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PARASITES AND BIOLOGICAL INVASIONS: ALIEN GREY SQUIRREL (*Sciurus carolinensis*) AND NATIVE RED SQUIRREL (*S. vulgaris*) AS MODEL SYSTEM

Ph.D. Thesis
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To you reader,

for being curious

Abstract

Parasites may play an important role in biological invasions through two main mechanisms: enemy release and apparent competition. First, alien species may lose part of their parasite fauna during the introduction process and this release from natural enemies may enhance their performances in the new range. Furthermore, parasites may mediate the competition between alien and native species: invaders may transmit alien parasites to naive native species (spillover) or acquire local parasites, increasing their environmental abundance and their impact on native hosts (spillback) and/or altering the pre-existent host-parasite dynamics. In this study, I investigate the above-mentioned processes, using native Eurasian red squirrels (*Sciurus vulgaris*) and North American Eastern grey squirrels (*S. carolinensis*) introduced to Italy as a model system.

First, I conducted a broad survey of the macroparasite fauna of native red squirrels over a wide geographic area and across different habitats. My results show that the native sciurid has a naturally poor parasite community, likely a consequence of both its arboreal habits and its isolation from other congeners. Both parasite richness and diversity are indeed low, especially for gastro-intestinal helminth fauna, dominated by a single nematode species, the oxyurid *Trypanoxyuris sciuri*. This finding highlights that the species may be particularly vulnerable to parasite spillover from the alien congener and other invasive species.

A parallel survey on the macroparasite fauna of grey squirrels was carried out to detect whether the alien host lost, acquired or introduced to Italy any parasite species. Through this investigation I demonstrated that grey squirrels lost part of their parasite fauna during the introduction process and, although they acquired some European parasites, their number does not compensate the number of species lost, with a resulting parasite richness in Italian populations much lower than in grey squirrels' native range. The helminth community of grey squirrels introduced to Italy is dominated by the North American nematode *Strongyloides robustus*, whereas the most common arthropod is the flea *Ceratophyllus sciurorum*, acquired from red squirrels. Hence, this part of the study gives support to the enemy release hypothesis and shows that this biological invasion holds the premises for both spillover and spill-back mechanisms towards native red squirrels to occur.

In the following part of the study, grey squirrels and their dominant nematode, *S. robustus*, were used as a model to assess the performance of indirect parasitological methods and the relationship between helminth fecundity and intensity. My results reveal that, while flotation is a valid method to survey infection status in living hosts, faecal egg counts do not provide a reliable estimate of *S. robustus* intensity of infection, since density-dependence in nematode fecundity leads to a non-linear relationship between the amount of eggs shed in faeces and parasite load.

Next, I investigated prevalence of alien *S. robustus* and local *T. sciuri* in living red squirrels to detect whether presence of grey squirrels affects the endo-macroparasite community of the native host. I used indirect methods (flotation and tape-tests) to compare infection status in populations of red squirrels living in presence and absence of the alien congener. Results show that *S. robustus* infection is linked to grey squirrel presence, thus confirming that red squirrels acquire this North American nematode via spillover from the invader. Interestingly, also prevalence of *T. sciuri* is significantly higher in red squirrels co-inhabiting with the alien species, suggesting that susceptibility to infection in red squirrels may increase as a consequence of higher stress levels induced by interspecific competition.

Finally, infections by Ljungan virus (a potential zoonoses) and adenoviruses (known to cause gastrointestinal disease and mortality in squirrels in Northern Europe) were investigated in both red and grey squirrels to shed some light on the role played by arboreal sciurids in microparasite circulation. I reported for the first time Ljungan virus in red squirrels, indicating that this infection is not limited to small ground-dwelling rodents, and extended the known distribution of adenoviruses in squirrels to Southern Europe. Besides, the low adenovirus prevalence found in grey squirrels confirms that the alien species is not the source of infection in red squirrels as had been previously presumed.

Overall, the present thesis highlights the importance of taking into account parasitological aspects when dealing with biological invasions. In particular, the model red-grey squirrel teaches that i) macroparasites have the potential to affect biological invasions as much as microparasites do; ii) an exhaustive knowledge of native species parasite fauna is fundamental to investigate apparent competition; iii) apart from introducing alien parasites, alien species may affect native species parasite communities through other mechanisms; iv) inference of parasitological parameters from indirect methods should always be considered carefully.

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CHAPTER 1

Introduction

"I keep six honest serving-men,
(They taught me all I knew);
Their names are What and Why and When
And How and Where and Who."

\- Rudyard Kipling, *The Elephant's Child* -

"*A good scientist is a person in whom*
the childhood quality of perennial curiosity lingers on.
Once he gets an answer, he has other questions."

\- Frederick Seitz -

1.1 Biological invasions: a growing threat

During last decades, as a consequence of increased mobility of people and goods, accidental or purposeful introductions of alien plants and animals outside their native range increased dramatically (Meyerson and Mooney 2007; Westphal et al. 2008; Hulme et al. 2009) and biological invasions are now considered the second cause of biodiversity loss worldwide after habitat destruction (Vitousek et al. 1997). Pimentel et al. (2005) quantified that 42% of endangered species in the United States are primarily threatened (among other causes) by introduced predators or competitors.

Alien species may impact directly on native species and community structure through trophic interactions (predation, grazing or parasitism), competitive exclusion or hybridization, or even affect the whole ecosystem, altering its structure and functions (reviewed in Mack et al. 2000). Some of these impacts are quite immediate and evident (e.g. impacts due to predation or interference competition), whereas others (e.g. impacts resulting from exploitation competition or hybridization) can be more subtle and difficult to recognise (Simberloff et al. 2013). Some species may even affect the whole habitat structure and ecosystem functionality: for example, many alien plants are known to profoundly alter soil composition and nutrient cycle, with complex cascade effects on the whole trophic web (Vilà et al. 2011; Simberloff 2011). Consequently, the effects of biological invasions on local biodiversity are often difficult to understand and predict: of the >11000 alien species introduced to Europe, only 11% has a proven ecological impact (Hulme et al. 2009).

In addition, aside from the evident direct damage to man-made structures or cultivated crops caused by some alien species, environmental impact of biological invasions has repercussions on human activities through the disruption of the so-called "ecosystem services" (Vilà et al. 2010). Economic costs of biological invasions in Europe (including costs for eradication and control and for research, prevention and monitoring) are estimated in 12.5 billions of Euros/year (Kettunen et al. 2008).

Finally, some introduced species may also represent a sanitary threat for human health, livestock and wildlife, acting as allergens (e.g. *Ambrosia* spp. introduced to Europe, Wopfner et al. 2005), as vectors or reservoirs for local infections or bringing with them novel parasites (see Par. 1.2.2). In the latter case, parasites themselves may be considered as invaders: parasite translocation (alone or along with their hosts) outside their range ("pathogen pollution", Daszak et al. 2000; Cunningham et al. 2003) is a growing phenomena and may lead to infectious disease emergence both in humans, wildlife and domesticated species (Daszak et al. 2000; Hatcher et al. 2012).

For all these reasons, interest about biological invasions is constantly growing and the need to understand mechanisms driving alien species settlement, their spread and their interactions with native species has become a priority in ecological research.

1.2 Parasites and biological invasions

In recent years several authors pointed out how parasites may play an important role in biological invasions via two main mechanisms that are explained in detail in the following : enemy release and apparent competition (reviewed in Prenter et al. 2004; Dunn et al. 2012). The term "enemy" broadly encompasses predators, grazers and parasites. I will focus on the role of parasites, hereafter using the term, according to Anderson and May (1992), to refer to both micro- (generally identified in viruses, bacteria and protozoan) and macroparasites (helminths and arthropods) and parasitoids as well.

1.2.1 The Enemy Release Hypothesis

Most alien species, once introduced to the new range, fail to establish a viable population, whereas others become invasive, spreading rapidly and causing extensive damage to native biodiversity (Williamson and Fitter 1996; Kolar and Lodge 2001). There can be many, and not mutually exclusive, explanations for the variable success of biological invasions that focus either on invasibility (the ensemble of ecosystem properties that determine its susceptibility to invasion, Lonsdale 1999) or invasiveness (the features of an alien species that define its ability to invade, Sakai et al. 2001). One of the proposed explanations for the varying degree of invasiveness observed in introduced species is the so-called Enemy Release Hypothesis (ERH) that predicts that i) alien species will likely lose part of their natural enemies (predators, grazers and parasites) during the introduction process and ii) they will benefit from this loss, showing enhanced performances in the new environment compared to their native range and attaining a competitive advantage on native species.

A loss of natural enemies in introduced species is indeed observed in several alien plants and animals (reviewed in: (Mitchell and Power 2003; Torchin et al. 2003; Torchin and Mitchell 2004). There can be several reasons behind the loss of parasites by alien species (MacLeod et al. 2010). First, some parasites may never reach the new environment as a consequence of stochastic founder effects (i.e. few introduced individuals may carry only a subset of native parasite communities) or, especially in case of exotic pets, housing conditions or medical treatments during captivity. Second, parasites may arrive in the new environment but fail to persist because of unsuitable abiotic conditions, absence of specific intermediate hosts or low transmission rates due to small population size (i.e. low propagule pressure, Lockwood et al. 2005) coupled with the absence of suitable alternative native hosts. Invaders may even acquire some local parasites, but the number of acquired species usually does not compensate the number of species lost (Torchin and Mitchell 2004, Fig. 1): as a result, in the area of introduction, populations of alien species often show impoverished parasite communities (in terms of richness and/or prevalence) compared to their native range.

As stated above, according to ERH, the consequence of this loss of natural enemies should be an increased demographic success for invaders. This second part of the hypothesis is based on the assumption that parasites reduce individual fitness, thus negatively affecting population dynamics. In particular, parasites may directly reduce host growth, fecundity and survival (reviewed in Tompkins and Begon 1999), or affect host fitness indirectly by increasing competition (intra- and interspecific) and vulnerability to predators (reviewed in Hatcher et al. 2006), or through the costs associated with defence mechanisms (Lochmiller and Deerenberg 2000; Rigby et al. 2002; Zuk and Stoehr 2002). Hence, theory suggests that a loss of enemies should result in a release from their detrimental effects, but despite the demonstrated importance of predators and parasites on population dynamics, an escape from natural enemies might still not translate in an effective advantage for invaders. For example, a loss of parasite species might result in reduced interspecific competition for the remaining parasites, thus leading to an increase in their abundance and impact (Lello et al. 2004).

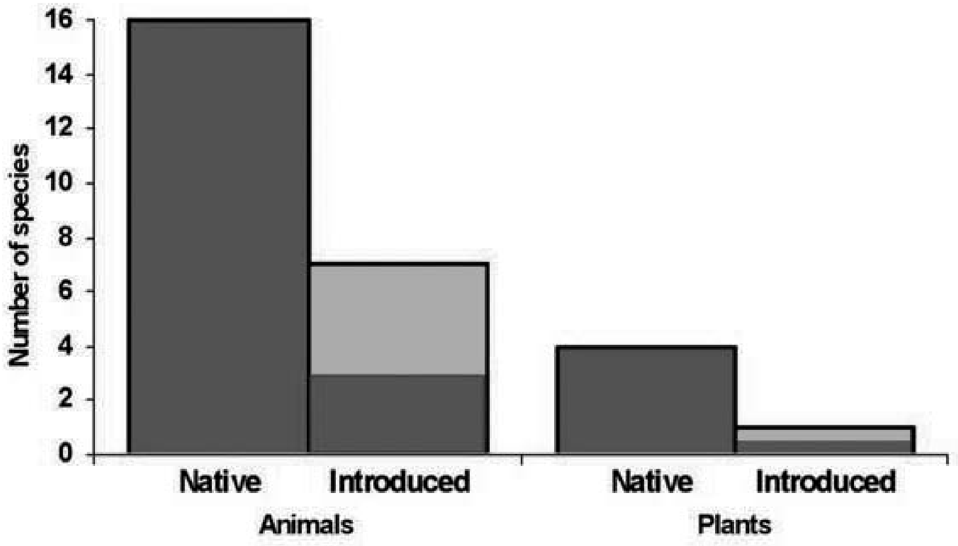

Figure 1 - Average number of parasite species infecting alien hosts in their native and introduced range. Dark bars indicate parasites introduced from the native range and light bars indicate local parasites acquired in the new range (from Torchin and Mitchell 2004).

Most of the studies supporting ERH are indeed comparative studies observing a loss of enemies after introduction, whereas studies effectively evaluating the effect of this loss on invaders are few, especially on animals. For example, at the biogeographical scale (i.e. comparing invader performance in the native and introduction range) Torchin et al. (2001) observed that European green crabs (*Carcinus maenas*) attain significantly greater body size in the introduction range, where parasitic castrators (negatively associated with body size in the native range) are absent. Also, at the community scale (i.e. comparing performances of native versus alien congeners), Roche et al. (2010) compared parasite loads of two competing cichlid fish in Panama and found that parasite abundance in the native species was significantly higher than in the invader and was negatively correlated with fish body condition, whereas in the introduced species no such association was found.

Furthermore, true experimental testing of ERH is still scarce: evidence for enemy release comes mostly from experiments of herbivore exclusion on invasive plants (e.g. DeWalt et al. 2004; Uesugi and Kessler 2013; Lakeman-Fraser and Ewers 2013), whereas, to my knowledge, only two studies experimentally tested ERH on invasive animal species. Prior and Hellmann (2013) surveyed the parasitoid community of the invasive gall wasp *Neuroterus saltatorius* in its native and introduction range, then conducted an enemy exclusion experiment in both ranges to compare the effects of parasitoid community on invader population dynamics. Their results show that some enemy loss occurred and that it may partially concur to the increased survivorship of the wasp in the new range, but suggest also that there are other, unidentified factors that may contribute more. At the community scale, Aliabadi and Juliano (2002) demonstrated that the release from a protozoan gut parasite leads to an increase in the competitive impact of the invasive mosquito *Aedes albopictus* on native *Ochlerotatus triseriatus*.

This general lack of experimental tests of ERH is probably due to the fact that such experiments, especially at the biogeographical scale, are time- and labour-demanding. Furthermore, quantifying parasite impact on fitness parameters in animals (especially vertebrates) may be a long and methodologically complex matter due to the low pathogenic effects of most parasites infections in wildlife. Similarly, even most of the observational studies comparing parasite communities of alien species in native and invaded ranges focus on plants or invertebrates. Excluding the meta analysis by Torchin et al. (2003), only a few authors surveyed parasites of alien vertebrates (e.g. Dove 2000; Marr et al. 2008; Roche et al. 2010; Marzal et al. 2011;

Lacerda et al. 2013). Again, the reason for this could be that sampling and reaching a sufficient sample size is more difficult than in plants.

Finally, there are even some cases in which enemies communities of invaders are richer in the new than in the native range (e.g. Dare and Forbes 2013) and this, coupled with the lack of experimental tests, has led to some criticism to ERH (see Colautti et al. 2004). As abovementioned, ERH is not the only proposed explanation for invasiveness: there may be some cases in which several factors contribute together to the complex scenario of the invasion process or others in which enemies do not play any role at all.

1.2.2 Parasite-mediated competition

As mentioned before, the introduction of alien species has been recognised as one of the major causes for Emerging Infectious Diseases (EIDs, i.e. diseases that have recently increased in incidence, impact, pathogenicity, geographical or host range, Daszak et al. 2003) in wildlife, humans and livestock (Daszak et al. 2000). Relatively to parasite transmission, two different events may occur when an alien species is introduced outside its native range: i) the invader carries along novel parasites and transmits them to resident species (spillover, Fig. 2); and/or ii) the invader acquires local parasites from native hosts and transmits it back to them (spillback, Fig. 2). Whatever the case (and whatever its outcome), susceptibility of alien and native hosts to shared parasites will likely be very different: we have a more tolerant host that is adapted to the parasite and a naive host that was never exposed to it and thus did not evolve any defence mechanisms. As a consequence, biological invasions are the ideal scenario for apparent competition to occur.

Apparent competition is a type of indirect interaction between two species defined as a negative effect of one species on the other, mediated through the action of shared natural enemies (Holt 1977; Price et al. 1986; Price et al. 1988). Again, the role of enemy may be played also by predators or herbivores, but here I will focus on parasites as natural enemies, hence on parasite-mediated competition (PMC). The necessary premise for PMC is a differential vulnerability of the two competing hosts to the shared parasite: the more tolerant species acts as a reservoir (i.e. a host which may independently maintain the parasite population in the environment) and transmits the parasite to the less tolerant species (Hudson and Greenman 1998).

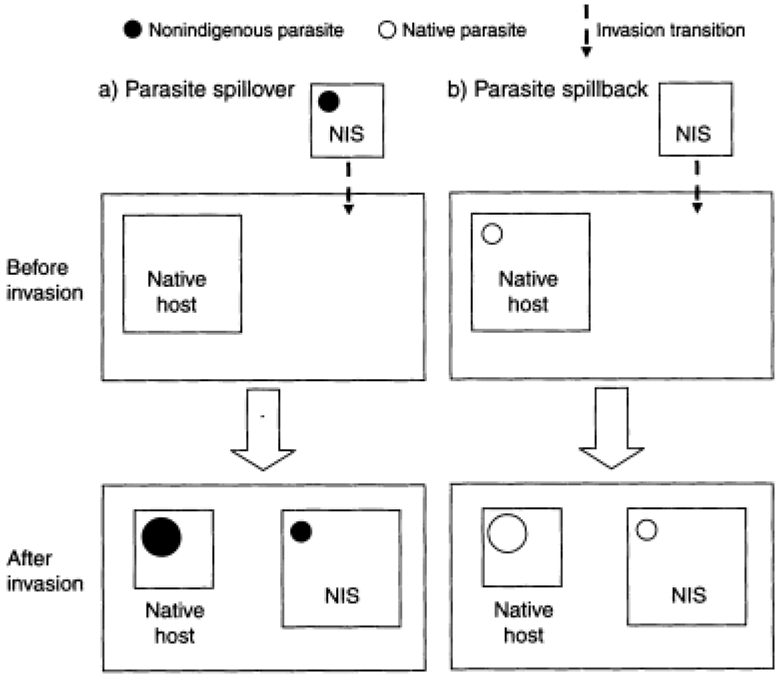

Figure 2 - Scheme illustrating spillover and spillback processes in native and introduced hosts (i.e. NIS, non-indigenous species). The size of the host box represents host population size and the size of the circle represents infection burden (from Kelly et al. 2009).

PMC, like interference or exploitation competition, may even lead to competitive exclusion of one host over the other as showed by Park (1948) in his classic experiment with flour beetles *Tribolium confusum* and *T. castaneum*. The former species was the superior competitor until a parasite was added to the system, reversing the outcome of the interaction and leading to *T. confusum* exclusion. Since parasites are important and ubiquitous regulators of population dynamics (Anderson and May 1978; May and Anderson 1978) and different host species inevitably have different resistance and tolerance to shared parasites (Woolhouse et al. 2001), PMC is likely as common an interaction as any other form of competition, only more difficult to detect and recognise, especially when involved parasites have low pathogenic effects.

However, when human intervention brings in contact hosts and parasites that have evolved separately, PMC may become more evident and its impact more severe. The first documented examples of PMC come indeed from biological invasions, such as the introduction, along with cattle, of rinderpest to Africa (Plowright 1982) or the spread of avian malaria in Hawaii together with European birds (Warner 1968). In a recent review, Strauss et al. (2012) use the term disease-mediated invasions (DMIs) to refer to biological invasions in which PMC benefits the invader and, depending on the process behind the competitive advantage, distinguish between spillover and spillback DMIs. Both spillover and spillback mechanisms are also consistent respectively with novel pathogen and endemic pathogen hypothesis proposed as causes for infectious disease emergence in wildlife (Rachowicz et al. 2005).

Spillover DMIs are probably the most well known of the two scenarios, since they may have a more immediate and visible impact on native biodiversity. In general, spillover is defined as the transmission of infectious agents from reservoir animal populations to sympatric wildlife (Daszak et al. 2000). In biological invasions, alien parasites carried by the invader may be considered as "biological weapons": those that successfully spillover to native species will likely have sub-lethal effects on their original host, but may be very detrimental to local naive species, leading to a competitive advantage for the invader. Besides, it is likely that parasite species that are highly pathogenic to the invader will not be introduced in the new range because infected individuals will die during translocation (Prenter et al. 2004; Strauss et al. 2012). The example above about avian malaria is a case of spillover DMI, but many other examples are known (reviewed in Tompkins and Poulin 2006; Dunn 2009; Strauss et al. 2012).

Spillback DMIs occurs when an introduced species successfully acquires a local parasite, is tolerant to it and acts as a competent reservoir, transmitting the parasite back to native hosts (Kelly et al. 2009). The presence of additional hosts may profoundly alter parasite epidemiology, leading to an increase in environmental abundance of parasite infective stages and in transmission rates toward native hosts.

This perturbation of pre-existent host-parasite dynamics may exacerbate the impact of the parasite on native hosts and, if the invader is less susceptible than native hosts, PMC will be in its favour and will facilitate the invasion (Strauss et al. 2012). Compared to spillover DMIs, there are less examples of spillback in literature, but some authors argue that this process may be as common as parasite spillover, since many EIDs associated with biological invasions and thought to be caused by introduced parasites, may be instead the result of spillback of previously rare, undocumented species (Tompkins and Poulin 2006; Kelly et al. 2009).

However, the acquisition of local parasites by the invader may have diverse outcomes than spillback, depending on the pathogenic effect of the parasite and on the suitability of the new host for its development and transmission. If the invader acquires the parasite, but has a low reservoir competence, it may act as a sink, reducing transmission and decreasing parasite abundance in the environment, with benefits for native species (dilution effect, e.g. Thieltges et al. 2009). On the other hand, the invader may be a competent host, but the acquired parasite may be lethal to it, leading to PMC favourable to native species and even preventing the establishment of the alien species (Hilker et al. 2005). Causes behind failed invasions are seldom investigated, hence there are few examples of introductions prevented by PMC, most of them about translocations of domesticated animals hindered by local diseases (e.g. Steverding 2008 on African trypanosomiasis). Similarly, neither the introduction of alien parasites, nor their spillover to native hosts do automatically lead to PMC, since a detrimental effect on the native host is likely, but is not certain.

Magnitude of both spillover and spillback processes may also be variable, depending on the characteristics of involved hosts and parasites. First, host-switching is facilitated between hosts sharing similar physiological and immunological characteristics, hence transmission risk is higher between closely-related species (Freeland 1983; Poulin and Mouillot 2003). The number of species that may be infected, depends also on the degree of specialisation of the parasite (Woolhouse et al. 2001): generalist parasites may more easily adapt to a wider host range and disentangling PMC in multiple-host system may get very complicated. In addition, the introduction of vectors or intermediate hosts for alien or even local parasites may further complicate the scenario. For example both avian pox and avian malaria were likely introduced to Hawaii along with domestic birds since the end of the 18th century, but the epizootics of both diseases are thought to have been triggered only later, after the introduction of the alien vector *Culex quinquefasciatus* (van Riper et al. 2002). Finally, the impact of microparasites (especially viruses) transmission to naive hosts is often evident and immediate, whereas macroparasites have generally sub-lethal effects that may be difficult to highlight and quantify (Dobson and Foufopoulos 2001), hence macroparasite DMIs may easily be overlooked.

1.3 Study aims and outline

This study is an attempt to investigate how parasites may mediate an invader's success and its interactions with native species, using two closely-related vertebrate hosts as model system. Object of the study are alien Eastern grey squirrels (*Sciurus carolinensis*) and native Eurasian red squirrels (*S. vulgaris*): parasite communities of both species in Italy were investigated to clarify whether there is any evidence and support for enemy release hypothesis or parasite-mediated competition in this biological invasion.

1.3.1. *Case of study: the red-grey squirrel system*

The grey squirrel is a North American species that has been introduced to Europe and whose detrimental impact on native biodiversity and human activities is widely recognised: the species is included in the IUCN list of world's worst invasive species (Lowe et al. 2000).

Grey squirrels were introduced to the British Isles since the end of the 19th century (Middleton 1930; Reynolds 1985). Following its introduction, the species has spread through Britain and Ireland, rapidly replacing red squirrels: today the native species survives mainly in South and Central Scotland and in large continuous populations in Northern Scotland (Lurz 2010). Local extinction of red squirrels is caused mainly by interspecific competition for resources which reduces female reproductive success and juvenile recruitment (Wauters et al. 2002b; Wauters et al. 2002a; Gurnell et al. 2004; Wauters et al. 2005), but it is also mediated by the presence of a shared microparasite, the squirrelpoxvirus (Tompkins et al. 2002). Grey squirrels act as healthy carriers for this virus, whereas squirrelpox is lethal for most infected red squirrels, causing lesions in the eye and mouth region, blindness and subsequent, rapid death by starvation or secondary infections (Tompkins et al. 2002). As a result, population modelling predicts that, where the virus is present, replacement of red squirrels by grey squirrels can be up to 25 times faster than in disease-free areas (Rushton et al. 2005). The disease was unrecorded prior to grey squirrel introduction and McInnes et al. (2006) found seropositive grey squirrel in their native range, suggesting that the virus was likely introduced from North America, along with its host. Hence, the alien species act as a reservoir from which the infection can spillover to the native host, accelerating native species decline and making this parasite-host system one of the best known and studied examples of disease-mediated invasions. Nevertheless, the role played by squirrelpox in the interaction between red and grey squirrels has been overlooked for long, likely because the high virulence made the virus scarcely visible: in England and Wales pox-like lesions in dead red squirrels were mentioned already in 1930, but the virus was not identified until the eighties (Bosch and Lurz 2012) and only in the late nineties some authors hypothesised that grey squirrels could be the source of the disease, making squirrelpox an important driver in the competition between the two species (Sainsbury and Gurnell 1995; Sainsbury et al. 2000). Similarly, the first cases of squirrelpox in red squirrels in Scotland and Ireland were recorded respectively in 2007 and 2011 (McInnes et al. 2009; McInnes et al. 2013).

During the second half of last century the grey squirrel was repeatedly introduced also to Northern and Central Italy (Fig. 3), becoming a threat to red squirrels in continental Europe (Martinoli et al. 2010; Bertolino et al. 2013).

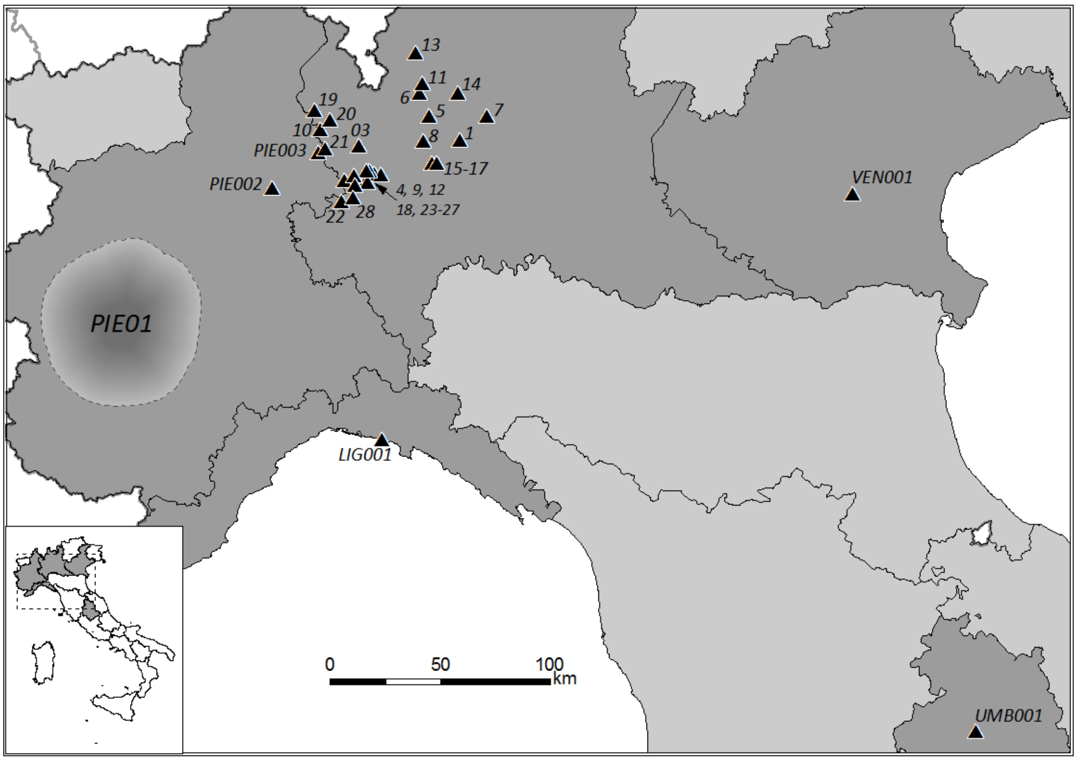

Figure 3 - Distribution of grey squirrels in Italy (from Martinoli et al. 2010).

Currently, the species is well established in the North-western part of the country with a large metapopulation in Piedmont (originated from the first introduction of four squirrels in 1948), a small urban population in Liguria and several populations of unclear origin that appeared in Lombardy during the last decades (Martinoli et al. 2010; Bertolino et al. 2013). More recently a new population in Central Italy, near the city of Perugia, has been reported (Martinoli et al. 2010) and this new introduction is particularly alarming since the species could spread rapidly along the continuous broadleaves forests on the Apennines, threatening two endemic red squirrel subspecies: *S. v. italicus* and *S. v. meridionalis*. As in the British Isles, in all the sites where the alien species is present, the red squirrel disappeared or is declining because of exploitation competition (Bertolino et al. 2013). To date, squirrelpoxvirus is unrecorded in Italy, but in the light of British experience, this may not mean that the virus is not present.

1.3.2. Outline of the study

Despite the attention received in recent years by the squirrelpox-red-grey system, to my knowledge, nobody investigated whether other parasites, in particular helminths, may play, or have played, a role in grey squirrel invasion and in its competition with the native congener. Hence, I will focus first of all on macroparasites, investigating the parasite fauna of grey squirrels introduced to Italy and comparing the parasite communities of native red squirrels' populations in presence of the alien congener and in grey-free areas. I have been able to obtain a large number of grey squirrels carcasses thanks to a EU/LIFE+ project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at removing the alien species, thus avoiding the problems of small sample size often encountered in field parasitological surveys. Endoparasite fauna of native red squirrels was investigated making use of road-killed individuals or, on living animals, indirect methods such as parasite egg count in faeces.

Literature information on red squirrels' macroparasite fauna, especially helminths, was particularly scarce. Hence, to be able to highlight any variation in the parasite community of red squirrels syntopic with the alien species, the first, necessary step has been to investigate macroparasite species naturally infecting red squirrels and the factors affecting their abundance (**Chapter 2**). Then, I explored parasite communities of several populations of alien grey squirrels in Northern Italy and compared it to literature data from their native range, in order to detect any loss, acquisition or introduction of parasite species that may support enemy release hypothesis or potentially lead to parasite-mediated competition (**Chapter 3**). In a subsample of grey squirrels, the results of parasitological, post-mortem examinations were compared to data obtained from coprological tests, primarily to understand whether faecal egg counts are a reliable measure to estimate parasite presence and parasite abundance in living squirrels (**Chapter 4**). Then, I analysed and compared macroparasite infection of red squirrels in red-only and red-grey sites, to detect whether spillover from grey squirrels occurs and whether the presence of the alien species affects local parasites prevalence (**Chapter 5**). Finally, I also investigated the presence of Ljungan virus (a potentially zoonotic disease) and Adenovirus (known to cause mortality in red squirrels around Europe) in both species to understand if the diseases are present in Italian squirrels and if the two species may play a role in their epidemiology (**Chapter 6**).

References

Aliabadi BW, Juliano SA (2002) Escape from Gregarine Parasites Affects the Competitive Interactions of an Invasive Mosquito. Biol Invasions 4:283–297. doi: 10.1023/A:1020933705556

Anderson RM, May RM (1992) Infectious Diseases of Humans: Dynamics and Control. Oxford University Press, Oxford

Anderson RM, May RM (1978) Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes. J Anim Ecol 47:219–247. doi: 10.2307/3933

Bertolino S, Montezemolo NC di, Preatoni DG, et al. (2013) A grey future for Europe: Sciurus carolinensis is replacing native red squirrels in Italy. Biol Invasions 1–10. doi: 10.1007/s10530-013-0502-3

Bosch S, Lurz PWW (2012) The Eurasian Red Squirrel. Westarp Wissenschaften, Hohenwarsleben

Colautti RI, Ricciardi A, Grigorovich IA, MacIsaac HJ (2004) Is invasion success explained by the enemy release hypothesis? Ecol Lett 7:721–733. doi: 10.1111/j.1461-0248.2004.00616.x

Cunningham AA, Daszak P, Rodríguez JP (2003) Pathogen pollution: defining a parasitological threat to biodiversity conservation. J Parasitol 89:S78–S83.

Dare OK, Forbes MR (2013) Do invasive bullfrogs in Victoria, British Columbia, Canada, show evidence of parasite release? J Helminthol 87:195–202. doi: 10.1017/S0022149X12000211

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging Infectious Diseases of Wildlife--Threats to Biodiversity and Human Health. Science 287:443–449. doi: 10.1126/science.287.5452.443

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Divers Distrib 9:141–150. doi: 10.1046/j.1472-4642.2003.00016.x

DeWalt SJ, Denslow JS, Ickes K (2004) Natural-Enemy Release Facilitates Habitat Expansion of the Invasive Tropical Shrub Clidemia hirta. Ecology 85:471–483. doi: 10.1890/02-0728

Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. Philos Trans R Soc Lond B Biol Sci 356:1001–1012. doi: 10.1098/rstb.2001.0900

Dove ADM (2000) Richness patterns in the parasite communities of exotic poeciliid fishes. Parasitology 120:609–623.

Dunn AM (2009) Chapter 7 Parasites and Biological Invasions. In: Joanne P. Webster (ed) Adv. Parasitol. Academic Press, pp 161–184

Dunn AM, Torchin ME, Hatcher MJ, et al. (2012) Indirect effects of parasites in invasions. Funct Ecol 26:1262–1274. doi: 10.1111/j.1365-2435.2012.02041.x

Freeland WJ (1983) Parasites and the coexistence of animal host species. Am Nat 121:223–236.

Gurnell J, Wauters LA, Lurz PWW, Tosi G (2004) Alien species and interspecific competition: effects of introduced eastern grey squirrels on red squirrel population dynamics. J Anim Ecol 73:26–35. doi: 10.1111/j.1365-2656.2004.00791.x

Hatcher MJ, Dick JTA, Dunn AM (2012) Disease emergence and invasions. Funct Ecol 26:1275–1287. doi: 10.1111/j.1365-2435.2012.02031.x

Hatcher MJ, Dick JTA, Dunn AM (2006) How parasites affect interactions between competitors and predators. Ecol Lett 9:1253–1271. doi: 10.1111/j.1461-0248.2006.00964.x

Hilker FM, Lewis MA, Seno H, et al. (2005) Pathogens can Slow Down or Reverse Invasion Fronts of their Hosts. Biol Invasions 7:817–832. doi: 10.1007/s10530-005-5215-9

Holt RD (1977) Predation, apparent competition, and the structure of prey communities. Theor Popul Biol 12:197–229. doi: 10.1016/0040-5809(77)90042-9

Hudson P, Greenman J (1998) Competition mediated by parasites: biological and theoretical progress. Trends Ecol Evol 13:387–390. doi: 10.1016/S0169-5347(98)01475-X

Hulme PE, Pyšek P, Nentwig W, Vilà M (2009) Will threat of biological invasions unite the European Union. Science 324:40–41.

Kelly DW, Paterson RA, Townsend CR, et al. (2009) Parasite spillback: A neglected concept in invasion ecology? Ecology 90:2047–2056. doi: 10.1890/08-1085.1

Kettunen M, Genovesi P, Gollasch S, et al. (2008) Technical support to EU strategy on invasive species (IS)—assessment of the impacts of IS in Europe and the EU (Final module report for the European Commission). 40 + Annexes.

Kolar CS, Lodge DM (2001) Progress in invasion biology: predicting invaders. Trends Ecol Evol 16:199–204. doi: 10.1016/S0169-5347(01)02101-2

Lacerda ACF, Takemoto RM, Poulin R, Pavanelli GC (2013) Parasites of the fish Cichla piquiti (Cichlidae) in native and invaded Brazilian basins: release not from the enemy, but from its effects. Parasitol Res 112:279–288. doi: 10.1007/s00436-012-3135-z

Lakeman-Fraser P, Ewers RM (2013) Enemy release promotes range expansion in a host plant. Oecologia 172:1203–1212. doi: 10.1007/s00442-012-2555-x

Lello J, Boag B, Fenton A, et al. (2004) Competition and mutualism among the gut helminths of a mammalian host. Nature 428:840–844. doi: 10.1038/nature02490

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98. doi: 10.1034/j.1600-0706.2000.880110.x

Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20:223–228. doi: 10.1016/j.tree.2005.02.004

Lonsdale WM (1999) Global Patterns of Plant Invasions and The Concept of Invasibility. Ecology 80:1522–1536. doi: 10.1890/0012-9658(1999)080[1522:GPOPIA]2.0.CO;2

Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the world's worst invasive alien species: a selection from the global invasive species database. Invasive Species Specialist Group Auckland,, New Zealand

Lurz PWW (2010) Red Squirrels: Naturally Scottish. Scottish Natural Heritage, Perth

Mack RN, Simberloff D, Lonsdale WM, et al. (2000) Biotic Invasions: Causes, Epidemiology, Global Consequences, and Control. Ecol Appl 10:689–710. doi: 10.1890/1051-0761(2000)010[0689:BICEGC]2.0.CO;2

MacLeod CJ, Paterson AM, Tompkins DM, Duncan RP (2010) Parasites lost - do invaders miss the boat or drown on arrival? Ecol Lett 13:516–527. doi: 10.1111/j.1461-0248.2010.01446.x

Marr SR, Mautz WJ, Hara AH (2008) Parasite loss and introduced species: a comparison of the parasites of the Puerto Rican tree frog, (Eleutherodactylus coqui), in its native and introduced ranges. Biol Invasions 10:1289–1298. doi: 10.1007/s10530-007-9203-0

Martinoli A, Bertolino S, Preatoni DG, et al. (2010) Headcount 2010: the multiplication of the grey squirrel populations introduced to Italy. Hystrix Ital. J. Mammal. 21:

Marzal A, Ricklefs RE, Valkiūnas G, et al. (2011) Diversity, Loss, and Gain of Malaria Parasites in a Globally Invasive Bird. PLoS ONE 6:e21905. doi: 10.1371/journal.pone.0021905

May RM, Anderson RM (1978) Regulation and Stability of Host-Parasite Population Interactions: II. Destabilizing Processes. J Anim Ecol 47:249–267. doi: 10.2307/3934

McInnes CJ, Coulter L, Dagleish MP, et al. (2009) First cases of squirrelpox in red squirrels (Sciurus vulgaris) in Scotland. Vet Rec 164:528–531. doi: 10.1136/vr.164.17.528

McInnes CJ, Coulter L, Dagleish MP, et al. (2013) The emergence of squirrelpox in Ireland. Anim Conserv 16:51–59. doi: 10.1111/j.1469-1795.2012.00570.x

McInnes CJ, Wood AR, Thomas K, et al. (2006) Genomic characterization of a novel poxvirus contributing to the decline of the red squirrel (Sciurus vulgaris) in the UK. J Gen Virol 87:2115–2125. doi: 10.1099/vir.0.81966-0

Meyerson LA, Mooney HA (2007) Invasive alien species in an era of globalization. Front Ecol Environ 5:199–208. doi: 10.1890/1540-9295(2007)5[199:IASIAE]2.0.CO;2

Middleton D (1930) The Ecology of the American Grey Squirrel (Sciurus carolinensis Gmelin) in the British Isles. Proc Zool Soc Lond 100:809–843. doi: 10.1111/j.1096-3642.1930.tb01000.x

Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral pathogens. Nature 421:625–627.

Park T (1948) Experimental studies of interspecific competition I. Competition between populations of the flour beetles, Tribolium confusum and Tribolium castaneum. Ecol Monogr 18:267–307. doi: 10.2307/1948641

Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52:273–288. doi: 10.1016/j.ecolecon.2004.10.002

Plowright W (1982) The effects of rinderpest and rinderpest control on wildlife in Africa. Symp Zool Soc Lond 50:1–28.

Poulin R, Mouillot D (2003) Parasite specialization from a phylogenetic perspective: a new index of host specificity. Parasitology 126:473–480. doi: 10.1017/S0031182003002993

Prenter J, MacNeil C, Dick JT., Dunn AM (2004) Roles of parasites in animal invasions. Trends Ecol Evol 19:385–390. doi: 10.1016/j.tree.2004.05.002

Price PW, Westoby M, Rice B, et al. (1986) Parasite Mediation in Ecological Interactions. Annu Rev Ecol Syst 17:487–505.

Price PW, Westoby M, Rice B (1988) Parasite-mediated competition: some predictions and tests. Am. Nat. 131:

Prior KM, Hellmann JJ (2013) Does enemy loss cause release? A biogeographical comparison of parasitoid effects on an introduced insect. Ecology 94:1015–1024. doi: 10.1890/12-1710.1

Rachowicz LJ, Hero J-M, Alford RA, et al. (2005) The Novel and Endemic Pathogen Hypotheses: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. Conserv Biol 19:1441–1448. doi: 10.1111/j.1523-1739.2005.00255.x

Reynolds JC (1985) Details of the Geographic Replacement of the Red Squirrel (Sciurus vulgaris) by the Grey Squirrel (Sciurus carolinensis) in Eastern England. J Anim Ecol 54:149. doi: 10.2307/4627

Rigby MC, Hechinger RF, Stevens L (2002) Why should parasite resistance be costly? Trends Parasitol 18:116–120. doi: 10.1016/S1471-4922(01)02203-6

Van Riper C, van Riper SG, Hansen WR, Hackett SJ (2002) Epizootiology and Effect of Avian Pox on Hawaiian Forest Birds. The Auk 119:929–942. doi: 10.1642/0004-8038(2002)119[0929:EAEOAP]2.0.CO;2

Roche DG, Leung B, Mendoza Franco EF, Torchin ME (2010) Higher parasite richness, abundance and impact in native versus introduced cichlid fishes. Int J Parasitol 40:1525–1530. doi: 10.1016/j.ijpara.2010.05.007

Rushton SP, Lurz PWW, Gurnell J, et al. (2005) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. Epidemiol Infect 134:521. doi: 10.1017/S0950268805005303

Sainsbury AW, Gurnell J (1995) An investigation into the health and welfare of red squirrels, Sciurus vulgaris, involved in reintroduction studies. Vet Rec 137:367–370. doi: 10.1136/vr.137.15.367

Sainsbury AW, Nettleton P, Gilray J, Gurnell J (2000) Grey squirrels have high seroprevalence to a parapoxvirus associated with deaths in red squirrels. Anim Conserv 3:229–233.

Sakai AK, Allendorf FW, Holt JS, et al. (2001) The Population Biology of Invasive Species. Annu Rev Ecol Syst 32:305–332.

Simberloff D (2011) How common are invasion-induced ecosystem impacts? Biol Invasions 13:1255–1268. doi: 10.1007/s10530-011-9956-3

Simberloff D, Martin J-L, Genovesi P, et al. (2013) Impacts of biological invasions: what's what and the way forward. Trends Ecol Evol 28:58–66. doi: 10.1016/j.tree.2012.07.013

Steverding D (2008) The history of African trypanosomiasis. Parasit Vectors 1:3. doi: 10.1186/1756-3305-1-3

Strauss A, White A, Boots M (2012) Invading with biological weapons: the importance of disease-mediated invasions. Funct Ecol 26:1249–1261. doi: 10.1111/1365-2435.12011

Thieltges DW, Reise K, Prinz K, Jensen KT (2009) Invaders interfere with native parasite–host interactions. Biol Invasions 11:1421–1429. doi: 10.1007/s10530-008-9350-y

Tompkins DM, Begon M (1999) Parasites Can Regulate Wildlife Populations. Parasitol Today 15:311–313. doi: 10.1016/S0169-4758(99)01484-2

Tompkins DM, Poulin R (2006) Parasites and Biological Invasions. In: Allen DRB, Lee DWG (eds) Biol. Invasions N. Z. Springer Berlin Heidelberg, pp 67–84

Tompkins DM, Sainsbury AW, Nettleton P, et al. (2002) Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. Proc R Soc Lond B Biol Sci 269:529–533.

Torchin ME, Lafferty KD, Dobson AP, et al. (2003) Introduced species and their missing parasites. Nature 421:628–630. doi: 10.1038/nature01346

Torchin ME, Lafferty KD, Kuris AM (2001) Release from Parasites as Natural Enemies: Increased Performance of a Globally Introduced Marine Crab. Biol Invasions 3:333–345. doi: 10.1023/A:1015855019360

Torchin ME, Mitchell CE (2004) Parasites, pathogens, and invasions by plants and animals. Front Ecol Environ 2:183–190. doi: 10.1890/1540-9295(2004)002[0183:PPAIBP]2.0.CO;2

Uesugi A, Kessler A (2013) Herbivore exclusion drives the evolution of plant competitiveness via increased allelopathy. New Phytol 198:916–924. doi: 10.1111/nph.12172

Vilà M, Basnou C, Pyšek P, et al. (2010) How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assessment. Front Ecol Environ 8:135–144. doi: 10.1890/080083

Vilà M, Espinar JL, Hejda M, et al. (2011) Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. Ecol Lett 14:702–708. doi: 10.1111/j.1461-0248.2011.01628.x

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM (1997) Human Domination of Earth's Ecosystems. Science 277:494–499. doi: 10.1126/science.277.5325.494

Warner RE (1968) The Role of Introduced Diseases in the Extinction of the Endemic Hawaiian Avifauna. The Condor 70:101–120. doi: 10.2307/1365954

Wauters L, Gurnell J, Martinoli A, Tosi G (2002a) Interspecific competition between native Eurasian red squirrels and alien grey squirrels: does resource partitioning occur? Behav Ecol Sociobiol 52:332–341. doi: 10.1007/s00265-002-0516-9

Wauters L, Tosi G, Gurnell J (2005) A review of the competitive effects of alien grey squirrels on behaviour, activity and habitat use of red squirrels in mixed, deciduous woodland in Italy. Hystrix Ital. J. Mammal. 16:

Wauters LA, Tosi G, Gurnell J (2002b) Interspecific competition in tree squirrels: do introduced grey squirrels (Sciurus carolinensis) deplete tree seeds hoarded by red squirrels (S. vulgaris)? Behav Ecol Sociobiol 51:360–367. doi: 10.1007/s00265-001-0446-y

Westphal MI, Browne M, MacKinnon K, Noble I (2008) The link between international trade and the global distribution of invasive alien species. Biol Invasions 10:391–398. doi: 10.1007/s10530-007-9138-5

Williamson M, Fitter A (1996) The Varying Success of Invaders. Ecology 77:1661–1666. doi: 10.2307/2265769

Woolhouse MEJ, Taylor LH, Haydon DT (2001) Population Biology of Multihost Pathogens. Science 292:1109–1112. doi: 10.1126/science.1059026

Wopfner N, Gadermaier G, Egger M, et al. (2005) The Spectrum of Allergens in Ragweed and Mugwort Pollen. Int Arch Allergy Immunol 138:337–346. doi: 10.1159/000089188

Zuk M, Stoehr AM (2002) Immune Defense and Host Life History. Am Nat 160:S9–S22. doi: 10.1086/342131

CHAPTER 2

Macroparasite community of the Eurasian red squirrel (*Sciurus vulgaris*): poor species richness and diversity

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ORIGINAL PAPER

Macroparasite community of the Eurasian red squirrel (*Sciurus vulgaris*): poor species richness and diversity

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Abstract The Eurasian red squirrel (*Sciurus vulgaris*) is the only naturally occurring tree squirrel throughout its range. We aim at improving current knowledge on its macroparasite fauna, expecting that it will have a poor parasite diversity because in species that have no sympatric congeners parasite richness should be lower than in hosts sharing their range with several closely related species, where host-switching events and lateral transmission are promoted. We examined gastro-intestinal helminth and ectoparasite communities (excluding mites) of, respectively, 147 and 311 red squirrel roadkills collected in four biogeographic regions in Italy and France. As expected, the macroparasite fauna was poor: we found five species of nematodes and some unidentified cestodes, three fleas, two sucking lice and two hard ticks. The helminth community was dominated by a single species, the oxyurid *Trypanoxyuris* (*Rodentoxyuris*) *sciuri* (prevalence, 87 %; mean abundance, 373±65 worms/host). Its abundance varied among seasons and biogeographic regions and increased with body mass in male hosts while decreased in females. The most prevalent ectoparasites were the flea *Ceratophyllus* (*Monopsyllus*) *sciurorum* (28 %), whose presence was affected by season, and the generalist tick *Ixodes* (*Ixodes*) *ricinus* that was found only in France (34 %). All the other helminths and arthropod species were rare, with prevalence below 10 %. However, the first record of *Strongyloides robustus*, a common nematode of North American Eastern grey squirrels (*S. carolinensis*), in two red squirrels living in areas where this alien species co-inhabits, deserves further attention, since low parasite richness could result in native red squirrels being particularly vulnerable to parasite spillover.

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Introduction

Parasite diversity in host communities is affected by many factors related to environmental, ecological, and evolutionary components of both host and parasite species (Poulin 1997; 2004). One of the observed patterns is the positive relationship between parasite richness in a host and the number of phylogenetically closely related host species living in the same area (Krasnov et al. 2006; Pisanu et al. 2009). Closely related species are likely to have similar immunological and physiological characteristics, thus parasites colonizing related hosts have to cope with a similar set of immune defences requiring less adaptations on their part (Poulin and Mouillot 2004). Moreover, contact and lateral transmission may be again facilitated since phylogenetic relatedness often reflects similar life-history traits, behaviour and ecological requirements (Brooks and McLennan 1991). Hence, in hosts sharing their range with several closely related species, host-switching events and lateral transmission could be promoted and parasite diversity should be higher than in species that have no sympatric congeners. Indeed, a positive relationship between presence of closely related hosts and parasite richness has been observed for example in rodents (Krasnov et al. 2004) and fish (Raibaut et al. 1998; Marques et al. 2011).

The Eurasian red squirrel (*Sciurus vulgaris*) is the only naturally occurring tree squirrel species throughout its range (Lurz et al. 2005). Locally, it shares its ecological niche only with night-active tree-dwelling rodents such as the edible dormouse (*Myoxus glis*) or the Siberian flying squirrel (*Pteromys volans*). Two more congeneric tree squirrels live in Palearctic region, *Sciurus anomalus* and *Sciurus lis*, but their range is restricted respectively to the Caucasian region and Southern Japanese islands (Gurnell 1987). In contrast with the red squirrel, in the Nearctic region, four species of tree squirrels belonging to the genus *Sciurus* (*Sciurus carolinensis*, *Sciurus niger*, *Sciurus griseus* and *Sciurus aberti*) share parts of their range among one another (Steele and Koprowski 2001). Moreover, in some areas, the range of these species extensively overlaps with the range of tree squirrels of the genus *Tamiasciurus*, which is phylogenetically close to *Sciurus* (Mercer and Roth 2003). The presence of closely related squirrels within the same forest results in high parasite richness, with many heteroxenous parasites infecting multiple hosts (see, e.g. Rausch and Tiner 1948 on parasitic helminths of Sciuridae).

Currently, there is little basic information on the macroparasite community of the Eurasian red squirrel. Actual knowledge about its ectoparasites and helminths comes from taxonomic studies about single parasite species or checklists regarding localized populations with small sample size (e.g. Shimalov and Shimalov 2002; Popiolek et al. 2009). Only Feliu et al. (1994) surveyed helminths of the red squirrel in many individuals over a wide area across the Iberian Peninsula.

Here, we aim at improving the knowledge of the Eurasian red squirrel macroparasite fauna, exploring both its ectoparasite and gastro-intestinal helminth fauna over a wide geographical area including diverse habitats and climatic regions. Following predictions by Krasnov et al. (2004, 2006), we expect red squirrels to harbour relatively few parasite species since the species is isolated from closely related squirrels. Moreover, the red squirrel is a solitary, arboreal species that spends a relatively small amount of time on the ground (Wauters and Dhondt 1987), therefore we also expect its helminths to be mainly specialists with direct life cycles. Last, we also explore environmental (biogeographic region and season) and host-related variables (gender and body mass) affecting the distribution and abundance of the dominant ecto- and endoparasites, to identify the major factors influencing the parasite communities of the red squirrel.

Finally, it must be stressed that in some European countries the survival of the red squirrel is at risk due to the introduction of the Eastern grey squirrel, Sciurus carolinensis (Martinoli et al. 2010; Bosch and Lurz 2012). Hence, a poor parasite community in red squirrels could be especially alarming since the species may be particularly vulnerable to spillover of new parasites from the alien congener (Tompkins and Poulin 2006; Pisanu et al. 2007).

Materials and methods

A total of 356 freshly roadkilled red squirrels were collected between 1999 and 2012 in Italy and France from four biogeographic regions (Atlantic, Continental, Alpine and Mediterranean) as defined by EU Habitats Directive (http://ec.europa.eu/environment/nature/natura2000/sites_hab/biogeog_regions/index_en.htm) (Fig. 1). Only complete carcasses were recovered and stored in individual plastic bags and frozen at −20 °C for later examination. For each animal we recorded sex and age class (juvenile or adult, Wauters et al. 1993). The latter was defined according to body mass, weighed to the nearest gram, and hind foot length measured to the nearest millimeter (Wauters and Dhondt 1989; Wauters et al. 2007). Out of the whole sample, 209 squirrels were examined only for ectoparasites, 45 only for gastrointestinal helminths and 102 for both. Ectoparasites (fleas, hard ticks and sucking lice) were collected by repeatedly grooming squirrels with a flea comb and carefully examining the body regions where they most commonly aggregate (e.g. behind the ears). Ectoparasites were then counted and stored in ethanol 70 % for later identification. We searched for gastrointestinal helminths in the stomach, small intestine, large intestine and rectum separately by washing each part with tap water. The content of each tract was then flushed through a 0.04-mm sieve and examined using a stereo-microscope. Helminths were counted and stored in lactophenol or ethanol

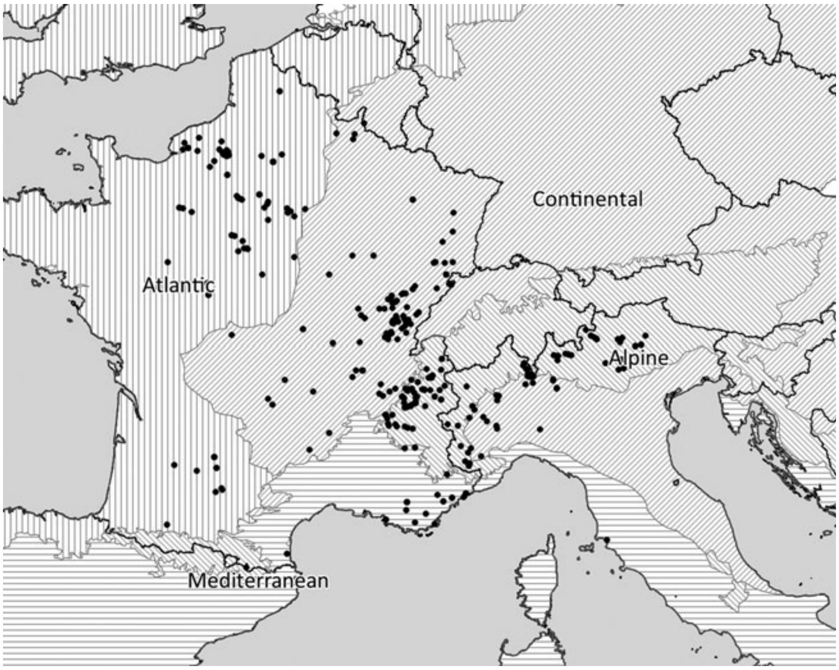

Fig. 1 Locations of the 356 red squirrel roadkills collected in France and Italy between 1999 and 2011 in Atlantic, Alpine, Continental and Mediterranean biogeographic region

70 % for later identification. Species identification of both ectoparasites and helminths was done morphologically, using a microscope equipped with camera lucida, and was based on the descriptions of Anoploura by Beaucournu (1968) and Beaucournu et al. (2008), of Siphonaptera by Beaucournu and Launay (1990), and of Ixodid ticks by Pérez-Eid (2007). Strongyloidid helminths refer to Chandler (1942) and Sato et al. (2007), Oxyurids to Hugot (1984), Trichostrongyloids genus *Trichostrongylus* Loos, 1905 to Durette-Desset (1983), and species to Audebert et al. (2003). Subfamily of Cyclophyllidean cestodes refers to Khalil et al. (1994).

Statistical analysis

The abundance (number of parasites/host) of the most common helminth and the prevalence (number of infested hosts) of the most common ectoparasite were analysed through generalized linear models to explore the influence of host characteristics and extrinsic factors on their distribution.

To deal with the aggregated distribution of parasites (Shaw et al. 1998), before the analysis the abundance of the dominant helminth was log-transformed (log $(x+1)$). After transformation the variable met the assumption for normality (Shapiro–Wilk test: $W>0.9$) and was analysed using linear models with Gaussian error. Only five specimens collected in the Mediterranean region were examined for helminths: due to the small sample size, these hosts were excluded from this part of the analysis. Variation in the presence of the most widespread species of ectoparasite was examined using logistic regression with a binomial error distribution and logit link function. We chose to analyse variation in presence rather than abundance because roadkilled squirrels were sometimes recovered several hours after their death, when part of the ectoparasites could have already left the host, resulting in a potential underestimation of parasite burdens. In all the models, the effects of sex, season, biogeographic region and body mass were explored. We defined season using the same categories, based on temporal changes in squirrel behaviour and food availability, described in previous studies (winter, December–February; spring March–May; summer June–August; autumn September–November, e.g. Wauters et al. 2007; Romeo et al. 2010). Since body mass was used as a parameter to separate juvenile from adult squirrels and since interactions of age with other fixed effects would result in extremely small sample size or missing data for some combinations, we did not include age as a factor. We also could not include year of collection as a fixed effect since sample size in different years was highly unequal. We first fitted saturated models including all fixed effects and their second-order interactions, then we obtained minimum adequate models through backward selection based on ΔAICc>2 (Burnham and Anderson 2004). Graphical checking showed no evidence of spatial or temporal autocorrelation in variance errors, confirmed variance homogeneity and normality of residuals in linear models with Gaussian error, and no overdispersion in binomial GLM (Zuur et al. 2010). Interpretation of final models was based on differences of least square means (DLSM). Parameter estimates are reported as mean (±SE).

All the statistical analysis were done using SAS/STAT 9.2 software (Copyright © 2009, SAS Institute Inc., Cary, NC, USA).

Results

Helminth community

A total of 5 nematodes species and 13 specimens of unidentified cestodes were found in 147 red squirrels (Table 1). No trematodes or acantocephalan species were found. The number of parasite species per host ranged between 0 and 2 with a mean value of 0.9 species/individual. The most common helminth, which was found in all biogeographic regions, was the oxyurid *Trypanoxyuris* (*Rodentoxyuris*) *sciuri* (Cameron 1932), with a total prevalence of 87.1 % and a mean abundance of 373±65 worms/host. *T.* (*R.*) *sciuri* abundance varied among biogeographic regions and seasons (Table 2). In addition, the effect of sex was found to depend on body mass: *T.* (*R.*) *sciuri* abundance increased with body mass in males (Fig. 2a), while it decreased with body mass in females (Fig. 2b). Squirrels from the Continental region were more infested than those from the Alpine and Atlantic regions (both DLSM: $p<0.0001$) whereas difference between *T.* (*R.*) *sciuri* abundance in the Atlantic and the Alpine region was not significant ($p=0.14$; Fig. 3a). Mean abundance of the parasite was significantly lower in specimens recovered in summer than in spring and autumn ($p=0.0002$ and $p=0.039$, respectively) while there was no difference between the other seasons (all $p>0.05$; Fig. 3b). The other five helminth taxa were rare, with prevalence below 5 %. Immature stages of 13 specimens of Cyclophyllidean cestodes, all belonging to the family Hymenolepididae (Ariola, 1899), were found in the small intestine of five squirrels (3.4 %). Also, an immature stage of a female of Capillariid nematode was found in the stomach of an adult female (0.7 %). One adult male of *Trichostrongylus vitrinus* (Loos, 1905) and another adult male of *Trichostrongylus* sp. (? *retortaeformis*) were found in the small intestine of two adult red squirrels (both 0.7 %). In Italy, 2 and 20 adult specimens of *Strongyloides robustus* (Chandler 1942) were identified in the small intestine of two adult squirrels (1.4 %).

Ectoparasite community

Seven ectoparasite species were found on 311 squirrels: three fleas, two sucking lice and two hard ticks (Table 3). Ectoparasite species richness ranged from 0 to 4, with a mean value of 0.7 species per host. The flea *Ceratophyllus* (*Monopsyllus*) *sciurorum sciurorum* (Schrank, 1803) was the most widespread parasitic arthropod, found in both countries and in all biogeographic regions, with a total prevalence of 27.6 %. *C.* (*M.*) *sciurorum* presence was affected only by season ($\chi^2_3=10.83$; $p=0.013$): prevalence were significantly lower in winter than in autumn and summer (DLSM: $p=0.026$ and $p=0.015$) and lower in spring than in autumn ($p=0.041$; Fig. 4). Another flea species, *Tarsopsylla octodecimdentata octodecimdentata* (Kolenati, 1863), was found on seven squirrels (1.9 %) collected at altitudinal levels ranging between 740 and 1,220 m a.s.l.. We also found a single specimen of *Dasypsyllus* (*Dasypsyllus*) *gallinulae gallinulae* (Dale, 1878), on a juvenile collected in France (0.3 %). Two species of sucking lice were found, the most common was *Neohaematopinus sciuri* (8.0 %), while *Enderleinellus nitzschi* was found only on three hosts (1.0 %). Finally, we found two species of hard ticks that showed a segregated distribution in the two countries: squirrels collected in France were frequently infested by *Ixodes* (*Ixodes*) *ricinus*

Table 1 Helminth species infecting red squirrels in four biogeographic regions

Helminth species	Continental		Alpine		Mediterranean		Atlantic	
	n (*p*)	mI±SE	*n* (*p*)	mI±SE	*n* (*p*)	mI±SE	*n* (*p*)	mI±SE
Juvenile	*N*=6		*N*=1		*N*=1		*N*=7	
Trypanoxyuris (*R.*) *sciuri*	5 (83 %)	923±232	0	–	1 (100 %)	*20*	3 (43 %)	*350*; *406*; *1,000*
Adult ♀	*N*=15		*N*=22		*N*=1		*N*=21	
Trypanoxyuris (*R.*) *sciuri*	14 (93 %)	554±300	22 (100 %)	225±96	1 (100 %)	*15*	14 (67 %)	262±161
Strongyloides robustus	1 (7 %)	*2*	0	–	0	–	0	–
Capillariinae [gen. sp.]	0	–	0	–	0	–	1 (4 %)	*1*
Hymnolepididae [gen. sp.]	0	–	0	–	0	–	1 (4 %)	*1*
Adult ♂	*N*=19		*N*=28		*N*=3		*N*=23	
Trypanoxyuris (*R.*) *sciuri*	19 (100 %)	777±281	26 (93 %)	114±41	3 (100 %)	*20*; *80*; *1867*	20 (87 %)	263±78
Strongyloides robustus	1 (5 %)	*20*	0	–	0	–	0	–
Trichostrongylus sp. (?*retortaeformis*)	0	–	1 (4 %)	*1*	0	–	0	–
Trichostrongylus vitrinus	0	–	0	–	0	–	1 (4 %)	*1*
Hymnolepididae [gen. sp.]	2 (11 %)	*2*; *4*	0	–	0	–	2 (9 %)	*3*; *3*

N number of host examined; *n* number of infected hosts; *p* prevalence; *mI* mean intensity (*n* parasites/infected hosts; when number of infected hosts<5, worm counts in italic)

Table 2 Generalized linear model exploring effects of host characteristics and environmental variables on *T.* (*R.*) *sciuri* abundance. Parameter estimates were significantly different from 0 (both $|t|>2.8$; $p<0.006$)

Source of variation	*F*	*df*	*p*	Parameter estimate (±SE)
Sex	17.9	1, 131	<0.0001	
Body mass	0.04	1, 131	0.8	
Season	4.9	3, 131	0.003	
Biogeographic region	14.7	2, 131	<0.0001	
Sex × body mass	19.2	1, 131	<0.0001	
M				0.014 (±0.004)
F				−0.013 (±0.005)

(prevalence in France: 34.0 %), whereas in Italy we only found specimens of *I.* (*I.*) *acuminatus*, on three adult hosts (prevalence in Italy: 6.5 %).

Discussion

As expected, Eurasian red squirrels have a poor macroparasite assemblage, with only three dominant species: one gastro-intestinal nematode and two arthropods. The composition of macroparasite communities is consistent across biogeographic regions/habitats.

In particular, the gastro-intestinal helminth community is dominated by a single species, *T.* (*R.*) *sciuri*, an oxyurid nematode specific to the red squirrel (Hugot 1984). As predicted, the host arboreal ecology seems to prevent infestation by helminths with indirect life-cycles and/or free-living stages. Most oxyurids are characterised by an over-infestation strategy, and are mainly vertically transmitted through the population, although some horizontal transmission can occur via physical contact or environmental contamination (Anderson 2000). Red squirrels are solitary and frequent contact between individuals occurs almost exclusively during the mating season (between January and May, Wauters and Dhondt 1989; Wauters et al. 1990). Moreover, horizontal transmission can occur in dreys, since the same nest can be used by different individuals on consecutive nights (Wauters and Dhondt 1990; Lurz et al. 2005). These changes in the probability of horizontal transmission could be the reason for the observed seasonal variation in levels of infestation by *T.* (*R.*) *sciuri*, with the highest peak in spring and the lowest abundance in summer, when contacts between individuals are rarer and squirrels spend less time in nests (Wauters and Dhondt 1987; Wauters 2000). This helminth was common in all the habitats, but its abundance was higher in the Continental biogeographic region. This could be related to red squirrels occurring at higher densities in deciduous or mixed broadleaf-pine woods with more predictable and higher food availability, and less harsh weather conditions, than in conifer forests in the Alpine region (Wauters et al. 2004, 2008; Lurz et al. 2005). The Continental region is also the most urbanized area in Europe and home range overlap between individual squirrels can increase in fragmented landscapes with small woodland patches (e.g. Verbeylen et al. 2009), possibly increasing the frequency of horizontal transmission of *T.* (*R.*) *sciuri*. Moreover, individuals living in disturbed habitats might be more susceptible to parasite infections because they are more stressed (Christe et al. 2006). Finally, gender differences in parasite infestations are commonly observed in many higher vertebrates due to sexual size dimorphism, testosterone immunodepressive effect and/or behavioural and ecological differences between males and females (see Poulin 1996; Shalk and Forbes 1997; Ferrari

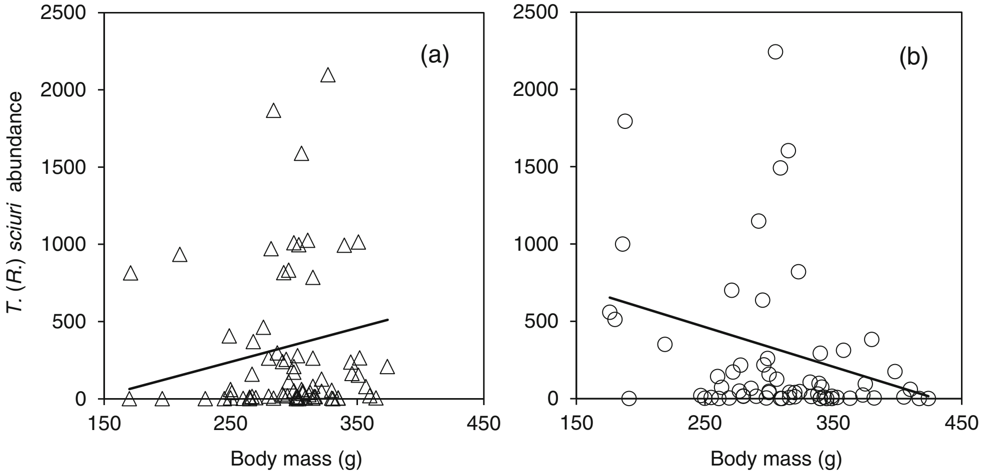

Fig. 2 Relationship between *T.* (*R.*) *sciuri* abundance and body mass in male (**a**) and female (**b**) squirrels

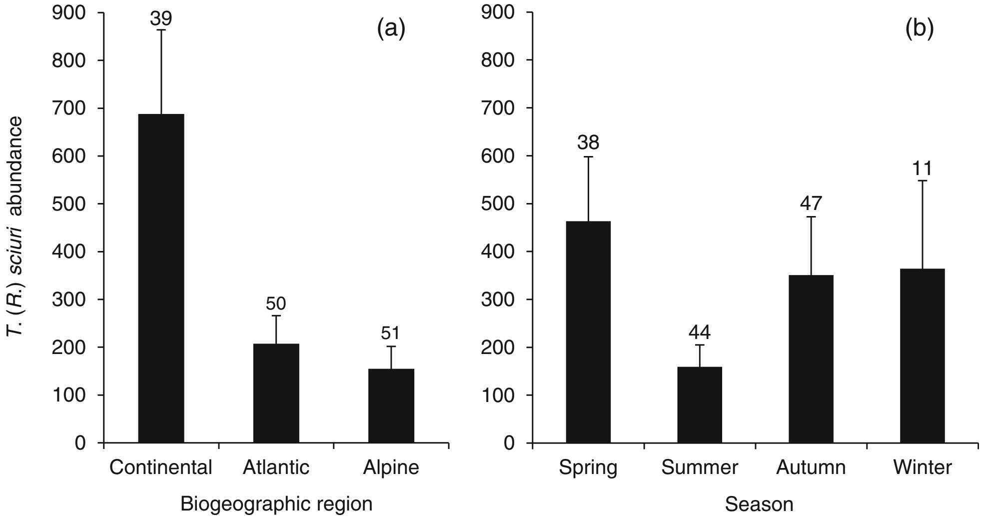

Fig. 3 Mean abundance (±SE) of the helminth *T.* (*R.*) *sciuri* by biogeographic region (**a**) and by season (**b**). *Sample size above error bars*

et al. 2007, 2010). For example, in several species of polygynous desert rodents, Krasnov et al. (2005) observed male-biased flea abundances during the mating season, when males' mobility and testosterone levels increase. In our case, although polygynous red squirrel males increase their mobility and home-range size during the mating season (Wauters et al. 1990; Di Pierro et al. 2008; Romeo et al. 2010), we did not observe any general nor seasonal differences in infestation between sexes. Yet, we found that the abundance of *T.* (*R.*) *sciuri* increased with body mass in males whereas females showed the opposite relationship. This interesting pattern could be linked to gender differences in behaviour or immune function and should be investigated more deeply.

Ectoparasite assemblage was richer than helminths assemblage, still it was dominated only by two species: the flea *C.* (*M.*) *sciurorum* and the tick *I.* (*I.*) *ricinus*. *C.* (*M.*) *sciurorum* is distributed throughout Europe, and its primary hosts are red squirrels (Beaucournu and Launay 1990), along with other

Table 3 Infestation by arthropod species in red squirrels from four biogeographic regions

Arthropod species	Continental		Alpine		Mediterranean		Atlantic	
	n	*p*	*n*	*p*	*n*	*p*	*n*	*p*
Juvenile	*N*=31		*N*=6		*N*=1		*N*=14	
Ceratophyllus (*M.*)*sciurorum*	8	26 %	4	67 %	0	–	6	43 %
Tarsopsylla o. octodecimdentata	0	–	1	17 %	0	–	0	–
Dasypsyllus (*D.*) *gallinulae*	0	–	0	–	0	–	1	7 %
Neohaematopinus sciuri	2	6 %	0	–	0	–	4	29 %
Ixodes (*I.*) *ricinus*	5	16 %	0	–	0	–	3	21 %
Adult ♀	*N*=45		*N*=29		*N*=11		*N*=31	
Ceratophyllus (*M.*)*sciurorum*	9	20 %	10	38 %	1	9 %	9	29 %
Neohaematopinus sciuri	1	2 %	2	7 %	0	–	4	13 %
Enderleinellus nitzschi	0	–	0	–	2	18 %	0	–
Ixodes (*I.*) *ricinus*	12	27 %	6	21 %	1	9 %	16	52 %
Ixodes (*I.*) *acuminatus*	0	–	1	3 %	0	–	0	–
Adult ♂	*N*=60		*N*=42		*N*=6		*N*=35	
Ceratophyllus (*M.*)*sciurorum*	15	25 %	10	24 %	1	17 %	12	34 %
Tarsopsylla o. octodecimdentata	1	2 %	3	7 %	1	17 %	0	–
Neohaematopinus sciuri	1	2 %	3	7 %	0	–	8	23 %
Enderleinellus nitzschi	0	–	1	2 %	0	–	0	–
Ixodes (*I.*) *ricinus*	16	27 %	10	24 %	1	17 %	20	57 %
Ixodes (*I.*) *acuminatus*	2	3 %	0	–	0	–	0	–

N number of host examined; *n* number of infested hosts; *p* prevalence

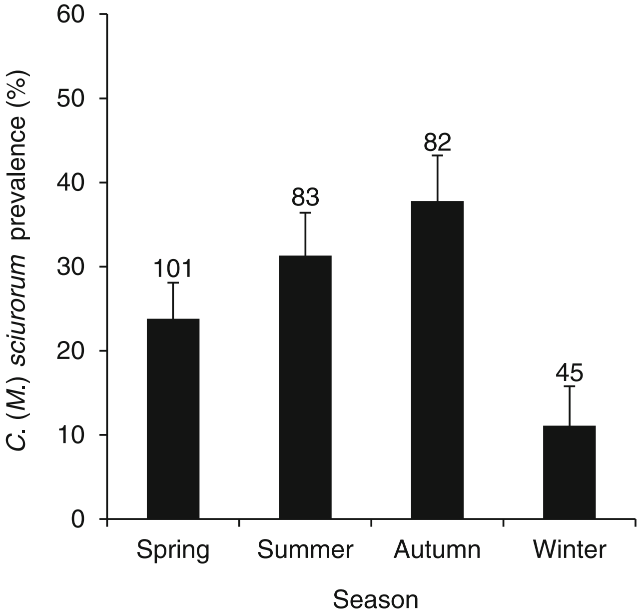

Fig. 4 Prevalence (±SE) of the flea *C.* (*M.*) *sciurorum* in different seasons. *Sample size above error bars*

aerial nesting mammals such as the edible dormouse (*Myoxus glis*) or the common dormouse (*Muscardinus avellanarius*) (Beaucournu and Launay 1990; Traub et al. 1983; Trilar et al. 1994). This flea has been found also on Siberian flying squirrels (*Pteromys volans*, Haukisalmi and Hanski 2007), on introduced grey squirrels in Britain (Smit 1957) and Siberian chipmunk in France (Pisanu et al. 2008), and occasionally on martens (*Martes* spp.) and several other small carnivores and birds (Beaucournu and Launay 1990; Smit 1957; Traub et al. 1983). Infestation by *C.* (*M.*) *sciurorum* did not show any spatial variability, but prevalence varied seasonally, with lowest values in winter. Lower levels of infestation in winter could be a consequence of reduced host density and activity, preventing flea transmission (Krasnov et al. 2002). The other main flea, *T. o. octodecimdentata*, is a Paleartic subspecies infecting mainly red squirrels, but also Siberian flying squirrels (Haukisalmi and Hanski 2007). This species is well adapted to cold climates; in fact, it replaces *C.* (*M.*) *sciurorum* at high altitudes in the Alps (Beaucournu and Launay 1990). It is a nest flea, thus usually found on hosts in small numbers (Smit 1957). Of the two sucking lice we found, *E. niztschi* is monoxenous whereas *N. sciuri* is a Holarctic species mainly occurring on Eurasian red and North American squirrels (Durden and Musser 1994). The only generalist parasite for which the red squirrel seems an important feeding host, at least in France, is the tick *I.* (*I.*) *ricinus*. Surprisingly, this species was found only in France, despite being known to occur in various habitats also in Italy (e.g. Dantras-Torres and Otranto 2013a; Dantas-Torres and Otranto 2013b). This dissimilarity may be due to differences in ungulate presence between collection sites in the two countries, since deer are primary hosts for the reproduction of *I.* (*I.*) *ricinus* (Pérez-Eid 2007). On specimens collected in Italy infestation by ticks was not relevant: red squirrels were only rarely infested by *I.* (*I.*) *acuminatus*, whose life-cycle takes place almost entirely inside burrows of ground-dwelling small mammals (Pérez-Eid 2007), limiting the opportunities for transmission to squirrels. The use of roadkilled animals may have led to an underestimation of ectoparasite richness, because some species leave carcasses earlier than others (e.g. Westrom and Yescot 1975), but ectoparasite screening on live-trapped red squirrels confirms that their macroparasite community in our study area is composed only by the above-mentioned arthropods (Romeo et al., unpublished data).

All other helminth and arthropod species found were rare and can be considered accidental (e.g. the nematode *T. vitrinus* and the flea *D. gallinulae*, specific to sheep and passerine birds, respectively), but particularly meaningful is the first record of *S. robustus* in Europe. This species was found in two roadkills collected from an area in Northern Italy where the introduced Eastern grey squirrel is present. This nematode is a common parasite of North American squirrels, mainly grey squirrels (e.g. Davidson 1976; Conti et al. 1984); hence, our finding suggests that this species may spillover from the alien species towards red squirrels.

In general, we found that the red squirrel's parasite fauna is composed by a limited number of species. This result is consistent with previous findings (Feliu et al. 1994) on helminths of the red squirrel in Spain, where, based on a large sample (N=248), the helminth community was also found to be dominated by auto-infective oxyurids: *T.* (*R.*) *sciuri* (prevalence: 17.8 %) and *Syphabulea mascomai* (39.3 %). The absence of *S. mascomai* in our survey seems to confirm that this species is endemic of the Iberian Peninsula (Hugot and Feliu 1990).

Ectoparasite richness in the Eurasian red squirrel is similar to the parasite diversity observed in congeners in the Nearctic region (e.g. Parker 1971; Coyner et al. 1996; Durden et al. 2004). However, the number of gastro-intestinal helminths species is much lower. For example, even excluding potentially accidental species (i.e. prevalence<5 %), Eastern grey squirrels (*S. carolinensis*) and fox squirrels (*S. niger*) in their native range are known to be infested by respectively 7 and 4 species of gastro-intestinal helminths (Chandler 1942; Rausch and Tiner 1948; Parker 1971; Davidson 1976; Conti et al. 1984; Coyner et al. 1996). In addition, most of these parasites are shared between these two hosts and also with other closely related tree squirrels such as the American red squirrel (*Tamiasciurus hudsonicus*) (Rausch and Tiner 1948; Eckerlin 1974; Flyer and Gates 1982). Unfortunately, we could not directly test the hypothesis that a poor parasite community is related to a poor host community because in Europe there are no areas where the species is (naturally) sympatric with other tree squirrels. However, the fact that the extension of the range and the diversity of habitats exploited by the Eurasian red squirrel and the North American species are similar, suggests that this difference in parasite richness is a specific result of the different structure of host communities in the two regions: one "isolated" host in the Palearctic and many closely related squirrels in the Nearctic. Hence, the comparison of our data with that of Nearctic squirrels, seems to support the hypothesis of a positive relationship between parasite richness in a host and the number of phylogenetically closely related host species living in the same area.

Finally, the low parasite richness observed in the gastrointestinal helminth fauna of the red squirrel could be particularly meaningful since impoverished parasite communities may show less plasticity towards environmental changes (Hudson et al. 2006) and be highly vulnerable to spillover from introduced alien species (especially closely related invaders). In recent years, there is growing concern about the role played by parasites in invasions by exotic plants and animals (e.g. Daszak et al. 2000; Cunningham et al. 2003; Prenter et al. 2004; Tompkins and Poulin 2006; Dunn 2009). One of the most cited examples concerns precisely the role of Squirrelpoxvirus in mediating the competition between Eurasian red squirrels and Eastern grey squirrels introduced to Great Britain and Ireland (Tompkins et al. 2002; Rushton et al. 2006). Considering that, apart from the grey squirrel, several squirrels species have been recently introduced to Europe (e.g. *Tamias sibiricus* and *Callosciurus* spp. in France and Italy, Chapuis 2005; Bertolino and Genovesi 2005; Bertolino and Lurz 2013), investigating the role of parasite-mediated competition in the interaction of this native species with other squirrels should be a priority in future research.

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References

Anderson RC (2000) Nematode parasites of vertebrates: their development and transmission, 2nd edn. CABI Publishing, Wallingford

Audebert F, Cassone J, Kerboeuf D, Durette-Desset M-C (2003) Development of *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus*, parasites of ruminants in the rabbit and comparison with *Trichostrongylus retortaeformis*. Parasitol Res 90:57–63

Beaucournu J-C (1968) Les Anoploures de Lagomorphes, Rongeurs et Insectivores dans la région paléarctique occidentale et en particulier en France. Ann Parasit Hum Comp 43:201–271

Beaucournu J-C, Launay H (1990) Les puces de France et du bassin méditerranéen occidental. Faune de France 76. Fédération Française des Sociétés de Sciences Naturelles, Paris

Beaucournu J-C, Pisanu B, Chapuis J-L (2008) *Enderleinellus tamiasis* Fahrenholz, 1916 (Anoploura: Enderleinellidae), espèce importée, implantée et nouvelle pour la faune de France. Parasite 15:175–178

Bertolino S, Genovesi P (2005) The application of the European strategy on invasive alien species: an example with introduced squirrels. Hystrix It J Mamm 16:59–69

Bertolino S, Lurz PWW (2013) *Callosciurus* squirrels: worldwide introductions, ecological impacts and recommendations to prevent the establishment of new invasive populations. Mammal rev 43:22–33

Bosch S, Lurz PWW (2012) The Eurasian red squirrel. Westarp Wissenschaften, Hohenwarsleben

Brooks DR, McLennan DA (1991) Phylogeny, ecology, and behavior. A research program in comparative biology. The University of Chicago Press, Chicago

Burnham KP, Anderson DA (2004) Multi-model inference: understanding AIC and BIC in model selection. Sociol Method Res 33:261–304

Cameron TW (1932) On a new species of *Oxyuris* from the grey squirrel in Scotland. J Helminthol 10:29–32

Chandler AC (1942) Helminths of tree squirrels in Southeast Texas. J Parasitol 28:135–140

Chapuis JL (2005) Répartition en France d'un animal de compagnie naturalisé, le tamia de sibérie (*Tamias sibiricus*). Rev Ecol (Terre Vie) 60:239–253

Christe P, Morand S, Michauux J (2006) Biological conservation and parasitism. In: Morand S, Krasnov BR, Poulin R (eds) Micromammals and macroparasites. Springer, Tokyo, pp 593–613

Conti JA, Forrester DJ, Frohlich RK, Hoff GL, Bigler WJ (1984) Helminths of urban gray squirrels in Florida. J Parasitol 70:143–144

Coyner DF, Wooding JB, Forrester DJ (1996) A comparison of parasitic helminths and arthropods from two species of fox squirrels (*Sciurus niger*) in Florida. J Wildlife Dis 32:492–497

Cunningham AA, Daszak P, Rodriguez JP (2003) Pathogen pollution: defining a parasitological threat to biodiversity conservation. J Parasitol 89:S78–S83

Dantas-Torres F, Otranto D (2013a) Seasonal dynamics of *Ixodes ricinus* on ground level and higher vegetation in a preserved wooded area in southern Europe. Vet Parasitol 192:253–258

Dantas-Torres F, Otranto D (2013b) Species diversity and abundance of ticks in three habitats in southern Italy. Ticks Tick-Borne Dis 4:251–255

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife—threats to biodiversity and human health. Science 287:443–449

Davidson WR (1976) Endoparasites of selected populations of Gray Squirrels (*Sciurus carolinensis*) in the southeastern United States. Proc Helminthol Soc Wash 43:211–217

Di Pierro E, Molinari A, Tosi G, Wauters LA (2008) Exclusive core areas and intrasexual territoriality in Eurasian red squirrels (*Sciurus vulgaris*) revealed by incremental cluster polygon analysis. Ecol Res 23:529–542

Dunn AM (2009) Parasites and biological invasions. Adv Parasitol 68:161–184

Durden LA, Musser GG (1994) The sucking lice (Insecta, Anoplura) of the world: a taxonomic checklist with records of mammalian hosts and geographical distributions. Bull Am Mus Nat Hist 218:1–90

Durden LA, Ellis BA, Bank CW, Crowe JD, Oliver JH Jr (2004) Ectoparasites of gray squirrels in two different habitats and screening of selected ectoparasites for bartonellae. J Parasitol 90:485–489

Durette-Desset M-C (1983) Keys to genera of the Superfamily *Trichostrongyloidea*. In: Anderson RC, Chabaud AG (eds) CIH Keys to the nematodes parasites of vertebrates. Cambridge Agricultural Bureau, Cambridge, pp 1–86

Eckerlin RP (1974) Studies on the life-cycle of *Strongyloides robustus* Chandler, 1942, and a survey of the helminths of Connecticut sciurids. PhD Dissertation, University of Connecticut

Feliu C, Torres J, Miquel J, Casanova JC (1994) The helminthfaunas of rodents of the Iberian Peninsula in relation to continental rodents: the case of *Sciurus vulgaris* Linnaeus, 1758 (Sciuridae). Res Rev Parasitol 54:125–127

Ferrari N, Rosà R, Pugliese A, Hudson PJ (2007) The role of sex in parasite dynamics: model simulations on transmission of *Heligmosomoides polygyrus* in populations of yellow-necked mice, *Apodemus flavicollis*. Int J Parasitol 37:341–349

Ferrari N, Rosà R, Lanfranchi P, Ruckstuhl KE (2010) Effect of sexual segregation on host–parasite interaction: model simulation for abomasal parasite dynamics in alpine ibex (*Capra ibex*). Int J Parasitol 40:1285–1293

Flyger V, Gates JE (1982) Fox and gray squirrel, *Sciurus niger*, *Sciurus caroliensis* and allies. Pine squirrell, *Tamiasciurus hudsonicus* and *T. douglasii*. In: Chapman JA, Feldhammer GA (eds) Wild mammals of North America: biology, management, and economics. The Johns Hopkins University Press, Baltimore, pp 209–238

Gurnell J (1987) The natural history of squirrels. Helm, London

Haukisalmi V, Hanski IK (2007) Contrasting seasonal dynamics in fleas of the Siberian flying squirrel (*Pteromys volans*) in Finland. Ecol Entomol 32:333–337

Hudson PJ, Dobson AP, Lafferty KD (2006) Is a healthy ecosystem one that is rich in parasites? TRENDS Ecol Evol 21:381–385

Hugot JP (1984) Sur le genre *Trypanoxyuris* (Oxyuridae, Nematoda) I. Parasites de Sciuriés: sous-genre *Rodentoxyuris*. Bull Mus Natl Hist Nat 6:711–720

Hugot JP, Feliu C (1990) Description de *Syphabulea mascomai* n. sp. et analyse du genre *Syphabulea*. Syst Parasitol 17:219–230

Khalil LF, Jones A, Bray RA (1994) Keys to the cestode parasites of vertebrates. CAB International, Wallingford

Krasnov BR, Khokhlova IS, Shenbrot GI (2002) The effect of host density on ectoparasite distribution: an example of a rodent parasitized by fleas. Ecology 83:164–175

Krasnov BR, Shenbrot GI, Khokhlova IS, Degen AA (2004) Flea species richness and parameters of host body, host geography and host 'milieu'. J Anim Ecol 73:1121–1128

Krasnov BR, Morand S, Hawlena H, Khokhlova IS, Shenbrot GI (2005) Sex-biased parasitism, seasonality and sexual size dimorphism in desert rodents. Oecologia 146:209–217

Krasnov BR, Poulin R, Morand S (2006) Patterns of macroparasite diversity in small mammals. In: Morand S, Krasnov BR, Poulin R (eds) Micromammammals and macroparasites. From evolutionary ecology to management. Springer, Tokyo, pp 197–231

Lurz PWW, Gurnell J, Magris L (2005) *Sciurus vulgaris*. Mammalian Species 769:1–10

Marques JF, Santos MJ, Teixeira CM, Batista MI, Cabral HN (2011) Host–parasite relationships in flatfish (Pleuronectiformes)—the relative importance of host biology, ecology and phylogeny. Parasitol 138:107–121

Martinoli A, Bertolino S, Preatoni DG, Balduzzi A, Marsan A, Genovesi P, Tosi G, Wauters LA (2010) Headcount 2010: the multiplication of the grey squirrel introduced in Italy. Hystrix It J Mamm 21:127–136

Mercer JM, Roth VL (2003) The effects of Cenozoic global change on squirrel phylogeny. Science 299:1568–1572

Parker JC (1971) Protozoan, helminth and arthropod parasites of the grey squirrel in south-western Virginia. PhD Dissertation, Virginia Polytechnic Institute

Pérez-Eid C (2007) Les tiques: identification, biologie, importance médicale et vétérinaire. Lavoisier, Paris

Pisanu B, Jerusalem C, Huchery C, Marmet J, Chapuis J-L (2007) Helminth fauna of the Siberian chipmunk, *Tamias sibiricus* Laxmann (Rodentia, Sciuridae) introduced in suburban French forests. Parasitol Res 100:1375–1379

Pisanu B, Marmet J, Beaucournu J-C, Chapuis J-L (2008) Diversité du cortège en Siphonaptères chez le Tamia de Sibérie (*Tamias sibiricus* Laxmann) introduit en forêt de Sénart (Ile-de-France). Parasite 15:35–43

Pisanu B, Lebailleux L, Chapuis J-L (2009) Why do Siberian chipmunks *Tamias sibiricus* (Sciuridae) introduced in French forests acquired so few intestinal helminth species from native sympatric Murids? Parasitol Res 104:709–714

Popiolek M, Hildebrand J, Zalesny J (2009) Morphology and taxonomy of *Rodentoxyuris sciuri* Quentin et Tenora, 1974 (Nematoda: Oxyurida: Enterobiinae) with notes on molecular phylogeny. Ann Zool 59:415–421

Poulin R (1996) Sexual inequalities in helminth infections: a cost of being male? Am Nat 147:287–295

Poulin R (1997) Species richness of parasite assemblages: evolution and patterns. Annu Rev Ecol Syst 28:341–358

Poulin R (2004) Macroecological patterns of species richness in parasite assemblages. Basic Appl Ecol 5:423–434

Poulin R, Mouillot D (2004) The relationship between specialization and local abundance: the case of helminth parasites of birds. Oecologia 140:372–378

Prenter J, MacNeil C, Dick JTA, Dunn AM (2004) Roles of parasites in animal invasions. TRENDS Ecol Evol 19:385–389

Raibaut A, Combes C, Benoit F (1998) Analysis of the parasitic copepod species richness among Mediterranean fish. J Mar Syst 15:185–206

Rausch R, Tiner JD (1948) Studies on the parasitic helminths of the North Central States. I. Helminths of Sciuridae. Am Midl Nat 39:728–747

Romeo C, Wauters LA, Preatoni D, Tosi G, Martinoli A (2010) Living on the edge: space use of Eurasian red squirrels in marginal high-elevation habitat. Acta Oecol 36:604–610

Rushton SP, Lurz PWW, Gurnell J, Nettleton P, Bruemmer C, Shirley MDF, Sainsbury AW (2006) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. Epid Infect 134:521–533

Sato H, Torii H, Une Y, Ooi H-K (2007) A new Rhabditoid nematode species in Asian sciurids, distinct from *Strongyloides robustus* in North American Sciurids. J Parasitol 93:1476–1486

Schalk G, Forbes MR (1997) Male biases in parasitism of mammals: effects of study type, host age and parasite taxon. Oikos 78:67–74

Shaw DJ, Grenfell BT, Dobson AP (1998) Patterns of macroparasite aggregation in wildlife host populations. Parasitology 117:597–610

Shimalov VV, Shimalov VT (2002) Helminth fauna of the red squirrel (*Sciurus vulgaris* Linnaeus, 1758) in Belorussian Polesie. Parasitol Res 88:1008

Smit FGAM (1957) Handbook for the Identification of British Insects, 1. Royal Entomological Society, London

Steele MA, Koprowski JL (2001) North American tree squirrels. Smithsonian Institution, Washington

Tompkins DM, Poulin R (2006) Parasites and biological invasions. In: Aleen RB, Lee WG (eds) Biological Invasions in New Zealand. Springer, Berlin, pp 67–82

Tompkins DM, Sainsbury AW, Nettleton P, Buxton J, Gurnell J (2002) Parapoxvirus causes a deleterious diseases in red squirrels associated with UK population declines. Proc Roy Soc B 269:529–533

Traub R, Rothschild M, Haddow JF (1983) The Rothschild collection of fleas. The Ceratophyllidae: key to the genera and host relationships. With notes on their evolution, zoogeography and medical importance. Academic, Orlando

Trilar T, Radulovic S, Walker DH (1994) Identification of anatural cycle involving *Rickettsia typhi* infection of *Monopsyllus sciurorum*

sciurorum fleas from the nests of the fat dormouse (*Glis glis*). Eur J Epidemiol 10:757–62

Verbeylen G, Wauters LA, De Bruyn L, Matthysen E (2009) Woodland fragmentation affects space use of Eurasian red squirrels. Acta Oecol 35:94–103

Wauters LA (2000) Squirrels—medium-sized Granivores in Woodland Habitats. In: Halle S, Stenseth NC (eds) Activity patterns in small mammals: a comparative ecological approach. ecological Studies 141, Springer, Heidelberg, pp 131–143

Wauters LA, Dhondt AA (1987) Activity budget and foraging behaviour of the red squirrel (*Sciurus vulgaris*, Linnaeus, 1758) in a coniferous habitat. Mamm Biol 52:341–352

Wauters LA, Dhondt AA (1989) Body weight, longevity and reproductive success in red squirrels (*Sciurus vulgaris*). J Anim Ecol 58:637–651

Wauters LA, Dhondt AA (1990) Nest-use by red squirrels (*Sciurus vulgaris* Linnaeus, 1758). Mammalia 54:377–389

Wauters LA, De Vos R, Dhondt AA (1990) Factors affecting male mating success in red squirrels (*Sciurus vulgaris*). Ethol Ecol Evol 2:195–204

Wauters LA, Bijnens L, Dhondt AA (1993) Body mass at weaning and juvenile recruitment in the red squirrel. J Anim Ecol 62:280–286

Wauters LA, Matthysen E, Adriaensen F, Tosi G (2004) Within sex-density dependence and population dynamics of red squirrels *Sciurus vulgaris*. J Anim Ecol 73:11–25

Wauters LA, Vermeulen M, Van Dongen S, Bertolino S, Molinari A, Tosi G, Matthysen E (2007) Effects of spatio-temporal variation in food supply on red squirrel *Sciurus vulgaris* body size and body mass and its consequences for some fitness components. Ecography 30:51–65

Wauters LA, Githiru M, Bertolino S, Molinari A, Tosi G, Lens L (2008) Demography of alpine red squirrel populations in relation to fluctuations in seed crop size. Ecography 31:104–114

Westrom D, Yescott R (1975) Emigration of ectoparasites from dead California ground squirrels *Spermophilus beecheyi* (Richardson). California Vector News: 97–103

Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Meth Ecol Evol 1:3–14

CHAPTER 3

Macroparasite fauna of alien grey squirrels (*Sciurus carolinensis*): composition, variability and implications for native species

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Macroparasite Fauna of Alien Grey Squirrels (*Sciurus carolinensis*): Composition, Variability and Implications for Native Species

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Abstract

Introduced hosts populations may benefit of an "enemy release" through impoverishment of parasite communities made of both few imported species and few acquired local ones. Moreover, closely related competing native hosts can be affected by acquiring introduced taxa (spillover) and by increased transmission risk of native parasites (spillback). We determined the macroparasite fauna of invasive grey squirrels (*Sciurus carolinensis*) in Italy to detect any diversity loss, introduction of novel parasites or acquisition of local ones, and analysed variation in parasite burdens to identify factors that may increase transmission risk for native red squirrels (*S. vulgaris*). Based on 277 grey squirrels sampled from 7 populations characterised by different time scales in introduction events, we identified 7 gastro-intestinal helminths and 4 parasite arthropods. Parasite richness is lower than in grey squirrel's native range and independent from introduction time lags. The most common parasites are Nearctic nematodes *Strongyloides robustus* (prevalence: 56.6%) and *Trichostrongylus calcaratus* (6.5%), red squirrel flea *Ceratophyllus sciurorum* (26.0%) and Holarctic sucking louse *Neohaematopinus sciuri* (17.7%). All other parasites are European or cosmopolitan species with prevalence below 5%. *S. robustus* abundance is positively affected by host density and body mass, *C. sciurorum* abundance increases with host density and varies with seasons. Overall, we show that grey squirrels in Italy may benefit of an enemy release, and both spillback and spillover processes towards native red squirrels may occur.

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Introduction

Biological invasions are one of the major causes for biodiversity loss worldwide [1–4], therefore the attention on mechanisms and processes driving alien species settlement, their spread and their subsequent impact on native ecosystems is constantly growing. In recent years several authors recognised that micro- and macroparasites can play an important role in biological invasions, affecting alien species settlement and mediating their interaction with native species (reviewed in: [5–7]). It has been observed in different taxa of both plants and animals, that invasive species often loose part of their parasite fauna during the introduction process and that, in most cases, this reduction in parasite species is not completely compensated by the acquisition of local parasites from the new environment [8,9]. Consequently, in a new settlement area, alien species are usually less heavily parasitised (both in terms of richness and prevalence) than in their native range. This release from natural "enemies" may increase individual viability and population growth rate, facilitating invaders' settlement and spread ([10–13], but see [14]).

Additionally, parasites that are successfully introduced by invaders may potentially become a major threat for native species and greatly affect the outcome of interspecific competition [15]. If spillover (i.e. transmission of infectious agents from reservoir populations to sympatric animals, [16]) between introduced and susceptible local hosts occurs, the parasite impact can indeed be very severe, since native species can be maladapted to alien parasites (e.g. [17]). Moreover, invaders may also successfully acquire local parasites, altering their epidemiology and increasing the abundance of infective stages in the environment (via the increased density of competent hosts). This acquisition may in turn exacerbate the impact of local parasites on native hosts (spill-back hypothesis, [18]) and also lead to an increase in health risks for humans [19]. On the contrary, if invaders are infected by local parasites but are not competent hosts, the alien species may act as a sink and lessen the parasite impact on native species (dilution effect, [20–22]).

One of the most cited examples of parasites playing a role in biological invasions, is the Squirrelpoxvirus (SQPV) mediating the competition between introduced North American Eastern grey squirrels (*Sciurus carolinensis*) and native Eurasian red squirrels (*Sciurus vulgaris*) in Great Britain and Ireland [23,24]. The alien species, introduced in Great Britain at the end of the 19th century, acts as healthy carrier for the SQPV, whereas the virus, in most cases, is lethal for red squirrels [25]. As a result, in sites where SQPV is present, the replacement of the native species by grey squirrels is much accelerated than when only food exploitation competition occurs [23].

During the second half of last century the grey squirrel was repeatedly introduced also to Northern Italy, becoming a threat to red squirrels in continental Europe [26,27]. In all the sites where the alien species is present, the red squirrel disappeared or is declining because of interspecific competition for resources which reduces female reproductive success and juvenile recruitment [28–31].

Despite the attention received by SQPV, there is a generalised lack of information about the potential role played by macroparasites in the competition between these two species. One of the reasons for this is that the effects of macroparasites are generally sublethal and more difficult to detect: most of the emerging infectious diseases reported for wildlife are caused by microparasites since they are more likely to produce massive mortality events associated with clearly recognisable symptoms [32]. Still, macroparasites can have a great impact on host population dynamics [33] and affect interspecific competition to the point of causing the exclusion of one host over the other, as shown for example by [34] between ring-necked pheasant and grey partridge in the U.K.

Hereafter, we investigate the composition of the macroparasite fauna of the grey squirrel in Northern Italy to inquire if this alien species introduced exotic parasites to Europe, or acquired local parasites (i.e., if there are any premises for parasite spillover and/or spill-back towards native species). We also compare macroparasite richness of grey squirrels introduced to Italy to what is known in the literature about their native range. We expect that richness in Italy will be lower than in North America, the more so in recently established populations (i.e. founded during the last two decades) compared to “older” populations (i.e. founded in 1948).

Finally, in order to point out potential extrinsic and intrinsic factors that may increase parasite transmission to red squirrels, we explored variation in burdens of dominant parasite taxa infesting grey squirrels according to season, host density, sex and body mass.

Materials and Methods

A total of 277 grey squirrel individuals were sampled between 2011 and 2013 in 7 study areas located in Northern Italy, four in Piedmont region and three in Lombardy. In all Piedmont sites, red squirrels went extinct between 1992 and 2000 and all the study areas are included in the metapopulation which originated from the release of 4 squirrels in 1948 [35]. On the contrary, Lombardy populations originated from independent releases that took place during the last 20 years [26] and red squirrels still persist at low numbers in some sites or are present with small populations nearby (Romeo et al., unpublished data). The number of founders of Lombardy nuclei is unclear, but each population was likely founded by few (<10) individuals (Romeo et al., unpublished data). Sampling was carried out specifically for scientific research on parasites and within a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at controlling or eradicating Italian grey squirrel populations. Squirrels were captured using Tomahawk live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) and immediately euthanised by CO_2 inhalation, following EC and AVMA guidelines [36–38] and with authorizations by Lombardy Region, Cuneo Province and Italian Institute for Environmental Protection and Research (ISPRA). In each sampling area, at least three trapping sessions (minimum 3 continuous days each) were carried out in different seasons. Traps were baited with a mixture of walnuts, hazelnuts and corn and, depending on day lenght, were checked two to three times a day to avoid animals from being overly stressed. For each individual we recorded sex, age class (juveniles or adults, based on weight and reproductive conditions), reproductive condition and body mass to the nearest gram. Each carcass was immediately placed in a sealed plastic bag and stored at $-20^{\circ}C$ for later examination.

Two hundred and fourteen grey squirrels were examined for both ectoparasites and gastro-intestinal helminths, 17 only for ectoparasites and 46 only for helminths. In the laboratory, defrozen carcasses were first combed on a white surface using a flea comb, to collect ectoparasites. Arthropods (fleas, ticks and sucking lice) were counted and stored in ethanol 70% for later identification. To search for helminths, the whole gastro-intestine from oesophagus to rectum was removed. Each tract (stomach, small intestine, caecum and colon-rectum) was dissected separately, washed with tap water and its content filtered through two sieves (lumen 0.40 and 0.03 mm, respectively). The content of each tract was then examined separately under a stereomicroscope (10× magnification) and helminths were counted and stored in lactophenol or ethanol 90% for identification. Morphological identification of both arthropods and helminths was carried out using a microscope equipped with camera lucida and was based on [39] for the genus *Trichuris* and [40] for the family Hymenolepididae. For details on the identification of the other taxa, see [41].

Statistical Analysis

To assess whether our sampling effort was adequate, we compared observed richness of both helminths and ectoparasites with estimated richness computed using EstimateS software (Version 9, R. K. Colwell, http://purl.oclc.org/estimates). The software estimates species richness extrapolating the asymptote of species accumulation curves (i.e. a plot of cumulative species richness against sampling effort) at each level of sampling effort. To avoid biases due to the order in which samples are drawn from the data set, the program averages richness estimates over many randomized runs (in our case, 100 runs). The program produces also several non-parametric estimators that add to the species richness an estimate based on the abundance of rare species. As suggested by [42], we chose the mean values of Chao2 estimator as it is the estimator that performs best with parasite distributions.

We explored the effect of host-linked factors (sex and body mass) and extrinsic factors (capture season and density of hosts) on the abundance (no. of parasites/host) of the most prevalent helminth and ectoparasite. Definition of seasons was based on temporal changes in tree squirrel behaviour and food availability, as described in previous studies (winter, December-February; spring, March-May; autumn, September-November, e.g. [43,44]). 15 individuals trapped in summer were excluded from the analysis to avoid problems with small sample size. To obtain density of hosts in each study site, we first estimated the population size using a catch-effort depletion model, assuming variable trapping effort, according to [45], as implemented in the R (R Core Team, http://www.r-project.org) package \texttt{fishmethods} [46]. This model improves standard linear regression methods to estimate the number of individuals present at the start of a series of trapping sessions (Y-variable), based on the number of animals trapped and removed (X-variable) in subsequent sessions, assuming a closed population during the entire trapping period [47]. We are confident about the assumption of closed populations sincee population size was estimated at the start of removal sampling, using sufficiently short trapping periods that did not include the autumn, the major period of dispersal in this species [48]. Furthermore, all our trapping sites are high-quality mixed broadleaf forest fragments far-between each other, surrounded by a low-quality matrix (i.e. cultivated land). Hence they are spatially distinct and partly-isolated from other sites with grey squirrels. Study sites were then classified according to relative density, calculated as population sizes divided by trapping areas, as follows: low-density sites (host density <3 squirrels/ha), medium-density sites (3< host density<7) and high-density sites (host density>7). Categories were set following available literature on grey squirrel population dynamics in different habitat types (e.g. [48,49]). Since all sampling sites had similar habitat conditions (i.e. mixed deciduous woods, low elevation, similar weather conditions), no other environmental variables were considered. Before the analysis, we examined all the explanatory variables for covariance and no major collinearity issues leading to statistical confounding effects were detected. The parasites considered in statistical analyses showed an aggregated distribution in the host population [50], thus variation in their abundance was analysed using Generalised Linear Models (GLMs) with negative binomial error distribution and log link-function. We first fitted full models with all fixed effects and their second order interaction and then obtained minimum models through backward elimination of non-significant factors. Interpretation of final models was based on pair-wise *t*-tests of Differences of Least Square Means (DLSM), applying sequential Bonferroni correction [51] for multiple comparisons.

Unless otherwise specified, all values and parameter estimates are reported as mean (±SE).

GLMs were performed using SAS/STAT 9.2 software (Copyright © 2009, SAS Institute Inc., Cary, NC, USA).

Ethics Statement

All sampling protocols were chosen to minimise animal stress and suffering. Traps were checked two to three times a day, depending on day length and handling time minimised to prevent animals from being overly stressed. Method of euthanasia (CO_2 inhalation) was chosen and performed according to what stated in AVMA guidelines [38] and also followed the guidelines determined by the EEC in Directives 86/609/EEC and 93/119/EEC and further developed in [36,37]. Euthanasia was carried out immediately on the field to avoid transportation and extended captivity of captured animals. Permits for trapping and culling grey squirrels were granted by Italian Institute for Environmental Protection and Research (ISPRA), Lombardy Region (Authorization No.: 3892, 02/05/2011) and Cuneo Province (Permit No.: 473, 12/05/2011).

Results

Parasite Fauna Composition

In 260 grey squirrels examined we identified a total of 6 gastro-intestinal nematode species and one cestode species (Table 1), with a resulting estimated richness of 7.0±0.4 SD species (Chao2 estimator). In addition, ten unidentified nematode specimens (7 larvae and 3 adult females) and one unidentified oxyurids were found in 11 different grey squirrels. No acantocephalan or trematode species were found. Individual richness ranged from 0 to 4 with a mean value of 0.8 species/host. The most abundant helminth was the nematode *Strongyloides robustus* with a total prevalence of 56.6% and a mean intensity (mI) of 16.9±2.1 worms infested/host. All the other identified nematodes were rare, with prevalence below 7% and most of them also with low intensities of infestation (Table 1). *Trichostrongylus calcaratus* was found in 17 hosts (6.5%, mI = 1.9±0.3), whereas 1 and 26 adult specimen of *T. retortaeformis* were found in 2 squirrels (0.8%). Adult males and immature females of *Trichuris muris* were found in 11 hosts (4.2%, mI = 1.3±0.2). In 4 grey squirrels (1.5%) we found respectively 1, 2, 2 and 4 specimens of *Aonchotheca annulosa*, and in 6 hosts (2.3%) we found the oxyurid *Trypanoxyuris sciuri* with intensities ranging from 1 to 379 worms. Finally, in one individual (0.4%) we found a single cestode specimen belonging to the family Hymenolepididae.

Excluding the single cestode specimen, richness in the two regions was consistent, with 5 nematodes each, since *T. muris* and *T. retortaeformis* were found only in Piedmont and Lombardy, respectively (see Table 1).

A total of 4 ectoparasite arthropod species was found on 231 grey squirrels: two fleas, one sucking louse and one ixodid tick (Table 2). The corresponding estimated richness was 4.0±0.5 SD species (Chao2 estimator). Individual richness ranged from 0 to 3, with a mean value of 0.5 species/host. The most prevalent species, found on 60 hosts (26.0%, mI = 2.7±0.3 parasites infested/host), was the flea *Ceratophyllus sciurorum*. The other most common arthropod was the sucking louse *Neohaematopinus sciuri* that was found on 41 hosts (17.7%; mI = 3.6±0.8). Finally, 4 specimens of the tick *Ixodes acuminatus* were found on 4 squirrels (1.7%) and a single specimen of the flea *Ctenocephalides felis* was found on one squirrel (0.4%). With the exception of the single specimen of *C. felis*, the other three ectoparasite species were found both in Piedmont and Lombardy (see Table 2).

Factors Affecting Parasite Infection

The most common helminth and arthropod infecting grey squirrels and thereby considered for abundance analysis were, respectively, *S. robustus* and *C. sciurorum*.

S. robustus abundance (number of worms/host) varied with density of hosts in the study site and host body mass (Table 3). Squirrels living in high-density sites were more infested than individuals living in medium- and low-density sites (both adjusted $p<0.0001$, Figure 1) and squirrels living in medium-density sites were more infested than in low-density sites (adjusted $p = 0.0008$). Host body mass had a positive effect on *S. robustus* abundance ($p = 0.0005$, Figure 2).

C. sciurorum abundance varied with season and density of hosts in the site (Table 3). Squirrels trapped in spring were more infested than in autumn and winter (both adjusted $p<0.0001$, Figure 3A), and animals living in high-density sites were more infested then those living in medium- and low-density populations (both adjusted $p<0.008$, Figure 3B).

Discussion

The parasite fauna of grey squirrels introduced to Northern Italy is poor, with 7 gastro-intestinal helminth species and 4 ectoparasite arthropod species. Observed richness of both helminth and ectoparasite species is consistent with richness computed using Chao2 estimator, indicating that the low number of species encountered is not a result of inadequate sampling effort.

The most abundant helminth is *S. robustus*, a North American nematode common in grey squirrels and other Nearctic sciurids in their native range (e.g. [52–54]). Also *T. calcaratus* is a Nearctic parasite commonly found in several squirrel species, but its primary host is the North American cotton-tail rabbit (*Sylvilagus*

Table 1. Helminth species infecting grey squirrels in Piedmont and Lombardy populations.

Helminth species	**Piedmont**		**Lombardy**		**Total**	
Host age	**n (p)**	**mI ± SE**	**n (p)**	**mI ± SE**	**n (p)**	**mI ± SE**
Juvenile	**N = 19**		**N = 14**		**N = 33**	
Strongyloides robustus	11 (58%)	6.7±2.4	6 (43%)	7.7±3.1	17 (52%)	7.1±1.9
Trichuris muris	3 (16%)	*1; 1; 1*	0	–	3 (9%)	*1; 1; 1*
Trypanoxyuris (*R.*) *sciuri*	0	–	1 (7%)	*1*	1 (3%)	*1*
Adult ♀	**N = 59**		**N = 54**		**N = 113**	
Strongyloides robustus	39 (66%)	15.4±3.5	30 (56%)	14.2±4.3	69 (61%)	14.9±2.7
Trichostrongylus calcaratus	12 (20%)	2.2±0.4	1 (2%)	*1*	13 (12%)	2.1±0.3
Trichuris muris	6 (10%)	1.5±0.3	0	–	6 (5%)	1.5±0.3
Aonchotheca annulosa	3 (5%)	*2; 2; 4*	1 (2%)	*1*	4 (4%)	*1; 2; 2; 4*
Trypanoxyuris (*R.*) *sciuri*	0	–	1 (2%)	*6*	1 (1%)	*6*
Trichostrongylus retortaeformis	0	–	1 (2%)	*26*	1 (1%)	*26*
Strongylida [gen. sp.]	3 (5%)	*1; 1; 1*	1 (2%)	*1*	4 (4%)	*1; 1; 1; 1*
Oxyurida [gen. sp.]	0	–	1 (2%)	*1*	1 (1%)	*1*
Adult ♂	**N = 63**		**N = 51**		**N = 114**	
Strongyloides robustus	33 (52%)	24.3±5.4	28 (55%)	19.3±5.8	61 (54%)	22.0±3.9
Trichostrongylus calcaratus	4 (6%)	*1; 1; 1; 2*	0	–	4 (4%)	*1; 1; 1; 2*
Trichuris muris	2 (3%)	*1; 1*	0	–	2 (2%)	*1; 1*
Trypanoxyuris (*R.*) *sciuri*	1 (2%)	*1*	3 (6%)	*1; 13; 379*	4 (4%)	*1; 1; 13; 379*
Trichostrongylus retortaeformis	0	–	1 (2%)	*1*	1 (1%)	*1*
Hymenolepididae [gen. sp.]	1 (2%)	*1*	0	–	1 (1%)	*1*
Strongylida [gen. sp.]	4 (6%)	*1; 1; 1; 1*	2 (4%)	*1; 1*	6 (5%)	1±0

N: number of host examined; n: number of infected hosts; p: prevalence; mI: mean intensity (no. parasites infected/hosts; when number of infected hosts <5, worm counts in italic).

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floridanus, [55]). Indeed, in Italy *T. calcaratus* is found only in areas were the introduced cotton-tail rabbit is present, suggesting that the lagomorph likely acts as primary host of this nematode. Both these North American helminths had already been reported in Europe: *T. calcaratus* in alien cotton-tail rabbits [56] and *S. robustus* in a few Italian red squirrels co-inhabiting with grey squirrels [41]. All the other nematodes we found are Eurasian species: *T. muris* and *T. retortaeformis* are common parasites of wood mice (*Apodemus* spp., [39]) and wild rabbits (*Oryctolagus cuniculus*, [57]) respectively, *A. annulosa* is a generalist nematode infesting a wide variety of mammals [58], including squirrels (e.g. [59,60]) and *T. sciuri* is the dominant helminth of Eurasian red squirrels [41,61]. *T. sciuri* was found only in few individuals and mostly in sites were red squirrels are still present or were present until a few years ago, suggesting that this parasite may not adapt well to grey squirrels and probably needs the presence of its native host to successfully persist. The most common ectoparasite species infesting grey squirrels in Italy are the flea *C. sciurorum* and the sucking louse *N. sciuri*. The first is the main flea species of Eurasian red squirrels [41,62], whereas *N. sciuri* is a species with a Holarctic distribution, commonly found both on the Eurasian red squirrel and on North American tree squirrels [63]. Interestingly, opposite to *T. sciuri*, *C. sciurorum* was found in all the sites, even in areas where the red squirrel went extinct decades ago, indicating that the flea got adapted to the new host and can complete its cycle without the native species being present. The other recorded arthropods are rare: the cosmopolitan flea *C. f. felis*, whose primary host is the domestic cat, and the Palearctic tick *I. acuminatus*, reported also rarely on red squirrels [41].

Only two helminths (*S. robustus* and *T. calcaratus*) and two arthropods (*C. sciurorum* and *N. sciuri*) have prevalence above 5% and only three of these species (*S. robustus*, *C. sciurorum* and *N. sciuri*) are present in all the sampled populations. All the other species are found only locally and are likely linked to the presence of other primary hosts. More important, contrary to our expectations, parasite species richness is consistent in Piedmont and Lombardy and does not seem to be affected by populations' origin or "age".

According to the available literature (excluding studies with sample size below 50 hosts), grey squirrels in their native range are parasitised by at least 8 gastro-intestinal helminth and 7 ectoparasite species (mites excluded) with prevalence above 5% (Table 4). Thus, compared to our results (we found only 2 helminths and 2 ectoparasites above the same prevalence), parasite richness reported for grey squirrels in their native range is higher, both for gastro-intestinal helminths and ectoparasites. This holds true even limiting the comparison to a smaller spatial scale, to studies carried out in the North Eastern part of the United States (i.e. the likely native range of the animals introduced to Italy): [64] and [65] reported respectively 4 helminths and 5 helminths and 5 arthropods infecting grey squirrels with prevalence above 5%. Moreover, we found only three of the species reported in North America (*S. robustus*, *T. calcaratus* and *N. sciuri*, the latter having a Holarctic distribution), whereas several parasites common in the Nearctic region are completely missing in Italy (e.g. the nematode *Citellinema bifurcatum* or the flea *Orchopeas howardii*). It is also interesting to notice that the parasite fauna of grey squirrels introduced to the U.K. is different than in Italy: for example in the U.K. the flea *O. howardii*, absent in Italy, is commonly observed

Table 2. Arthropod species infesting grey squirrels in Piedmont and Lombardy populations.

Arthropod species	**Piedmont**		**Lombardy**		**Total**	
Host age	**n (p)**	**ml ± SE**	**n (p)**	**ml ± SE**	**n (p)**	**ml ± SE**
Juvenile	**N = 17**		**N = 12**		**N = 29**	
Neohaematopinus sciuri	7 (41%)	3.7±0.9	0	–	7 (24%)	3.7±0.9
Ceratophyllus sciurorum	6 (35%)	3.2±1.3	1 (8%)	*3*	7 (24%)	3.1±1.1
Adult ♀	**N = 62**		**N = 44**		**N = 106**	
Neohaematopinus sciuri	13 (21%)	2.4±0.5	2 (4%)	*2; 10*	15 (14%)	3.0±0.7
Ceratophyllus sciurorum	16 (26%)	2.5±0.4	6 (14%)	3.0±1.4	22 (21%)	2.6±0.5
Ctenocephalides felis felis	1 (2%)	*1*	0	–	1 (1%)	*1*
Ixodes acuminatus	0	–	1 (2%)	*1*	1 (1%)	*1*
Adult ♂	**N = 54**		**N = 42**		**N = 96**	
Neohaematopinus sciuri	16 (30%)	4.1±1.7	3 (7%)	*1; 2; 3*	19 (20%)	3.9±1.5
Ceratophyllus sciurorum	22 (41%)	2.4±0.5	9 (21%)	3.0±0.5	31 (32%)	2.6±0.4
Ixodes acuminatus	1 (2%)	*1*	2 (5%)	*1; 1*	3 (3%)	*1; 1; 1*

N: number of host examined; n: number of infested hosts; p: prevalence; ml: mean intensity (no. parasites infested/hosts; when number of infested hosts < 5, worm counts in italic).

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(e.g. [66]), on the contrary, the nematode *S. robustus*, the most abundant helminth in Northern Italy, has never been reported.

Abundance of both the main helminth, *S. robustus*, and the main ectoparasite, *C. sciurorum*, in Italian grey squirrel populations varied with density of grey squirrels in the study sites. Abundance of both parasites was significantly higher in squirrels living in high-density populations. This result is not surprising since positive density dependence in parasite transmission is expected from theoretical studies [67] and a positive relationship between host density and abundance has indeed been observed in several taxa [68–70]. This pattern could also explain why *C. sciurorum* abundance varied also with seasons and was higher in spring than in autumn or winter. The peak in infestations levels occurs after the first breeding period of grey squirrels [48], when population density and contact among individuals increase and presence of potential hosts for fleas is higher. On the contrary, [41] reported an abundance peak of *C. sciurorum* in Eurasian red squirrels in autumn, after their second reproduction. Red squirrels are known to delay or even skip spring reproduction in different forest types, and often reach maximum population density in autumn rather than in spring [71,72]. Hence, this difference in seasonal abundance of the flea between the two hosts could be particularly alarming since, in areas where the two species co-inhabit, the normal seasonal distribution of *C. sciurorum* could be altered by the presence of grey squirrels, with an increased risk of transmission on red squirrels during spring. Furthermore, *S. robustus* abundance varied positively with host body mass. This result may be partly due to age growth [73], but may also be a consequence of the fact that larger animals offer a wider skin surface for *S. robustus* larvae to penetrate [68]. Moreover, in many tree squirrel species, body mass is positively correlated with dominance rank and/or home range size (e.g. [74–76]). Thus, parasite abundance may be related to individual boldness: having larger home ranges and engaging more in explorative and mating behaviour, larger, dominant squirrels may have higher exposure to infestation [77].

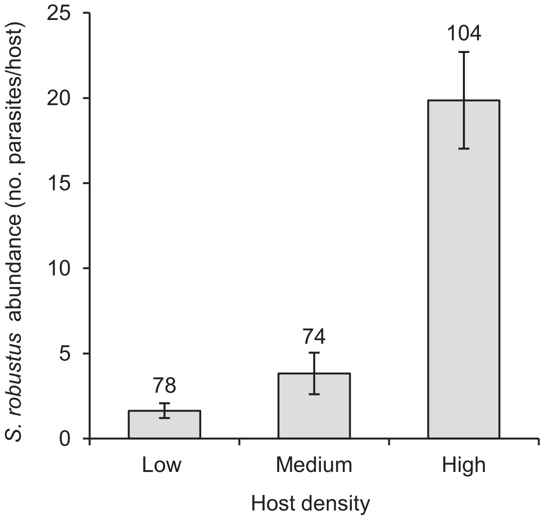

Figure 1. Variation of *S. robustus* abundance by host density. Mean abundance of *S. robustus* (sample size above standard error bars) varied with density of hosts in the site ($p<0.0001$). Squirrels living in high-density sites were more infested than individuals living in medium- and low-density sites (both sequential Bonferroni adjusted $p<0.0001$) and squirrels living in medium-density sites were more infested than in low-density sites (adjusted $p = 0.0008$).
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Hence, first of all, our findings demonstrate that grey squirrels introduced to Italy lost part of their original parasite fauna (even several dominant species), and although they acquired some Palearctic parasites, their number does not compensate the number of species lost. This holds true even when we compare

Table 3. Minimum selected model of the effects of host characteristics and environmental variables on parasite abundance (no. of parasites/host).

Dependent variable	**Source of variation**	**χ^2**	**df**	**P**	**Parameter estimate (±SE)**
***S. robustus* abundance**	Host density	95.3	2	<0.0001	
	Body mass	12.2	1	0.0005	0.0059±0.0017
***C. sciurorum* abundance**	Host density	18.5	2	<0.0001	
	Season	39.4	2	<0.0001	

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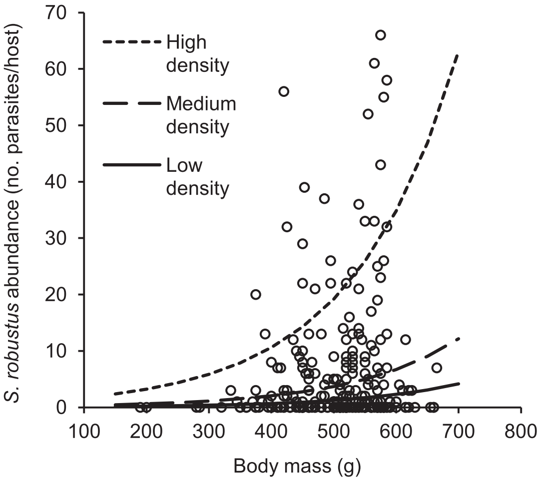

Figure 2. Variation of *S. robustus* abundance by host body mass. Relationship between *S. robustus* abundance and host body mass: observed values (blank circles) and values predicted by the model at different host densities (lines). Host body mass had a positive effect on *S. robustus* abundance ($p = 0.0005$; parameter estimate: 0.0059 ± 0.0017 SE).
doi:10.1371/journal.pone.0088002.g002

Table 4. Most prevalent gastro-intestinal helminths and arthropods (excluding mites) parasitizing grey squirrels in North America.

Parasite species	Prevalence	Sample size	Reference
Gastro-intestinal helminths			
Strongyloides robustus	28%–86%	62–270	[64,65,81]
Citellinema bifurcatum	35%–45%	62–270	[65,81,82]
Bohmiella wilsoni	14%–29%	175–270	[65,81]
Heligmodendrium hassalli	7%–92%	53–270	[52,64,65,81,82]
Capillaria americana	7%–14%	62–270	[64,65,81]
Trichostrongylus calcaratus	4%–16%	175–270	[65,81]
Syphacia thompsoni	5%	175–270	[65,81]
Enterobius sciuri (*T. bicristata?*)	2%–26%	175–270	[64,65,81]
Arthropods			
Neohaematopinus sciuri	33%–81%	53–106	[52,65,83]
Hopopleura sciuricola	32%–55%	53–106	[52,65,83]
Enderleinellus longiceps	2%–68%	67–106	[65,83]
Orchopeas howardii	51%–76%	53–106	[52,65,83]
Amblyomma americanum	22.4%–32.8%	67	[83,84]
Ixodes scapularis	1.5%–47.8%	67	[83,84]
Dermacentor variabilis	4.5–8.9	67	[83,84]

Only parasites that were recorded by more than one author and with maximum prevalence >5% are reported. Studies with sample size <50 hosts were excluded.
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our data only with studies carried out in a small portion of grey squirrel's native range and exclude parasite species that were only reported by a single author, making our conclusion conservative. Since all Italian grey squirrel populations were founded by a small number of individuals (i.e. had a low "propagule pressure", [78]), it is likely that some parasite species never reached the new range due to stochastic founder effects or were lost during the initial stages of invasion due to low host-densities insufficient for their transmission and persistence [79]. To test whether grey squirrels actually benefit from this parasite loss (i.e. whether the enemy release hypothesis holds true) further research is needed. Our results also suggest that *S. robustus* was introduced to Italy with the grey squirrel and that red squirrels likely acquired it by spillover from the alien species [41]. It should be noted that *S. robustus* is also suspected to mediate the competition between two species of North-American flying squirrels (*Glaucomys* spp.: [80]). We also show that the opposite process occurs: grey squirrels acquired the flea *C. sciurorum* and, to a lesser extent, the oxyurid nematode *T. sciuri* from red squirrels. Examining whether the acquisition of these parasites by the grey squirrels is altering their epidemiology

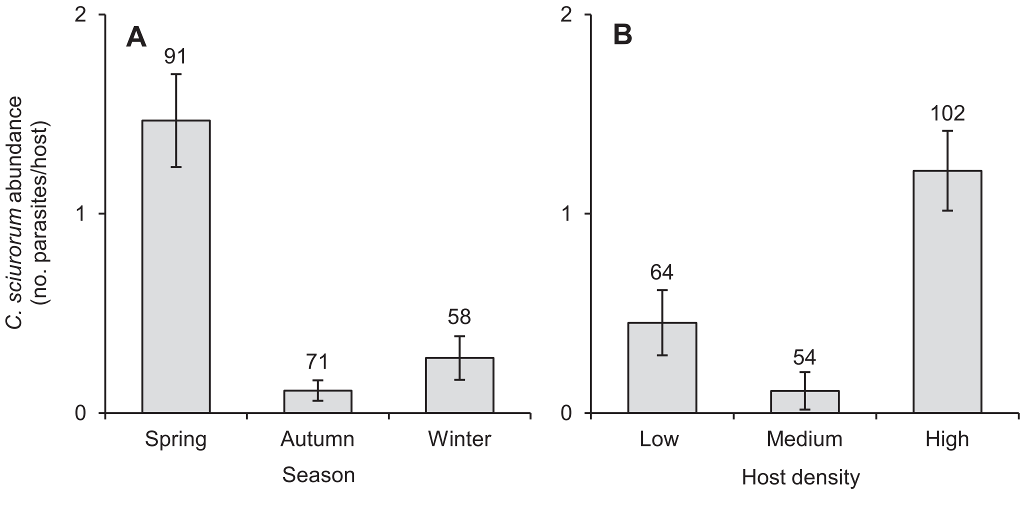

Figure 3. Variation of *C. sciurorum* abundance by season (A) and host density (B). Mean abundance of *C. sciurorum* (sample size above standard error bars) varied during different seasons ($p<0.0001$) and at different host densities ($p<0.0001$). Squirrels trapped in spring were more infested than in autumn and winter (both sequential Bonferroni adjusted $p<0.0001$) and animals living in high-density sites were more infested then those living in medium- and low-density populations (both adjusted $p<0.008$).
doi:10.1371/journal.pone.0088002.g003

with repercussions for red squirrels and investigating the consequences of *S. robustus* spillover for the native species are both priorities and specific aims of ongoing research.

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Author Contributions

Conceived and designed the experiments: CR LAW NF PL AM NS. Performed the experiments: CR LAW NF BP. Analyzed the data: CR LAW NF DGP BP. Contributed reagents/materials/analysis tools: PL AM NS DGP. Wrote the paper: CR.

References

1. Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E (1998) Quantifying Threats to Imperiled Species in the United States. BioScience 48: 607–615. doi:10.2307/1313420.
2. Clavero M, Garciaberthou E (2005) Invasive species are a leading cause of animal extinctions. Trends Ecol Evol 20: 110–110. doi:10.1016/j.tree.2005.01.003.
3. Hulme PE, Pyšek P, Nentwig W, Vilà M (2009) Will threat of biological invasions unite the European Union. Science 324: 40–41.
4. Vilà M, Basnou C, Pyšek P, Josefsson M, Genovesi P, et al. (2010) How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assessment. Front Ecol Environ 8: 135–144. doi:10.1890/080083.
5. Dunn AM, Perkins SE (2012) Editorial: Invasions and infections. Funct Ecol 26: 1234–1237. doi:10.1111/1365-2435.12022.
6. Dunn AM, Torchin ME, Hatcher MJ, Kotanen PM, Blumenthal DM, et al. (2012) Indirect effects of parasites in invasions. Funct Ecol 26: 1262–1274. doi:10.1111/j.1365-2435.2012.02041.x.
7. Strauss A, White A, Boots M (2012) Invading with biological weapons: the importance of disease-mediated invasions. Funct Ecol 26: 1249–1261. doi:10.1111/1365-2435.12011.
8. Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral pathogens. Nature 421: 625–627.
9. Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. Nature 421: 628–630. doi:10.1038/nature01346.
10. Torchin ME, Lafferty KD, Kuris AM (2001) Release from Parasites as Natural Enemies: Increased Performance of a Globally Introduced Marine Crab. Biol Invasions 3: 333–345. doi:10.1023/A:1015855019360.
11. DeWalt SJ, Denslow JS, Ickes K (2004) Natural-Enemy Release Facilitates Habitat Expansion of The Invasive Tropical Shrub Clidemia Hirta. Ecology 85: 471–483. doi:10.1890/02-0728.
12. Vilà M, Maron JL, Marco L (2005) Evidence for the enemy release hypothesis in Hypericum perforatum. Oecologia 142: 474–479. doi:10.1007/s00442-004-1731-z.
13. Blumenthal D, Mitchell CE, Pyšek P, Jarošík V (2009) Synergy between pathogen release and resource availability in plant invasion. Proc Natl Acad Sci 106: 7899–7904. doi:10.1073/pnas.0812607106.
14. Colautti RI, Ricciardi A, Grigorovich IA, MacIsaac HJ (2004) Is invasion success explained by the enemy release hypothesis? Ecol Lett 7: 721–733. doi:10.1111/j.1461-0248.2004.00616.x.
15. Hudson P, Greenman J (1998) Competition mediated by parasites: biological and theoretical progress. Trends Ecol Evol 13: 387–390. doi:10.1016/S0169-5347(98)01475-X.
16. Daszak P, Cunningham AA, Hyatt AD (2000) Emerging Infectious Diseases of Wildlife– Threats to Biodiversity and Human Health. Science 287: 443–449. doi:10.1126/science.287.5452.443.
17. Taraschewski H (2006) Hosts and parasites as aliens. J Helminthol 80: 99–128. doi:10.1079/JOH2006364.
18. Kelly DW, Paterson RA, Townsend CR, Poulin R, Tompkins DM (2009) Parasite spillback: A neglected concept in invasion ecology? Ecology 90: 2047–2056. doi:10.1890/08-1085.1.
19. Marsot M, Chapuis J-L, Gasqui P, Dozières A, Masséglia S, et al. (2013) Introduced Siberian Chipmunks (Tamias sibiricus barberi) Contribute More to Lyme Borreliosis Risk than Native Reservoir Rodents. PLoS ONE 8: e55377. doi:10.1371/journal.pone.0055377.
20. Keesing F, Holt RD, Ostfeld RS (2006) Effects of species diversity on disease risk. Ecol Lett 9: 485–498. doi:10.1111/j.1461-0248.2006.00885.x.
21. Thieltges DW, Reise K, Prinz K, Jensen KT (2009) Invaders interfere with native parasite–host interactions. Biol Invasions 11: 1421–1429. doi:10.1007/s10530-008-9350-y.
22. Poulin R, Paterson RA, Townsend CR, Tompkins DM, Kelly DW (2011) Biological invasions and the dynamics of endemic diseases in freshwater ecosystems. Freshw Biol 56: 676–688. doi:10.1111/j.1365-2427.2010.02425.x.
23. Rushton SP, Lurz PWW, Gurnell J, Nettleton P, Bruemmer C, et al. (2005) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. Epidemiol Infect 134: 521. doi:10.1017/S0950268805005303.
24. McInnes CJ, Coulter L, Dagleish MP, Deane D, Gilray J, et al. (2013) The emergence of squirrelpox in Ireland. Anim Conserv 16: 51–59. doi:10.1111/j.1469-1795.2012.00570.x.
25. Tompkins DM, White AR, Boots M (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. Ecol Lett 6: 189–196.
26. Martinoli A, Bertolino S, Preatoni DG, Balduzzi A, Marsan A, et al. (2010) Headcount 2010: the multiplication of the grey squirrel populations introduced to Italy. Hystrix Ital J Mammal 21. Available: http://www.italian-journal-of-mammalogy.it/article/view/4463. Accessed 16 October 2013.
27. Bertolino S, Montezemolo NC di, Preatoni DG, Wauters LA, Martinoli A (2013) A grey future for Europe: Sciurus carolinensis is replacing native red squirrels in Italy. Biol Invasions: 1–10. doi:10.1007/s10530-013-0502-3.
28. Wauters LA, Tosi G, Gurnell J (2002) Interspecific competition in tree squirrels: do introduced grey squirrels (Sciurus carolinensis) deplete tree seeds hoarded by red squirrels (S. vulgaris)? Behav Ecol Sociobiol 51: 360–367. doi:10.1007/s00265-001-0446-y.
29. Wauters L, Gurnell J, Martinoli A, Tosi G (2002) Interspecific competition between native Eurasian red squirrels and alien grey squirrels: does resource partitioning occur? Behav Ecol Sociobiol 52: 332–341. doi:10.1007/s00265-002-0516-9.
30. Gurnell J, Wauters LA, Lurz PWW, Tosi G (2004) Alien species and interspecific competition: effects of introduced eastern grey squirrels on red squirrel population dynamics. J Anim Ecol 73: 26–35. doi:10.1111/j.1365-2656.2004.00791.x.
31. Wauters L, Tosi G, Gurnell J (2005) A review of the competitive effects of alien grey squirrels on behaviour, activity and habitat use of red squirrels in mixed, deciduous woodland in Italy. Hystrix Ital J Mammal 16. Available: http://www.italian-journal-of-mammalogy.it/index.php/hystrix/article/viewArticle/4340. Accessed 9 July 2013.
32. Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. Philos Trans R Soc Lond B Biol Sci 356: 1001–1012. doi:10.1098/rstb.2001.0900.
33. Tompkins DM, Arneberg P, Begon ME, Cattadori IM, Greenman JV, et al. (2002) Parasites and host population dynamics. In: Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek JAP, Dobson AP, editors. The ecology of wildlife diseases. Oxford: Oxford University Press. 45–62. Available: http://eprints.lancs.ac.uk/9036/. Accessed 1 August 2013.
34. Tompkins DM, Draycott RAH, Hudson PJ (2000) Field evidence for apparent competition mediated via the shared parasites of two gamebird species. Ecol Lett 3: 10–14. doi:10.1046/j.1461-0248.2000.00117.x.
35. Bertolino S, Genovesi P (2005) The application of the European strategy on invasive alien species: an example with introduced squirrels. Hystrix Ital J Mammal 16. Available: http://www.italian-journal-of-mammalogy.it/index.php/hystrix/article/viewArticle/4343. Accessed 19 December 2013.
36. Close B, Banister K, Baumans V, Bernoth E-M, Bromage N, et al. (1996) Recommendations for euthanasia of experimental animals: Part 1. Lab Anim 30: 293–316. doi:10.1258/002367796780739871.
37. Close B, Banister K, Baumans V, Bernoth E-M, Bromage N, et al. (1997) Recommendations for euthanasia of experimental animals: Part 2. Lab Anim 31: 1–32. doi:10.1258/002367797780600297.
38. Leary SL, American Veterinary Medical Association (2013) AVMA guidelines for the euthanasia of animals: 2013 edition. Available: https://www.avma.org/KB/Policies/Documents/euthanasia.pdf. Accessed 12 November 2013.
39. Feliu C, Spakulová M, Casanova JC, Renaud F, Morand S, et al. (2000) Genetic and Morphological Heterogeneity in Small Rodent Whipworms in Southwestern Europe: Characterization of *Trichuris Muris* and Description of *Trichuris Arvicolae* N. SP. (Nematoda: Trichuridae). J Parasitol 86: 442–449. doi:10.1645/0022-3395(2000)086[0442:GAMHIS]2.0.CO;2.
40. Czaplinski B, Vaucher C (1994) Family Hymenolepididae Ariola, 1899.: 595–663.
41. Romeo C, Pisanu B, Ferrari N, Basset F, Tillon L, et al. (2013) Macroparasite community of the Eurasian red squirrel (Sciurus vulgaris): poor species richness and diversity. Parasitol Res 112: 3527–3536. doi:10.1007/s00436-013-3535-8.
42. Walther BA, Morand S (1998) Comparative performance of species richness estimation methods. Parasitology 116: 395–405.
43. Wauters LA, Vermeulen M, Van Dongen S, Bertolino S, Molinari A, et al. (2007) Effects of spatio-temporal variation in food supply on red squirrel Sciurus

vulgaris body size and body mass and its consequences for some fitness components. Ecography 30: 51–65. doi:10.1111/j.2006.0906-7590.04646.x.

44. Romeo C, Wauters LA, Preatoni D, Tosi G, Martinoli A (2010) Living on the edge: Space use of Eurasian red squirrels in marginal high-elevation habitat. Acta Oecologica 36: 604–610. doi:10.1016/j.actao.2010.09.005.
45. Gould WR, Pollock KH (1997) Catch-–effort maximum likelihood estimation of important population parameters. Can J Fish Aquat Sci 54: 890–897. doi:10.1139/f96-327.
46. Nelson GA (2013) fishmethods: Fishery Science Methods and Models in R. Available: http://cran.r-project.org/web/packages/fishmethods/index.html.Accessed 12 November 2013.
47. Leslie PH, Davis DHS (1939) An Attempt to Determine the Absolute Number of Rats on a Given Area. J Anim Ecol 8: 94–113. doi:10.2307/1255.
48. Koprowski JL (1994) Sciurus carolinensis. Mamm Species: 1. doi:10.2307/3504224.
49. Gurnell J (1996) The Effects of Food Availability and Winter Weather on the Dynamics of a Grey Squirrel Population in Southern England. J Appl Ecol 33: 325. doi:10.2307/2404754.
50. Shaw DJ, Grenfell BT, Dobson AP (1998) Patterns of macroparasite aggregation in wildlife host populations. Parasitology 117: 597–610.
51. Holm S (1979) A Simple Sequentially Rejective Multiple Test Procedure. Scand J Stat 6: 65–70.
52. Harkema R (1936) The Parasites of Some North Carolina Rodents. Ecol Monogr 6: 151. doi:10.2307/1943242.
53. Chandler AC (1942) Helminths of tree squirrels in southeast Texas. J Parasitol 28: 135–140.
54. Rausch R, Tiner JD (1948) Studies on the Parasitic Helminths of the North Central States. I. Helminths of Sciuridae. Am Midl Nat 39: 728. doi:10.2307/2421532.
55. Wiggins JP, Cosgrove M, Rothenbacher H (1980) Gastrointestinal parasites of the eastern cottontail (Sylvilagus floridanus) in central Pennsylvania. J Wildl Dis 16: 541–544.
56. Tizzani P, Menzano A, Catalano S, Rossi L, Meneguz PG (2011) First report of Obeliscoides cuniculi in European brown hare (Lepus europaeus). Parasitol Res 109: 963–966. doi:10.1007/s00436-011-2375-7.
57. Audebert F, Hoste H, Durette-Desset MC (2002) Life cycle of Trichostrongylus retortaeformis in its natural host, the rabbit (Oryctolagus cuniculus). J Helminthol 76: 189–192. doi:10.1079/JOH2002126.
58. Moravec F (2000) Review of capillariid and trichosomoidid nematodes from mammals in the Czech Republic and the Slovak Republic. Acta Soc Zool Bohemicae 64: 271–304.
59. Pisanu B, Jerusalem C, Huchery C, Marmet J, Chapuis J-L (2007) Helminth fauna of the Siberian chipmunk, Tamias sibiricus Laxmann (Rodentia, Sciuridae) introduced in suburban French forests. Parasitol Res 100: 1375–1379. doi:10.1007/s00436-006-0389-3.
60. Pisanu B, Lebailleux L, Chapuis J-L (2009) Why do Siberian chipmunks Tamias sibiricus (Sciuridae) introduced in French forests acquired so few intestinal helminth species from native sympatric Murids? Parasitol Res 104: 709–714. doi:10.1007/s00436-008-1279-7.
61. Hugot J-P (1984) Sur le genre Trypanoxyuris (Oxyuridae, Nematoda). I: Parasites de Sciuridés: sous-genre Rodentoxyuris. Bull Muséum Natl Hist Nat Sect Zool Biol Écologie Anim 6: 711–720.
62. Traub R, Rothschild M, Haddow JF (1983) The Rothschild collection of fleas. The Ceratophyllidae: key to the genera and host relationships.: xv +288pp.
63. Durden LA, Musser GG (1994) The sucking lice (Insecta, Anoplura) of the world?: a taxonomic checklist with records of mammalian hosts and geographical distributions. Bulletin of the AMNH?; no. 218. Sucking lice and hosts. Available: http://digitallibrary.amnh.org/dspace/handle/2246/825. Accessed 16 October 2013.
64. Eckerlin RP (1974) Studies on the life cycles of Strongyloides robustus Chandler 1942, and a survey of the helminths of Connecticut sciurids [PhD Dissertation]. Storrs: University of Connecticut.
65. Parker JC (1971) Protozoan, helminth and arthropod parasites of the gray squirrel in southwestern Virginia [PhD Dissertation]. Blacksburg: Virginia Polytechnic Institute.
66. Shorten M (1954) Squirrels. New Nat - Squirrels: xii +212 pp.
67. Anderson RM, May RM (1978) Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes. J Anim Ecol 47: 219–247. doi:10.2307/3933.
68. Arneberg P, Skorping A, Grenfell B, Read AF (1998) Host densities as determinants of abundance in parasite communities. Proc R Soc Lond B Biol Sci 265: 1283–1289. doi:10.1098/rspb.1998.0431.
69. Arneberg P (2001) An ecological law and its macroecological consequences as revealed by studies of relationships between host densities and parasite prevalence. Ecography 24: 352–358. doi:10.1034/j.1600-0587.2001.240313.x.
70. Arneberg P (2002) Host population density and body mass as determinants of species richness in parasite communities: comparative analyses of directly transmitted nematodes of mammals. Ecography 25: 88–94. doi:10.1034/j.1600-0587.2002.250110.x.
71. Wauters LA, Matthysen E, Adriaensen F, Tosi G (2004) Within-sex density dependence and population dynamics of red squirrels Sciurus vulgaris. J Anim Ecol 73: 11–25. doi:10.1111/j.1365-2656.2004.00792.x.
72. Wauters LA, Githiru M, Bertolino S, Molinari A, Tosi G, et al. (2008) Demography of alpine red squirrel populations in relation to fluctuations in seed crop size. Ecography 31: 104–114. doi:10.1111/j.2007.0906-7590.05251.x.
73. Hudson PJ, Dobson AP (1995) Macroparasites: observed patterns. In: Grenfell BT, Dobson AP, editors. Ecology of infectious diseases in natural populations. Cambridge: Cambridge University Press. 144–176.
74. Don BAC (1983) Home range characteristics and correlates in tree squirrels. Mammal Rev 13: 123–132. doi:10.1111/j.1365-2907.1983.tb00273.x.
75. Wauters L, Dhondt AA (1989) Body Weight, Longevity and Reproductive Success in Red Squirrels (Sciurus vulgaris). J Anim Ecol 58: 637–651. doi:10.2307/4853.
76. Wauters L, Dhondt AA (1992) Spacing behaviour of red squirrels, Sciurus vulgaris: variation between habitats and the sexes. Anim Behav 43: 297–311. doi:10.1016/S0003-3472(05)80225-8.
77. Boyer N, Réale D, Marmet J, Pisanu B, Chapuis J-L (2010) Personality, space use and tick load in an introduced population of Siberian chipmunks Tamias sibiricus. J Anim Ecol 79: 538–547. doi:10.1111/j.1365-2656.2010.01659.x.
78. Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20: 223–228. doi:10.1016/j.tree.2005.02.004.
79. MacLeod CJ, Paterson AM, Tompkins DM, Duncan RP (2010) Parasites lost - do invaders miss the boat or drown on arrival? Ecol Lett 13: 516–527. doi:10.1111/j.1461-0248.2010.01446.x.
80. Krichbaum K, Mahan CG, Steele MA, Turner G, Hudson PJ (2010) The potential role of Strongyloides robustus on parasite-mediated competition between two species of flying squirrels (Glaucomys). J Wildl Dis 46: 229–235.
81. Davidson WR (1976) Endoparasites of selected populations of gray squirrels (Sciurus carolinensis) in the southeastern United States. Proc Helminthol Soc Wash 43. Available: http://agris.fao.org/agris-search/search/display.do?f=2012/OV/OV201203331003331.xml;US19770205345. Accessed 29 July 2013.
82. Katz JS (1938) A survey of the parasites found in and on the fox squirrel, Sciurus niger rufiventer Geoffroy, and the southern gray squirrel, Sciurus carolinensis carolinensis Gmelin, in Ohio [MS thesis]. Columbus: Ohio State University.
83. Durden LA, Ellis BA, Banks CW, Crowe JD, Oliver Jr JH (2004) Ectoparasites of gray squirrels in two different habitats and screening of selected ectoparasites for bartonellae. J Parasitol 90: 485–489.
84. Schulze TL, Bowen GS, Lakat MF, Parkin WE, Shisler JK (1986) Seasonal Abundance and Hosts of Ixodes Dammini(Acari: Ixodidae) and Other Ixodid Ticks from An Endemic Lyme Disease Focus in New Jersey, USA. J Med Entomol 23: 105–109.

CHAPTER 4

Faecal egg counts from field experiment reveal density-dependence in helminth fecundity: *Strongyloides robustus* infecting grey squirrels (*Sciurus carolinensis*)

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Manuscript

Faecal egg counts from field experiment reveal density-dependence in helminth fecundity: *Strongyloides robustus* infecting grey squirrels (*Sciurus carolinensis*)

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Abstract Investigation of endo-macroparasite infections in living animals relies mostly on indirect methods aimed to detect parasite eggs in hosts' faeces. However, flotation technique is appropriate for preliminary screening, but does not provide quantitative information on parasite loads, whereas Faecal Egg Count (FEC) methods may not be reliable to estimate parasite intensity, since egg production may be affected by density-dependent effects on helminth fecundity. We used alien grey squirrels (*Sciurus carolinensis*) and their gastro-intestinal nematode *Strongyloides robustus* as a model system to assess the performance of coprological techniques and to investigate factors affecting parasite fecundity. We compared results of gut examination, flotation and MacMaster FECs in 65 culled grey squirrels. Sensitivity and specificity of flotation were respectively 81,2% (CI: 54,3% - 95,9%) and 85,7% (CI: 72,7% - 94,1%), resulting in low positive predictive values when prevalence of infection is low. Hence, flotation may be applied to perform a first screening of infection status, but should be followed by more specific methods to identify truly infected hosts. The predictive model explaining variation in *S. robustus* intensity retained as explanatory variable the second-order polynomial EPG (eggs/gram of faeces), leading to a non-linear relationship between parasite load and FECs. Parasite mean fecundity showed indeed a negative relationship with parasite load: individual egg production decreases with increasing values of parasite intensity. Furthermore, we did not find any effect of the amount of analysed faeces on FECs nor any seasonal variation in egg production, indicating that the observed reduction in helminth fecundity is caused exclusively by density dependent process such as competition among worms or host immune response. As a consequence, FECs are not a reliable method to estimate *S. robustus* intensity, since diverse values of EPG may correspond to the same number of parasites.

Introduction

Endo-macroparasite (i.e. helminths) infections affect host fitness by reducing its fecundity or survival and may thus act as important regulators of host population dynamics (reviewed in Tompkins and Begon 1999).

To investigate endo-macroparasite infections in wildlife, direct, post-mortem methods may be used when ad hoc sampling of dead animals is feasible (e.g. when dealing with game animals, alien species object of control programs or through opportunistic collection of animals found dead). However, the diagnosis of helminth infections in living hosts relies mostly on indirect, non-invasive methods aimed to assess the presence of eggs or larvae in hosts' faeces. Qualitative methods, such as faecal flotation, are routinely used in clinical diagnostics. This kind of methods are appropriate for preliminary screening, since they are fast and have a high diagnostic sensitivity, but they do not provide information on quantitative shedding of parasite eggs. On the contrary, Faecal Egg Count (FEC) methods are widely used in parasitological studies to estimate intensity of infection (i.e. no. parasites/infected hosts) since a positive, direct relationship between the number of eggs in faeces and parasite abundance has been observed in several host-parasite systems (e.g. Elkins et al. 1991; Sithithaworn et al. 1991; Seivwright et al. 2004).

The importance of quantitatively establishing a measure of parasite load resides primarily on the fact that pathological effects of macroparasites are proportional to the number of parasites infecting the host (Hudson and Dobson 1995). Hence, knowledge of intensity of infection is essential to make inference about parasite impact on host population dynamics. Moreover, individual intensity measures may add important details on parasite distribution through the host population, increasing our understanding of parasite transmission and epidemiology (Perkins et al. 2003).

However, some authors advise caution on the use of FECs to estimate intensity (e.g. Michael and Bundy 1989; Gillespie 2006) because the inference of the number of adult parasites from egg counts may not always be straightforward. Several factors may indeed influence the rate of eggs produced (i.e. fecundity) and expelled with the faeces. For example, when helminth fecundity varies seasonally (Shaw and Moss 1989) or sex-ratio is female-biased (Poulin 1997), egg production may not be representative of the number of adult worms infecting the host. But, in particular, parasite fecundity may be affected by density-dependent processes: as the number of parasites increases, the rate of egg production per individual proportionally decreases (e.g. Anderson and Schad 1985; Smith et al. 1987; Tompkins and Hudson 1999). Such a negative relationship between intensity of infection and individual fecundity means that the number of eggs expelled with the faeces may not increase linearly with the number of infecting parasites. As a result, FECs may not be representative of the infection status.

These density-dependent effects may arise after reaching a threshold of parasite abundance, as a consequence of competition among parasites (Tompkins and Hudson 1999) or indirectly through the action of the host immune response elicited by high infection intensities (Keymer 1982; Quinnell et al. 1990; Hudson and Dobson 1997). Moreover, host-related factors can indirectly influence parasite fecundity: for example we know that host gender may greatly affect resistance towards infection while host age incorporates the cumulated past exposure to parasites, thus reflecting changes in immune response (reviewed in Wilson et al. 2002). Hence, the analysis of factors affecting parasite fecundity may provide insights into the biology of host-parasite interactions.

The aim of the present study is to assess the performance of indirect methods of analysis (flotation and FEC) and to investigate potential density-dependent effects on parasite fecundity, using Eastern grey squirrels (*Sciurus carolinensis*) and their gastrointestinal parasite *Strongyloides robustus* as a model system. *S. robustus* is a parthenogenic, directly transmitted nematode common in many species of Nearctic tree squirrels (e.g. Rausch and Tiner 1948; Davidson 1976). Romeo et al. (2014) found that

S. robustus has been also introduced to Italy along with its host and represents the main gastro-intestinal helminth infecting the alien species in the new range (prevalence: 57%; N = 260). Moreover, *S. robustus* is also known to spill over to native naive Eurasian red squirrels (*S. vulgaris*) (Romeo et al. 2013). Hence, defining quick, effective and reliable methods for indirect screening of endo-macroparasite infections in sciurids will help to survey health status of the native species in the field, since culling endangered red squirrels is not an option.

We will first assess the performance of the flotation technique as a test to detect parasite presence, then evaluate whether FECs may give a reliable estimate of true infection intensity by comparing results of both coprological methods with results obtained by direct examination of gut content. Finally, we will analyse variability in *S. robustus* fecundity in order to determine whether host-related factors (host sex, age and body mass), seasonality and/or density-dependent processes affect egg production and emission.

Material and Methods

Parasitological analysis

A total of 65 grey squirrel individuals were examined for presence of the nematode S. robustus, both through coprological techniques and direct gut examination.

All the animals were collected throughout the year 2011 (from January to September) from a single introduced population located in Northern Italy. Sampling was carried out specifically for scientific research on parasites with authorizations by Cuneo Province and Italian Institute for Environmental Protection and Research (ISPRA). Squirrels were captured using live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) and immediately euthanised by CO_2 inhalation following EC and AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary 2013). Traps were checked two to three times a day, depending on day length, and starting at 10:00 a.m. For each individual we recorded sex, age class (subadults or adults, based on weight and reproductive conditions) and body mass to the nearest gram. Each carcass was immediately placed in a sealed plastic bag and stored at -20 °C for later examination. Faeces were collected from the trap floor, placed dry in Eppendorf tubes and stored at 4°C until analysis, which was always carried out within 1 day after sampling to avoid hatching of eggs (Seivwright et al. 2004).

Table 1. Factors affecting the probability of being positive to the flotation test

Factor	**Effect**	**df**	**Deviance**	**P value**
Infection status	44.64	1	13.30	<0.001
EPG	0.62	1	34.50	<0.001

To search directly for adult helminths, the whole gastro-intestine from oesophagus to rectum was removed during post-mortem examination. Each tract (stomach, small intestine, caecum and colon-rectum) was dissected separately, washed with tap water and its content filtered through two sieves (lumen 0.40 and 0.03 mm, respectively). The content of each tract was then examined under a stereo-microscope (10x magnification) and adult *S. robustus* were counted.

Faecal samples were analysed both through qualitative (flotation) and quantitative methods (McMaster technique, MAFF 1977). First, faeces were weighted to the nearest centigram, then diluted with 10 ml/g of saturated NaCl solution (1200 g/l). To amalgamate the faeces with NaCl solution, they were then crushed gently in a mortar. The obtained solution was filtered through a sieve to remove the biggest particles and placed in a closable tube that was shaken gently for one minute to homogenise the sample. Both chambers of a McMaster slide were then filled at once with the solution using a disposable Pasteur pipette. After waiting 3 minutes to let eggs float to the surface of the chambers, the slide was examined under a microscope (100x magnification). To obtain the number of eggs per gram of faeces (EPG) the total number of eggs counted under both grids (each one containing 0.15 ml of solution) was multiplied by the dilution factor (i.e. x 33).

The remaining faeces-NaCl solution was used for the flotation technique: it was poured in a 15 ml centrifuge tube and fresh NaCl solution was added until the rim of the tube was reached. After 30 minutes, necessary to let the eggs float to the surface (Dunn and Keymer 1986), a cover slip was leaned on the solution meniscus to collect floating eggs, put on a slide and examined under the microscope (40x magnification) to detect *S. robustus* eggs.

Table 2. Factors affecting *S. robustus* intensity (no. worms/host)

Factor	Effect	df	Deviance	P value
Age		1	15,99	<0.001
Ad	0			
SubAdult	-0.209			
Body Mass	0.007	1	6,19	0.012
EPG	0.004	1	8,27	0.004
EPG^2	-1.8e-06	1	6,50	0.010

Flotation performance

The performance of the flotation technique was assessed by calculating the test sensitivity (Se) and specificity (Sp) which are respectively the probability to correctly identify an infected individual as positive and a healthy individual as negative. The true infection status was considered to be reflected by the gastrointestinal analysis. To provide diagnostic interpretation of flotation results we computed the positive predictive value (PPV) and negative predictive value (NPV) of the test which represent the probability that an individual which tested positive or negative with an imperfect test is respectively infected and healthy (Thrusfield 2013). Since these measures are influenced by the population prevalence, we calculated both predictive values first using an hypothetical prevalence of 1% and then considering as a reference value 69% prevalence obtained through gastrointestinal analyses on a wider host sample (Romeo et al., 2014).

We also analysed the outcome of the flotation test through logistic regression, considering the true infection status, EPG and analysed grams of faeces as explanatory variables for the probability to obtain a positive result from flotation. Grams of faeces were included to account for the small amount of faeces that may fail to include eggs, since eggs may be distributed unevenly within faeces (Sinniah 1982; Brown et al. 1994).

Relationship between FECs and S. robustus intensity

To verify the predictive value of EPG on *S. robustus* intensity, we fitted a Generalised Linear Model (GLM) considering true parasite intensity (obtained from post-mortem analysis) as a response variable and EPG, host body mass, sex, age, trapping season and weight of faeces analysed as explanatory variables. Second order interactions of EPG with all the additional covariates were included to account for the effects of host-related and extrinsic factors on egg shedding. Moreover, we included a second order polynomial effect of EPG to test whether a non-linear convex relationship between the number of eggs in faeces and *S. robustus* was present.

To investigate factors affecting parasite fecundity, we fitted a second model testing the effects of *S. robustus* intensity, trapping season, weight of faeces examined and host sex, body mass and age on helminth mean fecundity, calculated as EPG/number of adult worms (Patterson & Viney, 2003).

For both models we used GLMs with negative binomial error distribution which fitted better the aggregated parasitological data. After running full models with all factors and their second order interactions, we obtained minimum models through backward elimination of non-significant effects ($p>0.05$). Statistical analysis was performed using the software package R 3.0.2 (R Core Team 2013).

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Results

Parasitological analysis

Adult parasites were found in 49 out of 65 examined squirrels corresponding to a prevalence of 75,3% (CI: 65,9% - 84,8%). Individual intensity of infected animals ranged from 1 to 109 adult worms with a mean value of 15.9 ± 3.1 SD worms/host. Grams of faeces available for faecal analysis ranged from 0.21 to 1.55. 45 out of 65 individuals were positive to the flotation test and McMaster FECs resulted in values ranging from 33 to 1815 EPG.

Flotation Performance

Parasite eggs were detected in 42 out of 49 infected individuals: 3 uninfected hosts tested positive and 7 infected animals were negative, leading to a test sensitivity of 85,7% (CI: 72,7% - 94,1%) and specificity of 81,2% (CI: 54,3% - 95,9%). PPV and NPV for populations with infection prevalence of 1% are respectively 4,4% and 99,8% whereas for populations with 69% prevalence they are 91% and 71,8%. The probability that the flotation test gives a positive result, besides being affected by the real infection status, increases with increasing values of EPG but is not influenced by the amount of faeces analysed (Table 1).

Relationship between FECs and S. robustus intensity

The minimal model predicting S. robustus intensity included a positive effect of EPG and its second order polynomial effect, leading to a convex, non-linear relationship between S. robustus and EPG (Fig. 1 and Tab. 2). Intensity of infection was also higher in adult than in subadult squirrels and increased with host body mass.

The minimal model explaining S. robustus fecundity retained only S. robustus intensity as explanatory variable (χ21=5.28; p=0.021). Parasite load had a negative effect on fecundity with increasing intensity of infection resulting in reduced fertility (Effect estimate= -0.026, Fig 2).

Discussion

The analysis of flotation performance indicates moderately high values of sensitivity and specificity that result in high predictive values (both positive and negative) when the test is applied to populations with high prevalence of infection, proving an accurate assessment of S. robustus presence/absence. However, when prevalence is low, flotation is still reliable to identify healthy animals, yet it may fail to correctly identify infected hosts. The probability of the test being positive is not affected by the weight of faeces analysed, indicating that in grey squirrels this method may be used also with the scant quantities of faecal material often collected during field sampling (i.e. less than 0.5 g).

Our results indicate also a low reliability of FECs to estimate true S. robustus intensity. In our predictive model we found a significant effect of the polynomial EPG term on S. robustus intensity, leading to a non-linear relationship between the two terms (Fig. 1).

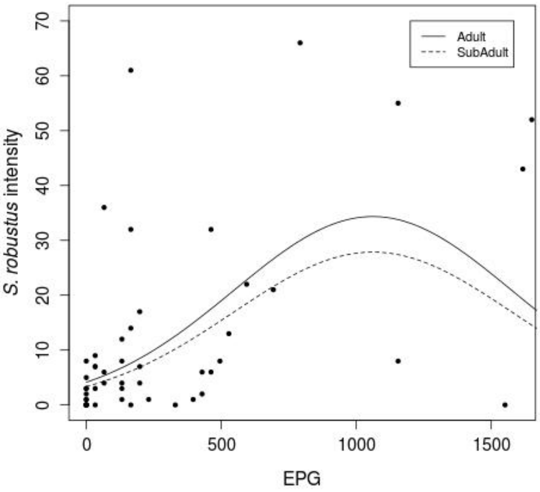

Figure 1. Relationship between EPG (eggs/gram of faeces) and *S. robustus* intensity (no. worms/host).

As a consequence, diverse values of EPG may actually correspond to the same parasite intensity. As observed on a wider data set (Romeo et al. 2014), parasite load was also positively affected by host body mass and age, but no significant interaction between EPG and the other covariates was detected, indicating that the relationship between egg production and intensity is not affected by other intrinsic nor extrinsic factors.

Moreover, the analysis of S. robustus fecundity shows that intensity has a negative effect on mean fecundity of adult female worms (Fig. 2).

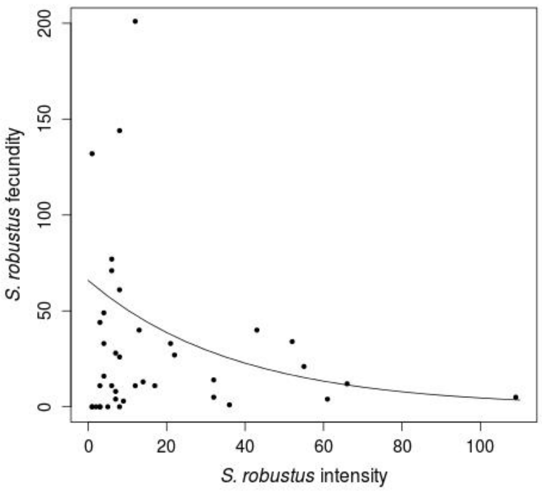

Figure 2. Effect of *S. robustus* intensity (no. worms/infected host) on *S. robustus* fecundity (no. eggs/adult female worm).

Density-dependent constraints on parasite growth, survival or reproduction have been observed in several host-parasite systems (e.g. Michael and Bundy 1989; Christensen et al. 1995; Roepstorff et al. 1996; Irvine et al. 2001; Dezfuli et al. 2002; Lowrie et al. 2004) and may be due to inter- or intraspecific competition between parasites, but also to the host immune response stimulated by high intensity of infection. In particular, Paterson and Viney (2002) demonstrated that density-dependence of *Strongyloides rattii* fecundity in experimentally infected mice is entirely caused by the host immune response acting to regulate infection.

Our field data do not allow us to separate these two effects, but the fact that other covariates had no effect on S. robustus fecundity, suggest that the observed density-dependence is not caused by seasonal variation in worm fecundity nor by host-related factors that may affect immune response (e.g. sex or age differences in immune function). However, in the congener nematode S. rattii, prior exposure of the host enhances the effect of immune responses on parasite establishment and survivorship, but has no effect on parasite fecundity (Paterson and Viney 2002). Hence, the absence of host-related effects (in particular host age) on S. robustus fecundity is not sufficient to exclude a role of immunity in mediating the observed density-dependent effect. Finally, egg shedding in rodents is known to be affected by circadian rhythms, with maxima in egg shedding coinciding with minima in faeces production (Brown et al. 1994). In our case the weight of analysed faeces held no significant effect in neither model, indicating that FECs are independent from the amount of faeces produced by the host.

We may thus conclude that FECs in this host-parasite system do not represent the most reliable measure to estimate intensity of infection. Nevertheless, the study of egg shedding remains a key point in investigating host-parasite dynamics since it may provide an insight in the infectiousness of individual hosts through the amount of infective stages that they produce. On the contrary, since flotation is quick, inexpensive and easy to carry out, its effectiveness is sufficient for performing a first screening of infection status in squirrels. In particular, flotation is mostly appropriate to screen for S. robustus infection in introduced grey squirrel populations where the observed prevalence of the parasite is high (Romeo et al, 2014). In addition, if we assume that the technique is equally effective in congeneric red squirrels, flotation may be a useful test for detecting S. robustus spillover to the native species. However, at the initial stages of grey squirrel invasion (i.e. when prevalence of infection in native host populations is still low) it should be followed by more specific and reliable tests (e.g. PCR) on positive samples, to determine truly infected animals.

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References

Anderson RM, Schad GA (1985) Hookworm burdens and faecal egg counts: an analysis of the biological basis of variation. Trans R Soc Trop Med Hyg 79:812–825. doi: 10.1016/0035-9203(85)90128-2

Brown E d., Macdonald D w., Tew T e., Todd I a. (1994) Rhythmicity of egg production by Heligmosomoides polygyrus in wild wood mice, Apodemus sylvaticus. J Helminthol 68:105–108. doi: 10.1017/S0022149X00013602

Close B, Banister K, Baumans V, et al. (1996) Recommendations for euthanasia of experimental animals: Part 1. Lab Anim 30:293–316. doi: 10.1258/002367796780739871

Close B, Banister K, Baumans V, et al. (1997) Recommendations for euthanasia of experimental animals:

Part 2. Lab Anim 31:1–32. doi: 10.1258/002367797780600297

Davidson WR (1976) Endoparasites of selected populations of gray squirrels (Sciurus carolinensis) in the southeastern United States. Proc. Helminthol. Soc. Wash. 43:

Dunn A, Keymer A (1986) Factors affecting the reliability of the McMaster technique. J Helminthol 60:260–262. doi: 10.1017/S0022149X00008464

Elkins DB, Sithithaworn P, Haswell-Elkins M, et al. (1991) Opisthorchis viverrini: relationships between egg counts, worms recovered and antibody levels within an endemic community in Northeast Thailand. Parasitology 102:283–288. doi: 10.1017/S0031182000062600

Gillespie TR (2006) Noninvasive Assessment of Gastrointestinal Parasite Infections in Free-Ranging Primates. Int J Primatol 27:1129–1143. doi: 10.1007/s10764-006-9064-x

Hudson PJ, Dobson AP (1995) Macroparasites: observed patterns. In: Grenfell BT, Dobson AP (eds) Ecol. Infect. Dis. Nat. Popul. Cambridge University Press, Cambridge, pp 144–176

Hudson PJ, Dobson AP (1997) Transmission Dynamics and Host-Parasite Interactions of Trichostrongylus tenuis in Red Grouse (Lagopus lagopus scoticus). J Parasitol 83:194. doi: 10.2307/3284438

Keymer A (1982) Density-dependent mechanisms in the regulation of intestinal helminth populations. Parasitology 84:573–587. doi: 10.1017/S0031182000052847

Leary SL, American Veterinary Medical Association (2013) AVMA guidelines for the euthanasia of animals: 2013 edition.

MAFF (1977) Manual of Veterinary Parasitological Laboratory Techniques. Her Majesty's Stationary Office, London

Michael E, Bundy D a. P (1989) Density dependence in establishment, growth and worm fecundity in intestinal helminthiasis: the population biology of Trichuris muris (Nematoda) infection in CBA/Ca mice. Parasitology 98:451–458. doi: 10.1017/S0031182000061540

Perkins SE, Cattadori IM, Tagliapietra V, et al. (2003) Empirical evidence for key hosts in persistence of a tick-borne disease. Int J Parasitol 33:909–917. doi: 10.1016/S0020-7519(03)00128-0

Poulin R (1997) Population abundance and sex ratio in dioecious helminth parasites. Oecologia 111:375–380. doi: 10.1007/s004420050248

Quinnell RJ, Medley GF, Keymer AE (1990) The Regulation of Gastrointestinal Helminth Populations. Philos Trans R Soc Lond B Biol Sci 330:191–201. doi: 10.1098/rstb.1990.0192

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria

Rausch R, Tiner JD (1948) Studies on the Parasitic Helminths of the North Central States. I. Helminths of Sciuridae. Am Midl Nat 39:728. doi: 10.2307/2421532

Romeo C, Pisanu B, Ferrari N, et al. (2013) Macroparasite community of the Eurasian red squirrel (Sciurus vulgaris): poor species richness and diversity. Parasitol Res 112:3527–3536. doi: 10.1007/s00436-013-3535-8

Romeo C, Wauters LA, Ferrari N, et al. (2014) Macroparasite Fauna of Alien Grey Squirrels (Sciurus carolinensis): Composition, Variability and Implications for Native Species. PLoS ONE 9:e88002. doi: 10.1371/journal.pone.0088002

Seivwright L j., Redpath S m., Mougeot F, et al. (2004) Faecal egg counts provide a reliable measure of Trichostrongylus tenuis intensities in free-living red grouse Lagopus lagopus scoticus. J Helminthol 78:69–76. doi: 10.1079/JOH2003220

Shaw JL, Moss R (1989) The role of parasite fecundity and longevity in the success of Trichostrongylus tenuis in low density red grouse populations. Parasitology 99:253–258. doi: 10.1017/S0031182000058704

Sinniah B (1982) Daily egg production of Ascaris lumbricoides: the distribution of eggs in the faeces and the variability of egg counts. Parasitology 84:167–175. doi: 10.1017/S0031182000051763

Sithithaworn P, Tesana S, Pipitgool V, et al. (1991) Relationship between faecal egg count and worm burden of Opisthorchis viverrini in human autopsy cases. Parasitology 102:277–281. doi: 10.1017/S0031182000062594

Smith G, Grenfell BT, Anderson RM (1987) The regulation of Ostertagia ostertagi populations in calves: density-dependent control of fecundity. Parasitology 95:373–388. doi: 10.1017/S0031182000057814

Thrusfield M (2013) Veterinary Epidemiology. John Wiley & Sons

Tompkins DM, Begon M (1999) Parasites Can Regulate Wildlife Populations. Parasitol Today 15:311–313. doi: 10.1016/S0169-4758(99)01484-2

Tompkins DM, Hudson PJ (1999) Regulation of nematode fecundity in the ring-necked pheasant (Phasianus colchicus): not just density dependence. Parasitology 118:417–423.

Wilson K, Bjørnstad ON, Dobson AP, et al. (2002) Heterogeneities in macroparasite infections: patterns and processes. Ecol Wildl Dis 6–44.

CHAPTER 5

Presence of alien grey squirrels affects helminth community of red squirrels: spillover of introduced *Strongyloides robustus* and increased prevalence of a local parasite

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Manuscript

Presence of alien grey squirrels affects helminth community of red squirrels: spillover of introduced *Strongyloides robustus* and increased prevalence of a local parasite

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Abstract

Parasite spillover from alien species may threaten naive native hosts and result in a competitive advantage for invaders, benefitting from a higher tolerance to the shared parasite. We compared gastro-intestinal helminth fauna of native Eurasian red squirrels (*Sciurus vulgaris*) in presence and absence of introduced Eastern grey squirrels (*S. carolinensis*), to detect any variation in parasite community composition in populations syntopic with the alien species. In particular, making use of non-invasive, indirect parasitological methods on living red squirrels, we investigated whether spillover of North American nematode *Strongyloides robustus* occurs, and whether prevalence of local oxyurid *Trypanoxyuris sciuri* is affected by grey squirrels presence. Prevalence of both parasites was significantly higher in red-grey sites (n=49 hosts examined), where 61% of red squirrels were infected by *S. robustus* and 90% by *T. sciuri*. Conversely, in red-only sites (n=60) the two parasites infected only 5% and 70% of red squirrels, respectively. Interspecific transmission of alien *S. robustus* is likely to occur through nest sharing: the nematode prevalence was indeed higher during colder season, when red squirrels spend more time inside their nest and are thus more exposed to infection. The increased *T. sciuri* prevalence in red squirrels co-inhabiting with grey squirrels is probably a result of stress-mediated effects linked with competitive pressure that induces a higher susceptibility to parasite infection. Further research on detrimental effects of *S. robustus* on naive red squirrels is needed. Nevertheless, our findings confirm that native red squirrels acquire *S. robustus* from the alien congener and show that alien species presence may also affect infection by local parasites, thus highlighting the importance of investigating variation in parasite communities of native species threatened by alien competitors.

Introduction

Biological invasions are recognised as one of the major causes for infectious disease emergence in humans, livestock and wildlife (Daszak et al. 2000). Alien species introduced outside their native range will indeed carry along with them at least some non-indigenous parasites (Torchin et al. 2003) that may spillover to naive native species. If transmission occurs, it is likely that alien parasites will have a detrimental effect on local species that did not evolve any defence mechanism to them (Strauss et al. 2012). Hence, the more tolerant host (i.e. the alien species) will act as a reservoir and transmit the parasite to the less tolerant species, thus gaining a competitive advantage over it (parasite-mediated competition, Hudson and Greenman 1998; Strauss et al. 2012).

Besides, native species closely-related to the invader may be more vulnerable to spillover, since host-switching is more likely to occur between two hosts that share at least some physiological and immunological characteristics (Poulin and Mouillot 2003).

One of the most studied examples of disease-mediated invasions is the Squirrelpoxvirus (SQPV) which strongly influences the outcome of interspecific competition between introduced North American Eastern grey squirrels (*Sciurus carolinensis*) and native Eurasian red squirrels (*S. vulgaris*) in the British Isles (Tompkins et al. 2003). Grey squirrels cause red squirrels local extinction mainly through exploitation competition, but where SQPV is present, the replacement process may be accelerated up to 25 times (Rushton et al. 2005).

Despite the attention received by SPQV, to our knowledge no one investigated whether macroparasites play a role in the competition between these two sciurids. Grey squirrels introduced to Italy are infected by two Nearctic nematode species (Romeo et al. 2014). In particular, their gastro-intestinal parasite community in Northern Italy is dominated by the directly transmitted nematode *Strongyloides robustus*, which is also one of the most common helminths infecting the species in its native range (Davidson 1976; Romeo et al. 2014). The same parasite, previously unreported in Europe, was found in two road-killed red squirrels living in contact with the alien congener (Romeo et al. 2013), suggesting that spillover from grey squirrels to the native species occurs. Moreover, the same study showed that the red squirrel has very poor gastro-intestinal helminth communities, dominated by the oxyurid *Trypanoxyuris sciuri*, and might thus be particularly vulnerable to endo-macroparasite spillover (Romeo et al. 2013). *S. robustus* is indeed suspected to mediate the competition between two species of North American flying squirrels (*Glaucomys volans* and *G. sabrinus*) by reducing *G. sabrinus* survival whereas *G. volans* acts as a more tolerant reservoir for the infection (Pauli et al. 2004; Weigl 2007; Krichbaum et al. 2010).

Our objective is therefore to detect variation in endo-macroparasite communities of native red squirrels caused by the presence of the alien congener. We have surveyed gastrointestinal helminth communities of living red squirrels, making use of indirect, non-invasive parasitological methods to compare infection status in presence and absence of grey squirrels. More specifically, we first aim to detect any *S. robustus* spillover and, secondly, to investigate host-related (host sex and body mass) and environmental factors (grey squirrel presence and season) affecting prevalence of infection by introduced *S. robustus* and local *T. sciuri* in red squirrel populations.

Materials and Methods

A total of 157 capture events were carried out between 2011 and 2013 in 8 red squirrel populations located in Northern Italy. The alien species was syntopic in 5 of these populations (hereafter, red-grey sites), whereas the 3 sites where no grey squirrels were trapped were considered as red-only sites. In each site, at least 3 trapping sessions of minimum 5 continuous days were carried out every year (in winter, spring, autumn and summer). Red squirrels were captured using live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA), baited with nuts and hazelnuts, that were checked two to three times a day, depending on day length. Animals were marked with metal ear tags (type 1003S, 10 by 2 mm, National Band and Tag, Newport, KY, USA) and immediately released after sample collection. Faecal samples for *S. robustus* egg detection were collected from the trap floor and stored dry at 4°C for later examination. To screen for *T. sciuri* presence we used adhesive tape tests since they are a more reliable method than coprological analysis for oxyurid eggs detection (Foreyt 2013). We collected tape tests by leaning for a few seconds 3-4 cm of adhesive, transparent tape on the perianus of captured squirrels and applying it on a microscope slide.

Coprological analysis were carried out within one day from sample collection to avoid eggs etching, making use of simple floatation technique with saturated NaCl solution (1200 g/l). To amalgamate the faeces with NaCl solution, they were crushed gently in a mortar. The obtained solution was then filtered through a sieve to remove the biggest particles, poured in a 15 ml centrifuge tube and fresh NaCl solution was added until the rim of the tube was reached. After 30 minutes, necessary to let the eggs float to the surface (Dunn and Keymer 1986), a cover slip was leaned on the solution meniscus to collect floating eggs and put on a slide. Both floatation and tape test slides were examined under a microscope (40x magnification) to detect *S. robustus* and *T. sciuri* eggs, respectively.

Table 1. Prevalence (expressed as % of infected hosts ± SE) of *S. robustus* and *T. sciuri* infecting red squirrels. N: number of host examined; n: number of infected hosts.

Parasite	**Red-only sites** (N=60)	**Red-grey sites** (N=49)	**Total** (N=109)
S. robustus	5.0 ± 2.8 (n=3)	61.2 ± 7.0 (n=30)	30.3 ± 4.4 (n=33)
T. sciuri	70.0 ± 5.9 (n=42)	89.8 ± 4.4 (n=44)	78.9 ± 3.9 (n=86)
S. robustus + *T. sciuri*	1.7 ± 1.7 (n=1)	55.1 ± 7.8 (n=27)	25.7 ± 4.2 (n=28)

We analysed factors affecting *S. robustus* and *T. sciuri* presence through logistic regression with binary response, using Generalised Linear Mixed Models (GLMMs) with repeated measures. In each model we considered area-type (i.e. red-only or red-grey), host sex, body mass, season and co-occurrence of the other parasite as explanatory variables. Capture year and site were included as random factors.

Table 1. Minimum models explaining observed variation in *S. robustus* and *T. sciuri* infection in red squirrels.

Dependent variable	Source of variation	χ^2	df	P	Parameter estimate (±SE)
S. robustus presence	area-type	14.06	1	0.0002	
	season	8.5	2	0.014	
T. sciuri presence	area-type	6.6	1	0.010	
	body mass	5.2	1	0.023	0.013 ± 0.006

Season were defined following the same categories, based on temporal changes in squirrel behaviour and food availability, described in previous studies (winter, December–February; spring March–May; autumn September–November, e.g. Wauters et al. 2007; Romeo et al. 2010). Since all sampling sites had similar habitat conditions (i.e. mixed deciduous woods, low elevation, similar weather conditions), no other environmental variables were considered. We first fitted full models with all the factors and their second-order interactions, then obtained minimum models through backward elimination of non-significant variables. Final results of significant factors with more than two levels were interpreted through pair-wise *t*-tests of Differences of Least Square Means (DLSM), applying sequential Bonferroni correction (Holm 1979) for multiple comparisons. Statistical analysis were carried out using PROC GLIMMIX in SAS/STAT 9.2 software (Copyright © 2009, SAS Institute Inc., Cary, NC, USA).

Results and discussion

We analysed 157 faecal samples and tape tests, corresponding to 60 different individuals captured in red-only sites and 49 in red-grey sites. Detailed results are given in Table 1. *S. robustus* infection status varied with area-type and season, whereas presence of *T. sciuri* was affected by area-type and increased with body mass (Table 2).

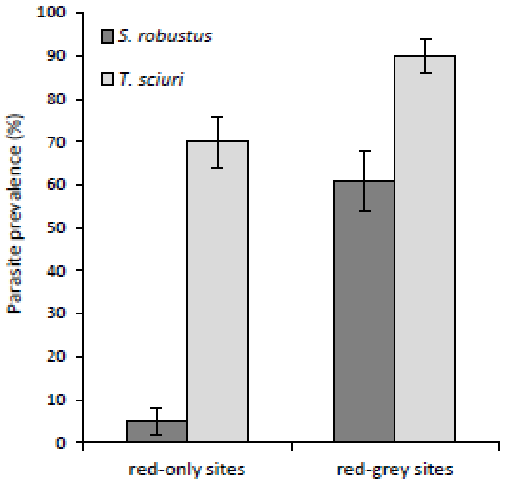

Figure 1. Prevalence (% of infected hosts) of *S. robustus* and *T. sciuri* infecting red squirrels in presence and absence of alien grey squirrels. Bars indicate standard error.

Prevalence of both parasites was significantly higher in red-grey sites (Figure 1). In particular, only three individuals were infected by *S. robustus* in red-only sites. All of them live in the same population located less than 5 km away from an area were grey squirrels are present, hence they probably had come in contact with dispersing individuals of the alien species. Moreover, we cannot exclude that the infected red squirrels dispersed from a nearby red-grey site. Hence, our findings confirm *S. robustus* infection in red squirrels is linked with the alien species presence. This nematode has a life cycle which includes a free-living part: since larvae development and survival are affected by environmental conditions (namely temperature and humidity), it has been suggested that squirrel nests could be the microenvironment where larvae development and transmission takes place most effectively (Wetzel and Weigl 1994; Bartlett 1995). In areas of co-occurrence, red and grey squirrels can have overlapping home ranges and some dreys and dens may be used by both species at different times (Wauters et al. 2000; Wauters et al. 2002). Interspecific transmission of *S. robustus* is thus likely to occur in nests shared by the two species: grey squirrels may contaminate nests with parasite eggs and red squirrels that subsequently use the same nests will be exposed to infection by developed, infective larvae. This hypothesis could also explain why *S. robustus* prevalence was lower during spring than in autumn and winter (both adjusted p<0.034, Figure 2). During colder months, squirrels reduce their activity and spend more time in their nests (Wauters and Dhondt 1988; Wauters et al. 1992), which may lead to higher exposure to infection. The prepatent period of this helminth is short (6 days according to Eckerlin 1974), therefore seasonal abundance should reflect conditions of the same season, without any time lag. Moreover, the observed prevalence of *S. robustus* in red squirrels syntopic with the alien species is similar to the prevalence reported in grey squirrels populations in Northern Italy (Romeo et al. 2014).

This data, together with the fact that the parasite is able to reproduce and shed viable eggs (i.e. eggs containing developed and living larvae) with red squirrels' faeces, demonstrates that *S. robustus* is well adapted to the new host. Hence, it is likely that grey squirrels are not the unique reservoir for the parasite and, once the infection is established in a red squirrel population, the alien nematode may successfully persist even if grey squirrels are removed.

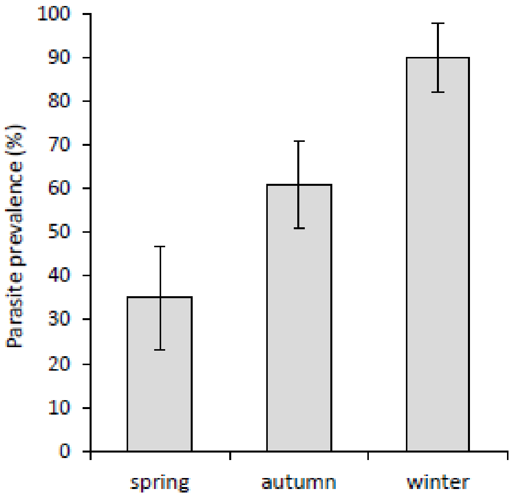

Figure 2. Prevalence (% of infected hosts) of *S. robustus* by season. Bars indicate standard error.

Interestingly, also *T. sciuri* prevalence is higher where grey squirrels are present. We may exclude that this result is a consequence of spill-back processes (see Kelly et al. 2009) from the alien congener, since prevalence of *T. sciuri* reported for grey squirrels is very low, suggesting that the alien species is not a suitable host for this parasite (Romeo et al. 2014).

Moreover, co-infection with *S. robustus* does not affect *T. sciuri* infection status. Hence, the higher prevalence of the local parasite in red-grey sites may be a consequence of stress-mediated mechanisms induced by the increased competitive pressure on the native host in presence of grey squirrels (Christe et al. 2006). In general, *T. sciuri* prevalence detected with tape tests is lower than values found in a wider sample of road-killed red squirrels by Romeo et al. (2013). This is not surprising, since, like other indirect test, tape test sensitivity is lower than direct gut examination (Hill et al. 2009). Romeo et al. (2013) found also a differential variation of *T. sciuri* abundance by body mass between the two sexes. In our case, prevalence of the parasite increased with body mass both in males and females, likely reflecting an age-related effect (Wilson et al. 2002).

Despite having usually low pathogenic effects that may easily be overlooked (Dobson and Foufopoulos 2001), helminths are important regulators of host population dynamics (Anderson and May 1978; May and Anderson 1978; Dobson and Hudson 1992), hence our results highlight the importance of investigating variation in helminth infections of native hosts caused by the presence of alien species, that may not only be a source of new infections, but also indirectly mediate prevalence of local parasites. However, further research to determine whether *S. robustus* spillover results in any detrimental effects on red squirrels fitness (e.g. survival or fecundity) is urgently needed.

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References

Anderson RM, May RM (1978) Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory

Processes. J Anim Ecol 47:219–247. doi: 10.2307/3933

Bartlett C (1995) Morphology, homogonic development, and lack of a free-living generation in Strongyloides robustus (Nematoda, Rhabditoidea), a parasite of North American sciurids. Folia Parasitol (Praha) 42:102–114.

Christe P, Morand S, Michaux J (2006) Biological conservation and parasitism. In: D SMP, D BRKP, D RPP (eds) Micromammals Macroparasites. Springer Japan, pp 593–613

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging Infectious Diseases of Wildlife-- Threats to Biodiversity and Human Health. Science 287:443–449. doi: 10.1126/science.287.5452.443

Davidson WR (1976) Endoparasites of selected populations of gray squirrels (Sciurus carolinensis) in the southeastern United States. Proc. Helminthol. Soc. Wash. 43:

Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. Philos Trans R Soc Lond B Biol Sci 356:1001–1012. doi: 10.1098/rstb.2001.0900

Dobson AP, Hudson PJ (1992) Regulation and Stability of a Free-Living Host-Parasite System: Trichostrongylus tenuis in Red Grouse. II. Population Models. J Anim Ecol 61:487–498. doi: 10.2307/5339

Dunn A, Keymer A (1986) Factors affecting the reliability of the McMaster technique. J Helminthol 60:260–262. doi: 10.1017/S0022149X00008464

Eckerlin RP (1974) Studies on the life cycles of Strongyloides robustus Chandler 1942, and a survey of the helminths of Connecticut sciurids. PhD Dissertation, University of Connecticut

Foreyt WJ (2013) Veterinary Parasitology Reference Manual. John Wiley & Sons

Hill WA, Randolph MM, Mandrell TD (2009) Sensitivity of Perianal Tape Impressions to Diagnose Pinworm (Syphacia spp.) Infections in Rats (Rattus norvegicus) and Mice (Mus musculus). J Am Assoc Lab Anim Sci JAALAS 48:378–380.

Holm S (1979) A Simple Sequentially Rejective Multiple Test Procedure. Scand J Stat 6:65–70.

Hudson P, Greenman J (1998) Competition mediated by parasites: biological and theoretical progress. Trends Ecol Evol 13:387–390. doi: 10.1016/S0169-5347(98)01475-X

Kelly DW, Paterson RA, Townsend CR, et al. (2009) Parasite spillback: A neglected concept in invasion ecology? Ecology 90:2047–2056. doi: 10.1890/08-1085.1

Krichbaum K, Mahan CG, Steele MA, et al. (2010) The potential role of Strongyloides robustus on parasite-mediated competition between two species of flying squirrels (Glaucomys). J Wildl Dis 46:229–235.

May RM, Anderson RM (1978) Regulation and Stability of Host-Parasite Population Interactions: II. Destabilizing Processes. J Anim Ecol 47:249–267. doi: 10.2307/3934

Pauli JN, Dubay SA, Anderson EM, Taft SJ (2004) Strongyloides robustus and the northern sympatric populations of northern (Glaucomys sabrinus) and southern (G. volans) flying squirrels. J Wildl Dis 40:579–582.

Poulin R, Mouillot D (2003) Parasite specialization from a phylogenetic perspective: a new index of host specificity. Parasitology 126:473–480. doi: 10.1017/S0031182003002993

Romeo C, Pisanu B, Ferrari N, et al. (2013) Macroparasite community of the Eurasian red squirrel (Sciurus vulgaris): poor species richness and diversity. Parasitol Res 112:3527–3536. doi: 10.1007/s00436-013-3535-8

Romeo C, Wauters LA, Ferrari N, et al. (2014) Macroparasite Fauna of Alien Grey Squirrels (Sciurus carolinensis): Composition, Variability and Implications for Native Species. PLoS ONE 9:e88002. doi: 10.1371/journal.pone.0088002

Romeo C, Wauters LA, Preatoni D, et al. (2010) Living on the edge: Space use of Eurasian red squirrels in marginal high-elevation habitat. Acta Oecologica 36:604–610. doi: 10.1016/j.actao.2010.09.005

Rushton SP, Lurz PWW, Gurnell J, et al. (2005) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. Epidemiol Infect 134:521. doi: 10.1017/S0950268805005303

Strauss A, White A, Boots M (2012) Invading with biological weapons: the importance of disease-mediated invasions. Funct Ecol 26:1249–1261. doi: 10.1111/1365-2435.12011

Tompkins DM, White AR, Boots M (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. Ecol Lett 6:189–196.

Torchin ME, Lafferty KD, Dobson AP, et al. (2003) Introduced species and their missing parasites. Nature 421:628–630. doi: 10.1038/nature01346

Wauters L, Gurnell J, Martinoli A, Tosi G (2002) Interspecific competition between native Eurasian red squirrels and alien grey squirrels: does resource partitioning occur? Behav Ecol Sociobiol 52:332–341. doi: 10.1007/s00265-002-0516-9

Wauters L, Swinnen C, Dhondt AA (1992) Activity budget and foraging behaviour of red squirrels (Sciurus vulgaris) in coniferous and deciduous habitats. J Zool 227:71–86. doi: 10.1111/j.1469-7998.1992.tb04345.x

Wauters LA, Dhondt AA (1988) The use of red squirrel Sciurus vulgaris dreys to estimate population density. J Zool 214:179–187. doi: 10.1111/j.1469-7998.1988.tb04995.x

Wauters LA, Lurz PWW, Gurnell J (2000) Interspecific effects of grey squirrels (Sciurus carolinensis) on the space use and population demography of red squirrels (Sciurus vulgaris) in conifer plantations. Ecol Res 15:271–284. doi: 10.1046/j.1440-1703.2000.00354.x

Wauters LA, Vermeulen M, Van Dongen S, et al. (2007) Effects of spatio-temporal variation in food supply on red squirrel Sciurus vulgaris body size and body mass and its consequences for some fitness components. Ecography 30:51–65. doi: 10.1111/j.2006.0906-7590.04646.x

Weigl PD (2007) The northern flying squirrel (Glaucomys sabrinus): a conservation challenge. J Mammal 88:897–907.

Wetzel EJ, Weigl PD (1994) Ecological Implications for Flying Squirrels (Glaucomys spp.) of Effects of Temperature on the In Vitro Development and Behavior of

Strongyloides robustus. Am Midl Nat 131:43. doi: 10.2307/2426607

Wilson K, Bjørnstad ON, Dobson AP, et al. (2002) Heterogeneities in macroparasite infections: patterns and processes. Ecol Wildl Dis 6–44.

CHAPTER 6

Ljungan virus and an Adenovirus in Italian Squirrel Populations

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ABSTRACT: We report Ljungan virus infection in Eurasian red squirrels (*Sciurus vulgaris*) for the first time, and extend the known distribution of adenoviruses in both native red squirrels and alien gray squirrels (*Sciurus carolinensis*) to southern Europe.The introduction of alien species is one of the major causes of infectious disease emergence in wildlife, representing a threat not only to biodiversity conservation, but also to domesticated animals and humans (Daszak et al., 2000). Alien species can introduce new pathogens, alter the epidemiology of local pathogens, become reservoir hosts and increase disease risk for native species (Prenter et al., 2004; Dunn, 2009).

As part of a broader project to study the role of infectious diseases and parasites in the competition between alien and native species, we investigated Ljungan virus (LV) and adenovirus infections in introduced North American Eastern gray squirrels (*Sciurus carolinensis*) and native Eurasian red squirrels (*Sciurus vulgaris*) in northern Italy. More specifically, we investigated whether arboreal sciurids are involved in LV circulation; and if adenovirus infection in squirrels is present in Italy.

Ljungan virus was isolated in 1999 (Niklasson et al., 1999), and has subsequently been detected in many species of small rodents, especially voles (Arvicolinae; Johansson et al., 2003; Hauffe et al., 2010; Salisbury et al., 2013). In northern Italy, this virus has been reported in a small sample of bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) with 50% and 10% prevalence, respectively (Hauffe et. al., 2010). Although its zoonotic potential is still debated, LV has been associated with type 1 diabetes (T1D), myocarditis, and several gestational diseases in humans (McDonald, 2009; Blixt et al., 2013). Experimentally infected laboratory mice develop signs of these same diseases, and LV-infected wild voles may also develop T1D-like syndromes (McDonald, 2009; Blixt et al., 2013).

Outbreaks of enteric adenovirus infections associated with gastrointestinal disease and mortality have been described in both free-living and captive red squirrels in Germany and the UK (e.g. Martínez-Jiménez et al., 2011; Peters et al., 2011), where subclinical adenovirus infections among introduced gray squirrels have also been reported (Everest et al., 2009).

We analyzed 232 gray squirrels from five populations, culled as part of a control program (2011/12), and 77 road-killed red squirrels for adenoviruses. All the specimens were collected in Piedmont and Lombardy regions (between 44°35'55 and 46°35'55' N; 7°37'41 and 10°32'08 W). For adenovirus analysis, sample sets of both squirrel species were heterogeneous for sex, age class and season of collection. For LV analysis, we used a subset of adult squirrels collected in autumn (49 gray squirrels and nine red squirrels), to maximize the chance of finding the virus, since infection is assumed to be correlated with small mammal (host) density and, therefore, probability of exposure (HCH and others, unpubl.).

For LV screening, total RNA was extracted from liver (stored at -80° C) using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). We performed a One-Step reverse transcriptase-PCR (Qiagen) in duplicate using primers described by Donoso Mantke et al. (2007). Amplicons were purified using the PureLink Quick gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA, USA) and directly sequenced using the Big Dye terminator cycle sequencing kit

(Applied Biosystems, Foster City, CA, USA) on an ABI 3130 sequencer. Sequences (189 base pairs) were checked using the basic local alignment search tool (NCBI, 2013) . For adenovirus screening, nucleic acid was extracted from spleen tissue (stored at -20° C), using the manufacturer's recommendations for the QIAamp DNA Mini kit (Qiagen). We performed nested PCR using primers described by Everest et al. (2012). To confirm the specificity of the primers, amplicons were recovered from agarose gels and purified using the Qiaquick Gel extraction kit (Qiagen), and then used as templates in direct dye-termination sequence reactions (Big Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems).

Two red squirrels (22%), but no gray squirrels, were infected with LV. An adenovirus was detected in 12 (16%) red squirrels and two (0.9%) gray squirrels. No cases of co-infection were detected.

To our knowledge this is the first record of LV in the Eurasian red squirrel, indicating that the infection is not limited to small, ground-dwelling rodents and extending the potential host-spectrum of LV to arboreal mammals. Little is known about the circulation of LV in the environment; however, one of our sequences was identical to a widespread haplotype found in several rodent species across Europe, and the other was identical to a haplotype carried by bank voles in Lombardy (Hauffe et al, 2010), suggesting that squirrels may play an active part in both intra- and interspecific LV circulation; further ecologic and phylogenetic analyses are underway to confirm this.

Poor preservation of road kills did not allow us to identify clinical signs of infection in adenovirus-positive red squirrels, whereas infected, freshly killed, gray squirrels did not show any abnormalities at postmortem examination. All the adenovirus-infected red squirrels were collected in areas where the alien species is not present. Moreover, the two positive gray squirrels lived in areas where the native species is still present or was present until recently. Our findings extend adenovirus distribution in red and gray squirrels to southern Europe, but the gray squirrel does not appear, from these results, to be the source of adenovirus infection in the native species. Our results are consistent with recent findings by Everest et al. (2013) suggesting that the infection could be maintained by the native species or by other sympatric woodland rodents such as wood mice (*Apodemus sylvaticus*).

Despite the limitations of this study (in particular, potential biases linked to convenience sampling and small sample sizes), we show for the first time that red squirrels can be infected with LV and one or more adenoviruses are present in southern Europe. We cannot determine whether squirrels are reservoir hosts of these infections, or whether these are results of spillover from other small mammal species (e.g., voles) in the same ecosystem. Transmission of infectious diseases in arboreal sciurids is still poorly understood; their role in disease emergence could be underestimated and further research to disclose their epidemiologic significance is needed.

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Literature cited

Blixt M, Sandler S, Niklasson B. 2013. Ljungan Virus and Diabetes. In: *Diabetes and Viruses*, Taylor K, Hyöty H, Toniolo A, Zuckerman AJ, editors. Springer, New York, New York, pp. 81–86.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife-- Threats to biodiversity and human health. *Science* 287: 443–449.

Donoso Mantke O, Kallies R, Niklasson B, Nitsche A, Niedrig M. 2007. A new quantitative real-time reverse transcriptase PCR assay and melting curve analysis for detection and genotyping of Ljungan virus strains. *J Virol Methods* 141: 71–77.

Dunn AM. 2009. Parasites and biological invasions. *Adv Parasitol*, 68: 161–184.

Everest DJ, Grierson SS, Stidworthy MF, Shuttleworth C. 2009. PCR detection of adenovirus in grey squirrels on Anglesey. *Vet Rec* 165: 482.

Everest DJ, Shuttleworth CM, Grierson SS, Duff JP, Jackson N, Litherland P, Kenward RE, Stidworthy MF. 2012. Systematic assessment of the impact of adenovirus infection on a captive reintroduction project for red squirrels (*Sciurus vulgaris*). *Vet Rec* 171: 176–176.

Everest DJ, Butler H, Blackett T, Simpson VR, Shuttleworth CM. 2013. Adenovirus infection in red squirrels in areas free from grey squirrels. *Vet Rec* 173: 199–200.

Hauffe HC, Niklasson B, Olsson T, Bianchi A, Rizzoli A, Klitz W. 2010. Ljungan virus detected in bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) from northern Italy. *J Wildl Dis* 46: 262–266.

Johansson ES, Niklasson B, Tesh RB, Shafren DR, da Rosa APAT, Lindberg AM. 2003. Molecular characterization of M1146, an American isolate of Ljungan virus (LV) reveals the presence of a new LV genotype. *J Gen Virol* 84: 837–844.

Martínez-Jiménez D, Graham D, Couper D, Benkö M, Schöniger S, Gurnell J, Sainsbury AW. 2011. Epizootiology and pathologic findings associated with a newly described adenovirus in the red squirrel, *Sciurus vulgaris*. *J Wildl Dis* 47: 442–454.

McDonald AG. 2009. Ljungan virus: An emerging zoonosis? *Clin Microbiol Newsl* 31: 177–182.

National Center for Biotechnology Information (NCBI). Basic local alignment search tool. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed December 2013.

Niklasson B, Kinnunen L, Hörnfeldt B, Hörling J, Benemar C, Olof Hedlund K, Matskova L, Hyypiä T, Winberg G. 1999. A new Picornavirus isolated from bank voles (*Clethrionomys glareolus*). *Virology* 255: 86–93.

Peters M, Vidovszky MZ, Harrach B, Fischer S, Wohlsein P, Kilwinski J. 2011. Squirrel adenovirus type 1 in red squirrels (*Sciurus vulgaris*) in Germany. *Vet Rec* 169: 182–182.

Prenter J, MacNeil C, Dick JT, Dunn AM. 2004. Roles of parasites in animal invasions. *Trends Ecol Evol* 19: 385–390.

Salisbury A-M, Begon M, Dove W, Niklasson B, Stewart JP. 2013. Ljungan virus is endemic in rodents in the UK. *Arch Virol*. DOI: 10.1007/s00705-013-1731-6.

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CHAPTER 7

Conclusions

"All models are wrong,
but some are useful".

- George E. P. Box -

"An approximate answer to the right problem
is worth a good deal more than
an exact answer to an approximate problem."

- John Tukey -

"Absolute certainty is a privilege
of uneducated minds-and fanatics.
It is, for scientific folk, an unattainable ideal."

- Cassius J. Keyser -

The aim of the present thesis is to shed light on host-parasite relationships in the specific context of biological invasions. I used Eurasian red squirrels (*Sciurus vulgaris*) and introduced Eastern grey squirrels (*S. carolinensis*) as a model to investigate how parasite communities of both native hosts and invaders are affected by the invasion process. In particular, I focused my attention on those mechanisms that may play an important role in determining the outcome of alien species introduction and their impact on native species such as enemy-release and apparent competition (see **Chapter 1**).

7.1. Summary of results

First, in **Chapter 2,** I present the results of a broad survey of the macroparasite fauna (gastro-intestinal helminths and arthropods) of the red squirrel which was investigated using road kills collected in diverse habitats and over a wide geographical area (in Italy and France). As expected, red squirrels macroparasite communities are composed by few and mainly specialised parasites. The poor parasite richness and diversity observed in our survey is likely a consequence of both i) the arboreal habits of the host that prevents infection by more generalist species with indirect life-cycles and ii) the absence of syntopic congeners or closely-related sciurids that, over evolutionary time scales, could have facilitated the enrichment of its parasite fauna via host-switching processes (e.g. Krasnov et al. 2004; Marques et al. 2011). In particular, in all the examined habitats, the gastro-intestinal helminth community was dominated by a single nematode (the oxyurid *Trypanoxyuris sciuri*, prevalence: 87%), with only an occasional co-occurence of some accidental species. This finding is alarming because, as a consequence, red squirrels could be particularly vulnerable to helminth spillover from alien species since they offer many free niches for the establishment of other gastro-intestinal parasites. In two road-killed red squirrels collected in areas co-inhabited by introduced grey squirrels, we found indeed adult individuals of the nematode *Strongyloides robustus*, a Nearctic parasite commonly infecting several North American squirrel species (Bartlett

1995). This is the first record of *S. robustus* in Europe and suggests that some spillover between the two congeneric sciurids is indeed occurring.

A parallel survey of gastro-intestinal helminths and parasite arthropods was then carried out on a wide sample of introduced grey squirrels collected in Northern Italy (**Chapter 3**) to detect whether the alien species lost, introduced or acquired any parasite species. We compared our findings with literature data from grey squirrels' native range, showing that many parasite species common in North America are completely missing in Italy. The alien host also acquired some Palearctic parasites, but their number does not compensate the number of species lost. Hence, this study supports the enemy release hypothesis (Torchin et al. 2003; see **Chapter 1**), since both helminth and ectoparasite richness in the introduced range are lower than in grey squirrels' native range. Interestingly, we also show that the alien host introduced to Italy the above-mentioned Nearctic nematode *S. robustus* and acquired the red squirrel flea *Ceratophyllus sciurorum* that successfully replaced the Nearctic flea *Orchopeas howardii*, absent in Italy. *S. robustus* and *C. sciurorum* were present in all the sampled populations, dominating grey squirrels' parasite community with a total prevalence of 57% and 26%, respectively. Moreover, in grey squirrels we observed a peak in *C. sciurorum* abundance in spring, whereas red squirrels are more infested in autumn (see **Chapter 2**), as a consequence, the presence of the alien species in areas inhabited by red squirrels could alter the usual seasonal distribution of the flea, with potential repercussions on the native sciurid. Hence, the study reveals that grey squirrels invasion holds the premises for both spillover and spill-back processes towards red squirrels to occur (Strauss et al. 2012; see **Chapter 1**).

In **Chapter 4**, grey squirrels and their dominant nematode *S. robustus* were used as a model to investigate factors affecting parasite fecundity and to assess the performance of indirect survey methods to estimate macro-endoparasites prevalence and intensity. We compared results of faecal flotation and faecal egg counts (FECs) to results obtained from direct examination of grey squirrels' gastro-intestine. Our findings reveal that flotation offers a reliable estimate of *S. robustus* prevalence in the host-population, independently from the amount of faecal material analysed. Hence, assuming similar performances in the native species, flotation may be a valid method to detect parasite spillover in living red squirrels co-inhabiting with the alien species. However, when true infection prevalence is low, flotation may lead to an overestimation of infected individuals, hence it should be followed by more specific tests to detect truly positive hosts. On the contrary, our predictive model of *S. robustus* intensity showed that the relationship between the nematode burden and the amount of eggs in faeces is not linear, hence FECs are not a reliable method to estimate worm intensity. The analysis of *S. robustus* fecundity indicated indeed that individual egg production decreases with increasing values of parasite intensity. Since no seasonal variation in helminth fecundity was detected, this result is likely a consequence of density-dependent processes due to competition among parasites (Tompkins and Hudson 1999) or, as observed for the congeneric nematode *S. rattii* in mice (Paterson and Viney 2002), to the host immune response elicited by high intensity of infection.

In **Chapter 5** the factors affecting macro-endoparasite community of native red squirrels were analysed to detect any variation due to grey squirrel presence. In particular, through indirect, non-invasive methods (flotation and tape-tests), we compared *S. robustus* and *T. sciuri* prevalence in red squirrels living in areas with and without alien grey squirrels (red-only and red-grey populations, respectively). Our results show that *S. robustus* infection in red squirrels is linked to grey squirrel presence, confirming that the native species acquires this nematode via spillover from the alien host. Both the life-cycle of the parasite and the fact that *S. robustus* prevalence increases during the cold season, suggest that inter-specific transmission occurs in nests shared, on different nights, between individuals of the two species. Moreover, we detected a significant increase in *T. sciuri* prevalence in areas co-inhabited by grey squirrels. Since prevalence of this oxyurid nematode in Italian populations of grey squirrels is very low (see **Chapter 3**), this result is not likely linked with spill-back processes (Kelly et al. 2009; see **Chapter 1**), but could be a consequence of the competitive pressure inducing an increase in stress levels that in turn lead to higher susceptibility to parasite infection.

Finally, in **Chapter 6**, we surveyed through PCR techniques Ljungan virus (LV) and adenovirus infections in culled grey squirrels and road-killed red squirrels. LV is a potential zoonoses (reviewed in McDonald 2009) that has been detected in several species of small rodents across Europe (Niklasson et al. 1999; Hauffe et al. 2010; Salisbury et al. 2013), but whose epidemiology is still unclear. Lethal outbreaks of enteric adenoviral infections are reported in red squirrels in Germany and the UK (Peters et al. 2011; Everest et al. 2012), where also subclinical infections in grey squirrels were detected (Everest et al. 2009). The aim of this study was thus to investigate whether arboreal sciurids are involved in LV circulation and if any adenovirus is present in Italian squirrel populations. We found that red squirrels in Italy are infected by LV (prevalence: 22%; N=9), whereas no positive grey squirrels were detected (N=49). Despite the small sample size of red squirrels examined, our result indicates that LV infection is not limited to small ground-dwelling rodents and that the virus has a wider host-spectrum than previously reported. Furthermore, our sequences were identical to haplotypes found in voles and mice, suggesting that arboreal squirrels may play an active role in inter-specific LV circulation. An adenovirus was detected both in red (16%; N=77) and grey squirrels (0.9%; N=232), thus extending the known distribution of this infection in squirrels to Southern Europe. Poor preservation of carcasses did not allow us to identify clinical signs of infection in road-killed red squirrels, whereas no signs were detected in positive grey squirrels. However, the low prevalence of adenovirus observed in the alien host seems to confirm that the species does not act as a reservoir for the virus as had been previously presumed (see also Everest et al. 2013).

7.2 Concluding remarks

Overall, the present thesis highlights the importance of taking into account parasitological aspects when dealing with biological invasions. In particular, despite evidence that helminths and arthropods may have a profound impact on host fitness, the role played by macroparasites in affecting host population dynamics is often neglected. Here I show that both helminths and arthropods represent a potential threat to native species and that their role should not be underestimated. I also tried to gain an exhaustive knowledge of red squirrel's parasite fauna, since I believe that a complete picture of parasites infecting native species is not only essential to detect alterations caused by invaders, but may also offer insights into their vulnerability to invasions, with host having poor parasite communities being more susceptible to competition mediated by parasites. Moreover, I demonstrated that the threat is not only posed by spillover of introduced parasites resulting in apparent competition, but that the presence of alien species may also alter pre-existent host-parasite dynamics and affect native hosts' response to local parasites, likely through stress-mediated effects on immune system induced by other forms of competition with invaders. Finally, I showed that host-parasite interactions may not always be straightforward and complex mechanisms may act to regulate parasite dynamics, highlighting how validation of indirect, diagnostic methods is fundamental and species-specific. Hence, the model red-grey squirrel teaches that i) macroparasites have the potential to affect biological invasions as much as microparasites do; ii) an exhaustive knowledge of native species parasite fauna is fundamental to investigate apparent competition; iii) apart from introducing alien parasites, alien species may affect native species parasite communities through other mechanisms; iv) inference of parasitological parameters from indirect methods should always be considered carefully.

This study also opens several questions that will need to be addressed in the future. First, having confirmed that *S. robustus* spills over to the native species, the next step should be to investigate its impact on red squirrel fitness (i.e. survival and/or fecundity) to assess whether the nematode mediates the competition between the two species. Unfortunately, the evaluation of *S. robustus* impact on living red squirrels will presents some difficulties since, as highlighted in this thesis, estimates of its intensity through FECs are unreliable and the sole variation of presence/absence may not reflect subtle changes in fitness parameters. Similarly, it would be interesting to test if the presence of grey squirrels leads to an alteration of *C. sciurorum* temporal distribution or to an increase of flea abundance in red squirrels. I also show that grey squirrels lost many parasite species during the introduction process, but does this loss translate into an effective advantage for the invading species? We may reasonably presume that the release from parasite pressure would result in increased host performances, but to truly answer this question, an experimental study at the biogeographical scale (i.e. comparing parasite impact on grey squirrels' fitness in their native and introduced range) would be the best approach.

Finally my results suggest that the role of arboreal small mammals in zoonotic diseases circulation could have been underestimated. Hence, a more exhaustive study on Ljungan virus and other microparasites infecting red squirrels would be needed. For example, red squirrels are already known to carry *Toxoplasma* spp. and *Borrelia* spp. (Jokelainen and Nylund 2012; Pisanu et al. 2014) and it would be interesting to examine in depth their role in the circulation of these zoonoses. Besides, we should not forget that introduced grey squirrels may act as reservoirs for the same microparasites, increasing their presence in the environment or altering their dynamics, thus representing a threat for public health. Hence, the role of the alien species in zoonotic diseases circulation should be examined more carefully.

I would like to conclude with a personal consideration on eco-parasitological studies. During my research I realised that studying host-parasite relationships is indeed a complex matter that requires competence in several fields of both ecology and veterinary sciences. Nowadays, it is widely recognised that parasites are an ubiquitous and important force able to affect host population dynamics, competitive interactions and many other evolutionary and ecological processes in natural populations. Nevertheless, I feel that studies dealing with parasites are often lacking a multi-disciplinary point of view, for example strictly focusing on parasitological aspects without adequately considering host ecology and population dynamics. Hence, I believe that, in the future, a more integrated approach to these topics will be beneficial to gain a more complete and effective understanding of host-parasite interactions.

References

Bartlett C (1995) Morphology, homogonic development, and lack of a free-living generation in Strongyloides robustus (Nematoda, Rhabditoidea), a parasite of North American sciurids. Folia Parasitol (Praha) 42:102–114.

Everest DJ, Butler H, Blackett T, et al. (2013) Adenovirus infection in red squirrels in areas free from grey squirrels. Vet Rec 173:199–200. doi: 10.1136/vr.f5304

Everest DJ, Grierson SS, Stidworthy MF, Shuttleworth C (2009) PCR detection of adenovirus in grey squirrels on Anglesey. Vet Rec 165:482.

Everest DJ, Shuttleworth CM, Grierson SS, et al. (2012) Systematic assessment of the impact of adenovirus infection on a captive reintroduction project for red squirrels (Sciurus vulgaris). Vet Rec 171:176–176. doi: 10.1136/vr.100617

Hauffe HC, Niklasson B, Olsson T, et al. (2010) Ljungan Virus Detected in Bank Voles (Myodes glareolus) and Yellow-Necked Mice (Apodemus flavicollis) from Northern Italy. J Wildl Dis 46:262–266.

Jokelainen P, Nylund M (2012) Acute Fatal Toxoplasmosis in Three Eurasian Red Squirrels (Sciurus vulgaris) Caused by Genotype II of Toxoplasma gondii. J Wildl Dis 48:454–457. doi: 10.7589/0090-3558-48.2.454

Kelly DW, Paterson RA, Townsend CR, et al. (2009) Parasite spillback: A neglected concept in invasion ecology? Ecology 90:2047–2056. doi: 10.1890/08-1085.1

Krasnov BR, Shenbrot GI, Khokhlova IS, Degen AA (2004) Flea species richness and parameters of host body, host geography and host "milieu."J Anim Ecol 73:1121–1128. doi: 10.1111/j.0021-8790.2004.00883.x

Marques JF, Santos MJ, Teixeira CM, et al. (2011) Host-parasite relationships in flatfish (Pleuronectiformes) – the relative importance of host biology, ecology and phylogeny. Parasitology 138:107–121. doi: 10.1017/S0031182010001009

McDonald AG (2009) Ljungan Virus: an Emerging Zoonosis? Clin Microbiol Newsl 31:177–182. doi: 10.1016/j.clinmicnews.2009.11.001

Niklasson B, Kinnunen L, Hörnfeldt B, et al. (1999) A New Picornavirus Isolated from Bank Voles (Clethrionomys glareolus). Virology 255:86–93. doi: 10.1006/viro.1998.9557

Paterson S, Viney ME (2002) Host immune responses are necessary for density dependence in nematode infections. Parasitology 125:283–292.

Peters M, Vidovszky MZ, Harrach B, et al. (2011) Squirrel adenovirus type 1 in red squirrels (Sciurus vulgaris) in Germany. Vet Rec 169:182–182. doi: 10.1136/vr.d2610

Pisanu B, Chapuis J-L, Dozières A, et al. (2014) High prevalence of Borrelia burgdorferi s.l. in the European red squirrel Sciurus vulgaris in France. Ticks Tick-Borne Dis 5:1–6. doi: 10.1016/j.ttbdis.2013.07.007

Salisbury A-M, Begon M, Dove W, et al. (2013) Ljungan virus is endemic in rodents in the UK. Arch Virol 1–5. doi: 10.1007/s00705-013-1731-6

Strauss A, White A, Boots M (2012) Invading with biological weapons: the importance of disease-mediated invasions. Funct Ecol 26:1249–1261. doi: 10.1111/1365-2435.12011

Tompkins DM, Hudson PJ (1999) Regulation of nematode fecundity in the ring-necked pheasant (Phasianus colchicus): not just density dependence. Parasitology 118:417–423.

Torchin ME, Lafferty KD, Dobson AP, et al. (2003) Introduced species and their missing parasites. Nature 421:628–630. doi: 10.1038/nature01346

Appendix

List of publications in ISI-ranked journals by Claudia Romeo.

Publications that I have co-authored **during my PhD** (the most recent Impact Factor of each journal is reported):

Romeo C., Wauters L.A., Ferrari N., Lanfranchi P., Martinoli A., Pisanu B., Preatoni D.G. and Saino N. (2014). Macroparasite fauna of alien grey squirrels (*Sciurus carolinensis*): composition, variability and implications for native species. *PloS ONE* 9: e88002. (IF: 3.73)

Romeo C., Ferrari N., Rossi C., Everest D.J., Grierson S.S., Lanfranchi P., Martinoli A., Saino N., Wauters L.A. and Hauffe H.C. (2014). Ljungan virus and an adenovirus in Italian squirrel populations. *Journal of Wildlife Diseases* 50: in press. (IF: 1.27)

Mori E., Ancillotto L., Menchetti M., **Romeo C.** and Ferrari N. (2013). Italian red squirrels and introduced parakeets: victims or perpetrators? *Hystrix Italian Journal of Mammalogy* 24: 195–196. (IF: 0.35)

Romeo C., Pisanu B., Ferrari N., Basset F., Tillon L., Wauters L.A., Martinoli A., Saino N. and Chapuis J.-L. (2013). Macroparasite community of the Eurasian red squirrel (*Sciurus vulgaris*): poor species richness and diversity. *Parasitology Research* 112: 3527–3536. (IF: 2.85)

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“Morning sir, or madam, or neuter," the thing said. "This your planet, is it?"
"Well, er. I suppose so," Newt said.
"Had it long, have we sir?"
"Not personally. I mean, as a species, about half a million years. I think."
The alien exchanged glances with its colleague.
"Been letting the old acid rain build up, haven't we sir," it said.
"Been letting ourselves go a bit with the old hydrocarbons, perhaps?"
"I'm sorry?"
"Well, I'm sorry to have to tell you, sir,
but your polar ice caps are below regulation size for a planet of this category, sir."
"Oh, dear," said Newt.
"We'll overlook it on this occasion, sir."
The smaller alien walked past the car.
"CO2 level up nought point five percent," it rasped, giving him a meaningful look.
"You do know you could find yourself charged with being a dominant species
while under the influence of impulse-driven consumerism, don't you?”

- Neil Gaiman & Terry Pratchett, *Good Omens* -

"The fact that we live at the bottom of a deep gravity well,
on the surface of a gas covered planet
going around a nuclear fireball 90 million miles away and think this to be normal
is obviously some indication of how skewed our perspective tends to be."

- Douglas Adams, *The Salmon of Doubt: Hitchhiking the Galaxy One Last Time -*