compensate for the feeding risk. Testing this hypothesis would require the monitoring of the sex ratios of immature developmental stages in nests over time. Unfortunately, sex identification of these stages cannot be done visually, at least in our study species. To our knowledge, molecular markers that allow sex determination in immature ticks are currently unavailable,

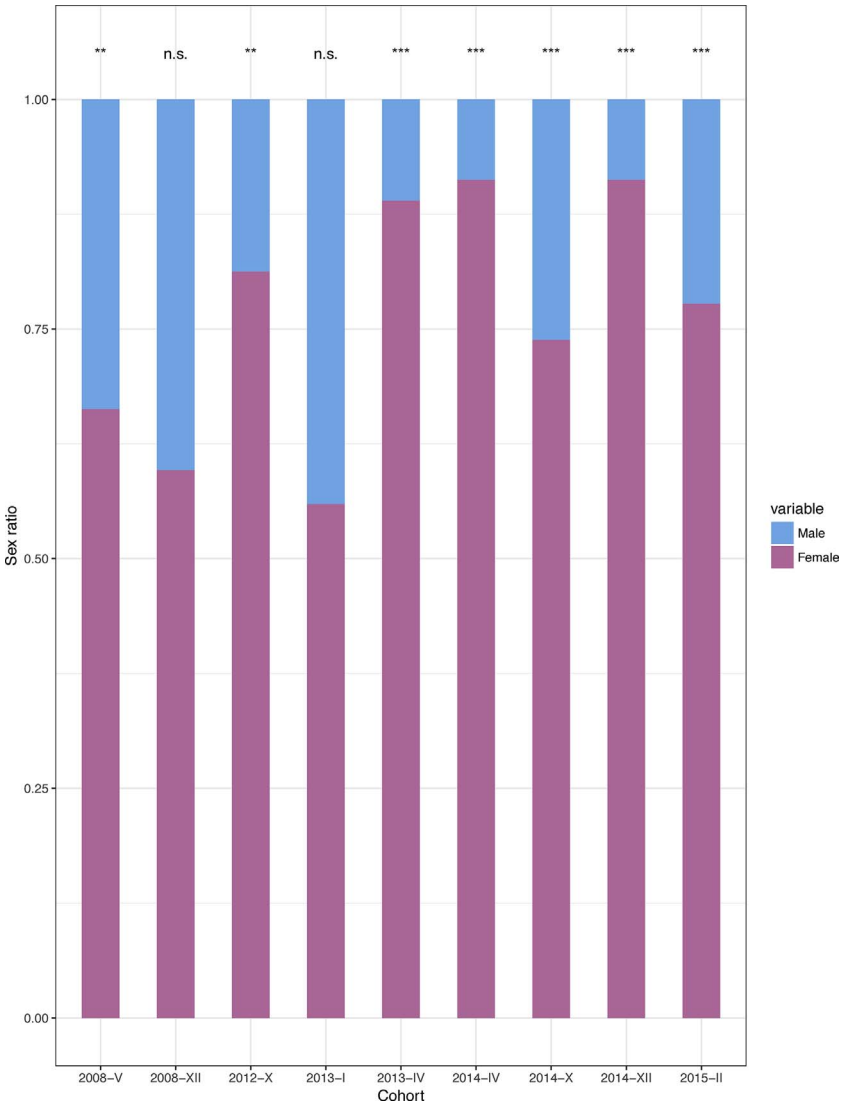

Fig. 1. Ratio of adult male (N = 160) and female (N = 558) *Ixodes arboricola* ticks across nine cohorts. n.s.: not significant; **: P = 0.001; ***: $P < 0.001$.

Table 3
Summary of the proportion of ticks infected with three reproductive parasite species, and generalised linear models on the sex-distorting bacteria screening, with a total of 93 ticks.

Genus	Proportion infected	χ^2	df	P
Arsenophonus	0			
Cardinium	0			
Wolbachia	0			
Rickettsia	0.645	0.406	1	0.524
Rickettsiella	0.022	0.877	1	0.349
Spiroplasma	0	0.010	1	0.920
Rickettsia:Rickettsiella	0.183	0.905	1	0.342
Rickettsia:Spiroplasma	0.118	0	0	1
Rickettsia:Spiroplasma:Rickettsiella	0.022	0	0	1
Location		1.444	4	0.837

and there have not been any molecular studies to tackle this problem as of yet.

The fact that in *I. arboricola* females are far more prevalent than males may have important consequences for disease ecology, because in the adult stage only females obtain a blood meal and are therefore liable to pathogen transmission (Heylen et al., 2012b; Van Oosten et al., 2016a). *Ixodes arboricola* is an important disease vector, carrying and transmitting tick-borne encephalitis (TBE) virus (Gresikova and Kaluzova, 1997; Hillyard, 1996; Lichard and Kozuch, 1967) and rickettsiae (Špitalská et al., 2011), and carrying several avian *Borrelia* genospecies (Heylen et al., 2012a; Špitalská et al., 2011). In general, when estimating pathogen transmission dynamics, it is therefore important to take into account the life history of the species under study.

The skewed sex ratios of *I. arboricola* do not seem to be a characteristic of nidicolous ticks in general. Firstly, in the nidicolous *I. angustus*, which infests various rodents and soricid insectivores in the nests, male-biased ratios are observed (Robbins and Keirans, 1992). As host dispersal may occur much more frequently than is the case in *I. arboricola*, it may not be adaptive to produce female-biased offspring in this species. Similarly, no sex bias is observed in the nidicolous seabird tick *I. uriae*, which has been found on more than 50 different seabird species (McCoy, pers. comm.). For hosts breeding in colonies on cliffs, ticks can move among nests independently, and, indeed, there is high within tick-population genetic variation (McCoy et al., 2003). Finally, in *I. hexagonus*, a species found inside nests of the European hedgehog (*Erinaceus europaeus*) (Pfäffle et al., 2011) – but occasionally on mustelid carnivores, red foxes (*Vulpes vulpes*) and pets too (Liebisch and Walter, 1986) – no sex-bias has been observed (Toutoungi et al., 1993). Whereas hosts of *I. arboricola* shun cavities for a long period in summer, during which most natal bird dispersal takes place, juvenile hedgehogs occupy nests immediately after dispersal (Reeve, 1994). This means attached ticks are more likely to end up in suitable habitat than is the case with *I. arboricola*.

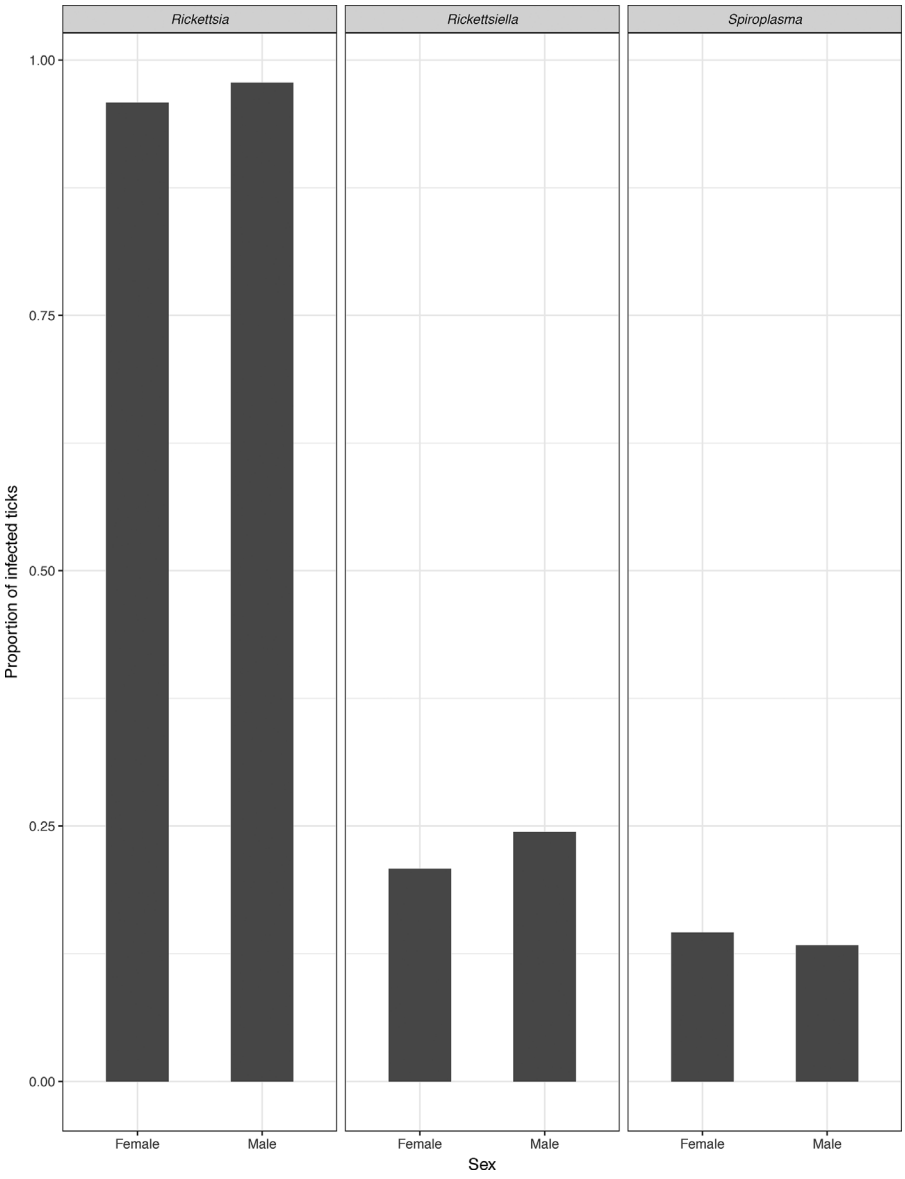

Fig. 2. Proportion of male (N = 45) and female (N = 48) *Ixodes arboricola* ticks infected with bacteria from six reproductive parasite genera.

We can conclude that sex ratios observed in nidicolous ticks vary among species and even among populations. Life-history characteristics likely play an important role, but in virtually all tick species survival analyses of sexes under natural conditions are lacking, which are key to elucidate general patterns. Sex ratios may be determined by micro-organisms, both endogenic (e.g., bacteria and viruses) and exogenic (e.g., fungi), but they may also be skewed as a result of natural selection. Future molecular techniques will provide new avenues to identify the broad spectrum of potential actors that shape sex ratios.

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References

Braig, H.R., Zhou, W., Dobson, S.L., O'Neill, S.L., 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180, 2373–2378.

Clobert, J., Danchin, É., Dhondt, A.A., Nichols, J.D., 2001. Dispersal. Oxford University Press, Oxford.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., Hurst, G.D., 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6, 27. http://dx.doi.org/10.1186/1741-7007-6-27.

Duron, O., Wilkes, T.E., Hurst, G.D.D., 2010. Interspecific transmission of a male-killing bacterium on an ecological timescale. Ecol. Lett. 13, 1139–1148. http://dx.doi.org/10.1111/j.1461-0248.2010.01502.x.

Duron, O., Jourdain, E., McCoy, K.D., 2014. Diversity and global distribution of the *Coxiella* intracellular bacterium in seabird ticks. Ticks Tick Borne Dis. 5, 557–563. http://dx.doi.org/10.1016/j.ttbdis.2014.04.003.

Duron, O., Noël, V., McCoy, K.D., Bonazzi, M., Sidi-Boumedine, K., Morel, O., Vavre, F., Zenner, L., Jourdain, E., Durand, P., Arnathau, C., Renaud, F., Trape, J.F., Biguezoton, A.S., Cremaschi, J., Dietrich, M., Léger, E., Appelgren, A., Dupraz, M., Gómez-Díaz, E., Diatta, G., Dayo, G.K., Adakal, H., Zoungrana, S., Vial, L., Chevillon, C., 2015. The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, Coxiella burnetii. PLoS Pathog. 11, 1–23. http://dx.doi.org/10.1371/journal.ppat.1004892.

Duron, O., Binetruy, F., Noël, V., Cremaschi, J., McCoy, K.D., Arnathau, C., Plantard, O., Goolsby, J., Pérez de León, A.A., Heylen, D.J.A., Van Oosten, A.R., Gottlieb, Y., Baneth, G., Guglielmone, A.A., Estrada-Peña, A., Opara, M.N., Zenner, L., Vavre, F., Chevillon, C., 2017. Evolutionary changes in symbiont community structure in ticks. Mol. Ecol. 33. http://dx.doi.org/10.1111/mec.14094.

Engelstädter, J., Hurst, G.D.D., 2009. The ecology and evolution of microbes that manipulate host reproduction. Annu. Rev. Ecol. Evol. Syst. 40, 127–149. http://dx.doi.org/10.1146/annurev.ecolsys.110308.120206.

Ferrari, J., Vavre, F., 2011. Bacterial symbionts in insects or the story of communities

affecting communities. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1389–1400. http://dx.doi.org/10.1098/rstb.2010.0226.
Fourie, L.J., Belozerov, V.N., Kok, D.J., 1996. Sex ratio characteristics in *Ixodes rubicundus* (Acari Ixodidae), the Karoo paralysis tick. Exp. Appl. Acarol. 20, 477–481.
Gosler, A., 1993. The Great Tit. Hamlyn, London, UK.
Gotoh, T., Noda, H., Ito, S., 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity (Edinb) 98, 13–20. http://dx.doi.org/10.1038/sj.hdy.6800881.
Gresikova, M., Kaluzova, M., 1997. Biology of tick-borne encephalitis virus. Acta Virol. 41, 115–124.
Hendriks, L., Goris, A., Debruyn, K., DeWachter, R., 1990. The nucleotide sequence of the small ribosomal subunit RNA of the yeast *Torulaspora delbrueckii*. Nucleic Acids Res. 18, 4611.
Hendriks, L., Debaere, R., Vandepeer, Y., Neefs, J., Goris, A., Dewachter, R., 1991. The evolutionary position of the rhodophyte *Porphyra umbilicalis* and the basidiomycete *Leucosporidium scottii* among other eukaryotes as deduced from complete sequences of small ribosomal subunit RNA. J. Mol. Evol. 32, 167–177.
Heylen, D.J.A., Matthysen, E., 2010. Contrasting detachment strategies in two congeneric ticks (Ixodidae) parasitizing the same songbird. Parasitology 137, 661–667. http://dx.doi.org/10.1017/S0031182009991582.
Heylen, D.J.A., Matthysen, E., 2011. Differential virulence in two congeneric ticks infesting songbird nestlings. Parasitology 138, 1011–1021. http://dx.doi.org/10.1017/S0031182011000618.
Heylen, D.J.A., Tijsse, E., Fonville, M., Matthysen, E., Sprong, H., 2012a. Transmission dynamics of *Borrelia burgdorferi* s.l. in a bird tick community. Environ. Microbiol. 29, 663–673. http://dx.doi.org/10.1111/1462-2920.12059.
Heylen, D.J.A., White, J., Elst, J., Jacobs, I., Van De Sande, C., Matthysen, E., 2012b. Nestling development and the timing of tick attachments. Parasitology 139, 766–773. http://dx.doi.org/10.1017/S0031182011002277.
Heylen, D.J.A., Matthysen, E., Fonville, M., Sprong, H., 2014a. Songbirds as general transmitters but selective amplifiers of *Borrelia burgdorferi* sensu lato genotypes in *Ixodes rinicus* ticks. Environ. Microbiol. 16, 2859–2868. http://dx.doi.org/10.1111/1462-2920.12304.
Heylen, D.J.A., Van Oosten, A.R., Devriendt, N., Elst, J., De Bruyn, L., Matthysen, E., 2014b. Seasonal feeding activity of the tree-hole tick, *Ixodes arboricola*. Parasitology 141, 1044–1051. http://dx.doi.org/10.1017/S0031182014000225.
Hillyard, P.D., 1996. Ticks of North-West Europe: Keys and Notes for Identification of the Species. Field Studies Council in association with the Natural History Museum, London.
Hudde, H., Walter, G., 1988. Verbreitung und Wirtswahl der Vogelzecke *Ixodes arboricola*. Vogelwarte 34, 201–207.
Kiszewski, A.E., Matuschka, F.R., Spielman, A., 2001. Mating strategies and spermiogenesis in ixodid ticks. Annu. Rev. Entomol. 46, 167–182. http://dx.doi.org/10.1146/annurev.ento.46.1.167.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Br. Bioinforma 5, 150–163. http://dx.doi.org/10.1093/bib/5.2.150.
Kurtti, T.J., Palmer, A.T., Oliver, J.H.J., 2002. *Rickettsiella*-like bacteria in *Ixodes woodi* (Acari: ixodidae). J. Med. Entomol. 39, 534–540. http://dx.doi.org/10.1603/0022-2585-39.3.534.
Lichard, M., Kozuch, O., 1967. Persistence of tick-borne encephalitis virus in nymphs and adults of *Ixodes arboricola* and its transmission to white mice. Acta Virol. 11, 480.
Liebisch, A., Walter, G., 1986. Untersuchungen von Zecken bei Haus- und Wildtieren in Deutschland: Zum Vorkommen und zur Biologie der Igelzecke (*Ixodes hexagonus*) und der Fuchszecke (*Ixodes canisuga*). Deut Tierarztl Woch 93, 447–450.
Liebisch, G., 1996. Biology and life cycle of *Ixodes* (*Pholeoixodes*) *arboricola* Schulze and Schlottke, 1929 (Ixodidae). In: In: Mitchell, R., Horn, D.J., Needham, G.R., Welbourn, W.C. (Eds.), Acarology IX, vol. 1. Ohio Biological Survey, Columbus, OH USA, pp. 453–455.
Matthysen, E., Adriaensen, F., Dhondt, A.A., 2001. Local recruitment of great and blue tits (*Parus major, P. caeruleus*) in relation to study plot size and degree of isolation. Ecography 24, 33–42. http://dx.doi.org/10.1034/j.1600-0587.2001.240105.x.
Matthysen, E., Adriaensen, F., Dhondt, A.A., 2011. Multiple responses to increasing spring temperatures in the breeding cycle of blue and great tits (*Cyanistes caeruleus, Parus major*). Glob. Chang. Biol. 17, 1–16. http://dx.doi.org/10.1111/j.1365-2486.2010.02213.x.
McCoy, K.D., Boulinier, T., Tirard, C., Michalakis, Y., 2003. Host-dependent genetic structure of parasite populations: differential dispersal of seabird tick host races. Evolution 57, 288–296. http://dx.doi.org/10.1554/0014-3820(2003)057[0288:HDGSOP]2.0.CO;2.
Moran, N.A., McCutcheon, J.P., Nakabachi, A., 2008. Genomics and evolution of heritable bacterial symbionts. Annu. Rev. Genet. 42, 165–190. http://dx.doi.org/10.1146/annurev.genet.41.110306.130119.
Novakova, M., Costa, F.B., Krause, F., Literak, I., Labruna, M.B., 2016. *Rickettsia vini* n. sp. (Rickettsiaceae) infecting the tick *Ixodes arboricola* (Acari: ixodidae). Parasit. Vectors 9, 469. http://dx.doi.org/10.1186/s13071-016-1742-8.
Palomar, A.M., Portillo, A., Santibáñez, P., Santibáñez, S., García-Álvarez, L., Oteo, J.A., 2012. Genetic characterization of *Candidatus* Rickettsia vini a new rickettsia amplified in ticks from La Rioja. Spain.Ticks Tick. Borne. Dis. 3, 319–321.
Petney, T.N., Pfäffle, M., Skuballa, J., 2011. An annotated checklist of the ticks of Germany. Syst. Appl. Acarol. 17, 115–170. http://dx.doi.org/10.11158/saa.17.2.2.
Pfäffle, M., Petney, T., Skuballa, J., Taraschewski, H., 2011. Comparative population dynamics of a generalist (*Ixodes ricinus*) and specialist tick (*I. hexagonus*) species from European hedgehogs. Exp. Appl. Acarol. 54, 151–164. http://dx.doi.org/10.1007/s10493-011-9432-x.
Poulin, R., 2007. Evolutionary Ecology of Parasites. Princeton University Press, Princeton, USA.
R Core Team, 2017. R: A Language and Environment for Statistical Computing.
Reeve, N., 1994. Hedgehogs, Poyser Natural History. T. & A.D. Poyser.
Robbins, R.G., Keirans, J.E., 1992. Systematics and Ecology of the Subgenus *Ixodiopsis* (Acari: Ixodidae: *Ixodes*). Entomological Society of America, Lanham, Maryland.
Sonenshine, D.E., 1991. Biology of Ticks. Oxford University Press, Oxford, UK.
Stouthamer, R., Breeuwer, J.A.J., Hurst, G.D.D., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53, 71–102. http://dx.doi.org/10.1146/annurev.micro.53.1.71.
Špitalská, E., Literák, I., Kocianová, E., Taragel'ová, V., 2011. The importance of *Ixodes arboricola* in transmission of *Rickettsia* spp., *Anaplasma phagocytophilum*, and *Borrelia burgdorferi* Sensu Lato; Czech republic, Central Europe. Vector-Borne Zoonotic Dis. 11, 1235–1241. http://dx.doi.org/10.1089/vbz.2010.0210.
Thompson, J.D., Gibson, T.J., Higgins, D.G., 2002. Multiple sequence alignment using ClustalW and ClustalX. Curr. Protoc. Bioinf. Chapter 2 (Unit 2.3). http://dx.doi.org/10.1002/0471250953.bi0203s00.
Toutoungi, L.N., Gem, L., Aeschlimann, A., 1993. Biology of *Ixodes (Pholeoixodes) hexagonus* under laboratory conditions. Part I. Immature stages. Exp. Appl. Acarol. 17, 655–662.
Tyller, Z., Paclík, M., Remeš, V., 2012. Winter night inspections of nest boxes affect their occupancy and reuse for roosting by cavity nesting birds. Acta Ornithol. 47, 79–85. http://dx.doi.org/10.3161/000164512X653944.
Van Oosten, A.R., Heylen, D.J.A., Jordaens, K., Backeljau, T., Matthysen, E., 2014. Population genetic structure of the tree-hole tick *Ixodes arboricola* (Acari: ixodidae) at different spatial scales. Heredity (Edinb). 113, 408–415. http://dx.doi.org/10.1038/hdy.2014.41.
Van Oosten, A.R., Matthysen, E., Heylen, D.J.A., 2016a. Mating strategies and multiple paternity, assessed by microsatellites, of the dispersal-limited, ectoparasitic tree-hole tick, *Ixodes arboricola*. Int. J. Parasitol. 46, 593–602. http://dx.doi.org/10.1016/j.ijpara.2016.05.004.
Van Oosten, A.R., Matthysen, E., Heylen, D.J.A., 2016b. The more the merrier − experimental evidence for density-dependent feeding facilitation in the bird-specialised tick *Ixodes arboricola*. Int. J. Parasitol. 46, 187–193. http://dx.doi.org/10.1016/j.ijpara.2015.11.002.
Walter, G., Liebisch, A., Streichert, J., 1979. Untersuchungen zur Biologie und Verbreitung von Zecken (Ixodoidea, Ixodidae) in Norddeutschland. Angew. Ornithol. 5, 65–73.
Wernegreen, J.J., Loram, J.E., Douglas, A.E., Bhattacharya, D., 2012. Endosymbiosis. Curr. Biol. 22, 555–561. http://dx.doi.org/10.1016/j.cub.2012.06.010.
Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751. http://dx.doi.org/10.1038/nrmicro1969.
Werren, J.H., 1980. Sex ratio adaptations to local mate competition in a parasitic wasp. Science 208 (4448), 1157–1159.
Wrensch, D.L., Ebbert, M., 1993. Evolution and Diversity of Sex Ratio in Insects and Mites. Chapman & Hall, New York, United States.
Zchori-Fein, E., Perlman, S.J., 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol. Ecol. 13, 2009–2016. http://dx.doi.org/10.1046/j.1365-294X.2004.02203.x.