University of Insubria
Department of Theoretical and Applied Sciences
PhD Program in Chemical and Environmental Science
XXX Cycle

Relationships among parasites, physiological stress and personality in the interactions between invasive alien and native species

PhD Thesis
Francesca Santicchia
709094

Tutor:
Prof. Adriano Martinoli

2016-2017

"Ecology has the distinction of being peculiarly confronted with uniqueness: millions of different species, countless billions of genetically distinct individuals, all living and interacting in a varied and ever-changing world..."

"T. H. Dobzhansky said, 'Nothing in biology makes sense, except in the light of evolution', then, equally, very little in evolution, and hence in biology as a whole, makes sense except in the light of ecology"

Bengon M., Townsend C. R and Harper J. L.
Ecology: From Individuals to Ecosystems

Table of contents

Abstract

Invasive alien species (IAS) are identified as one of the major environmental research themes in the EU Horizon 2020 framework; indeed, recent studies have revealed that they represent a worldwide threat to biodiversity through different ecological processes. Biological invasion success and impact can be determined, positively and negatively, by parasitic infections known to have an important, but often unclear, role over a range of biological scales from processes within the original host to population-level dynamics which can have consequences for other components in the "invaded" ecosystem.

In general, parasite transmission, and therefore disease incidence, is determined by a combination of exposure to infected hosts, vector or infective stages, and host susceptibility. Recent studies showed how parasites and animal behavior are inextricably linked and that animal personality can create between individual heterogeneities in the acquisition and spread of parasites. Furthermore, it has been frequently suggested that personality may play a role in natural invasions with successful invaders being characterized as bolder, more aggressive or more prone to dispersal.

The presence of IAS may also affect native species susceptibility, impairing their immune response by increasing individual physiological stress, thereby facilitating parasitic infections. Several studies highlight how invasive propensity and, consequently, successful invasion are favored in (host) species that preferentially allocate resources to growth and reproductive effort, such as number of offspring per breeding season, over costly immune defence mechanisms.

The aim of the present study is to investigate the interactions between Invasive Alien Species (IAS) and native species, and improve the knowledge on biological invasions impact in the light of potential influence of parasitic infections, physiological stress and personality on individual fitness (reproductive success/investment).

In particular, this study investigated the interspecific competition between the North American invasive Eastern grey squirrel (*Sciurus carolinensis*) and the native Eurasian red squirrel (*Sciurus vulgaris*).

First, I conducted the validation of an enzyme immunoassay to measure Fecal Glucocorticoid Metabolites (FGM) in Eurasian red squirrel, my results showed that the peak excretion time is between 24-32 h after the stressor and that this assay can detect changes in FGM concentrations in response to changes in reproductive condition or seasonal/habitat factors. Thus, measuring FGM concentrations can be a useful tool to detect physiological stress in wildlife.

Next, I investigated native species (*Sciurus vulgaris*) physiological stress in relation to invasive species (*Sciurus carolinensis*) presence. My results showed how the presence of an invasive competitor altered the glucocorticoid (GC) response of individuals of a native species and that, in the two woodlands colonized by grey squirrels during the study, FGM concentrations in the native red squirrels increased with respect to pre-colonization concentrations. This was further tested by a grey squirrel removal experiment that confirmed how FGM concentrations in syntopic red squirrels significantly decreased after the removal of the majority of grey squirrels.

In the following part of the study, I used capture-mark-recapture data of Eurasian red squirrels, to measure indirect indices of personality. My results showed that scores derived from the number of times a squirrel is trapped (trappability) and from the number of different traps visited (trap diversity), during the first one or two years of presence, are repeatable across years and therefore can be used as indices of an individual's personality. Then, I explored whether indices of boldness (individual willingness to take risks) and exploration (propensity to explore and acquire information about the environment) were correlated with local survival in either of the sexes and with a measure of reproductive success in females, under different conditions of food availability. Bold squirrels, both males and females, survived better than shy ones in Norway spruce forests, while in Scots pine or mixed spruce-fir forests the opposite trend occurred with higher local survival for shy than for bold red squirrels. Conversely, exploration was negatively correlated with local survival in all habitats and in both sexes. In addition, across habitat types, female reproductive success increased with body mass and decreased with the tendency to explore, and bolder females did not have any reproductive advantage in years of poor food availability.

Then, in grey squirrels, I demonstrated that indirect indices of personality are consistent with direct measurements obtained with arena tests. Hence, both methods produced consistent measures of the personality trait activity-exploration. Furthermore, my results showed that Eastern grey squirrel personality differences affected individual variation in endoparasite infection, with bolder-explorative animals more heavily infected by *Strongyloides robustus* than shy grey squirrels. However, I also found that host personality mainly influences the probability of acquiring *S. robustus*, whereas it has no effect on parasite intensity in already infected hosts, which is instead affected by host body mass.

Successively, I presented a staining technique, which allows to distinguish the number of uterine scars of different litters of female grey squirrels in N. Italy. My results showed that female fecundity over an entire year was slightly larger than in populations in the native range, adding knowledge on Eastern grey squirrel population growth, thus IAS spreading capacity.

Next, I explored relationships between parasite load, physiological stress, reproductive success/investment and the potential mediatory role of personality. My results showed that variation in four parasite infections predictors (*S. robustus* abundance, prevalence, intensity and helminths richness) is not affected by individual variation in levels of physiological stress. Furthermore, Eastern grey squirrel FGM concentrations are influenced by body mass, vary between seasons and, in males, in relation to reproductive condition. Investigating whether the increase in physiological stress and/or higher parasite loads were associated with lower reproductive success/investment in grey squirrels, I found that female reproductive success is affected only by body mass and there is no effect of FGM concentrations or of parasite infection. Conversely, male testes mass is positively related to body mass but negatively to parasite infections and there is no effect of FGM concentrations, suggesting that reproductive investment of male grey squirrels is related to a cost represented by high *S. robustus* abundance and intensity. In general, all models reported an effect of body mass that positively influenced FGM concentrations, female reproductive success, males reproductive investment and parasite infections (abundance and intensity).

Finally, I investigated the prediction that personality would not affect directly reproductive success/investment, but instead indirectly through the influence on FGM concentration and/or parasite load. My results showed that personality score did not affect female reproductive success, male reproductive investment or individual variation in levels of physiological stress.

Overall, the present thesis highlights that Eastern grey squirrel impact on Eurasian red squirrel populations occurs also through increase in physiological stress in the native species. Moreover, this study shows that individual condition (body mass) is a prominent factor that drives reproductive output of the invasive species. However, this relationship can be influenced by parasites (in males), which are confirmed to have a role in biological invasion, and the body mass – parasite load interactions can be mediated by differences in personality.

Introduction

Invasive alien species (IAS) are identified as one of the major environmental research themes in the EU Horizon 2020 framework; indeed, recent studies have revealed that they represent a worldwide threat to biodiversity through different ecological processes (Clavero and García-Berthou 2005; Keller et al. 2011). Well documented studies shown that IAS impact on native species occurs through diverse mechanisms, such as interspecific competition (Cadi and Joly 2003; Gurnell et al. 2004), predation (Berger et al. 2007; Banks and Dickman 2007), transmission of infectious diseases (Daszak et al. 2000; Strauss et al. 2012) and even changes in ecosystem functioning (Ehrenfeld 2010; Strayer 2012); which, ultimately, can lead to extinction of native species (Keller et al. 2011). Although extinction is often the end result of invasions, other ecological and evolutionary consequences are less understood (Mooney and Cleland 2001).

For instance, biological invasion success and impact can be determined, positively and negatively, by parasitic infections known to have an important, but often unclear, role over a range of biological scales from processes within the original host to population-level dynamics which can have consequences for other components in the "invaded" ecosystem (Dunn and Perkins 2012; Dunn et al. 2012). Parasites, indeed, have been documented to influence the outcome and impact of invasions or may themselves be invasive species (Dunn 2009; Tompkins et al. 2011; Hatcher et al. 2012). Moreover, they can exert subtle forces in structuring communities manipulating and mediating interspecific and intraspecific interactions both directly and indirectly (Tompkins et al. 2011; Roy and Handley 2012; Telfer and Brown 2012).

In general, parasite transmission, and therefore disease incidence, is determined by a combination of exposure to infected hosts, vector or infective stages, and host susceptibility (Anderson and May 1978; Telfer and Brown 2012). Recent studies showed how parasites and animal behavior are inextricably linked (Boyer et al. 2010; Ezenwa et al. 2017) and that animal personality can create between individual heterogeneities in the acquisition and spread of parasites (VanderWaal and Ezenwa 2016). For instance, individual personality differences may affect, at least partly, exposure with the general idea that bold, explorative, active and social animals that interact more with conspecific have a high probability of encountering parasite infective stages, hence are more likely to be infected and, consequently, transmit parasitic infections to other individuals. Furthermore, it has been frequently suggested that personality may play a role in natural invasions (Cote et al. 2010; Réale et al. 2010) with successful invaders being characterized as bolder, more aggressive or more prone to dispersal (Cote et al. 2010; Gonzalez-Bernal et al. 2014). Thus, associations between parasitism and host behavior may further complicate the role of parasites in the spread and impact of invasive species.

The presence of IAS may also affect native species susceptibility, impairing their immune response by increasing individual physiological stress (Busch and Hayward 2009; Martin 2009; Brearley et al. 2013), thereby facilitating parasitic infections (Wilson et al. 1993; Dunn et al. 2012; Poulin 2013; St. Juliana et al. 2014). In particular, high physiological stress may induce the suppression of the host immune response through the action of glucocorticoid hormones (see Padgett and Glaser 2003; Martin 2009) that are involved in many body functions, including the expression of behavior, the response to infection and diseases, and even the regulation of reproduction and development (Dantzer et al. 2014).

Several studies highlight how invasive propensity and, consequently, successful invasion are favored in (host) species that preferentially allocate resources to growth and reproductive effort, such as number of offspring per breeding season, over costly immune defence mechanisms (Kolar and Lodge 2001; Sakai et al. 2001; Lee and Klasing 2004; Telfer and Brown 2012). Only recently, variation in individual fitness (e.g. reproduction and survival) has been investigated in the light of relationships with direct or cumulative effects/influences of physiological stress, behavior and parasitic infections (Sih et al. 2004; Both et al. 2005; Morand et al. 2006; Hillegass et al. 2010; Dantzer et al. 2010, 2014; Bosson et al. 2013). However, disentangling all these factors and the cause-consequence pathways is a complex matter.

1 Study aims and model system

Once adapted to the new environment, the success of IAS depends strongly on population growth, hence on survival and reproductive rate, and on interspecific interactions with native species. Parasites have a key role in invasion processes but still little is known about their effect on invasive and native host populations. Moreover, exploring the interactions between parasite infections and other factors, such as behavior and/or physiological stress, is challenging but may evidence new insights in the ecology of biological invasions. In addition, measuring their cumulative influence on individual fitness is essential to significantly improve our knowledge on invasive species ecology.

In this study, I have investigated the interspecific competition between Eastern grey squirrel (*Sciurus carolinensis*) and Eurasian red squirrel (*Sciurus vulgaris*), in terms of parasite infections, physiological stress and the role individual variation in personality might play in mediating host-parasite interactions.

Tree squirrels are often successful invaders and several biological characteristics determine their success as invasive species: high reproductive potential, high vagility, diverse food habits, ability to construct nests, and plasticity in human-impacted landscapes (Palmer et al. 2007; Bertolino 2009; Di Febbraro et al. 2016). The Eastern grey squirrel is an invasive species that, since the first introductions in Great Britain and in Northern Italy, has expanded its range, replacing native red squirrels (Bertolino et al. 2014; Gurnell et al. 2015). The rapid spread of the invasive *S. carolinensis* and subsequent decline and widespread extinction of the native *S. vulgaris* has been documented in many studies. The two species have similar space use and activity patterns, and compete for food resources, resulting in smaller body size, reduced female reproduction and juvenile recruitment in red squirrels in woodlands shared with the alien congener (Wauters et al. 2002a, b; Gurnell et al. 2004, 2015). On the British Isles, disease-mediated competition occurs with grey squirrels acting as a healthy reservoir transmitting a squirrelpox virus (SQPV) which, in most cases, is lethal to infected red squirrels (Mc Innes et al. 2013; Shuttleworth et al. 2014; White et al. 2016).

Previous studies in Northern Italy, where SQPV does not occur (Romeo et al. submitted), have attributed the replacement of red by alien grey squirrels to competition for high-energy food resources (Wauters et al. 2002a, b; Gurnell et al. 2004). However, recent studies suggest that grey squirrels have lost most of their parasite fauna from the native range (parasite release) and that spillover of macroparasites to *S. vulgaris* occurs (Romeo et al. 2013, 2014a, 2015).

Aside from being one of the best documented cases of ecological replacement of a native species by a IAS (both in UK and Italy), the system is ideally suited to study the potential relationships between physiological stress, personality and parasite infection for the following reasons: (i) their macroparasite fauna has been studied in detail (Romeo et al. 2013; 2014a, b, 2015); (ii) their distribution is well known allowing to distinguish between stable populations, colonizing populations on the edge of its range; and populations where both species co-occur (Bertolino et al. 2014) and thus are subject to inter-specific competition; (iii) grey squirrels are controlled for the conservation of the native squirrel, giving access to large numbers of carcasses to study endoparasites and reproductive conditions; (iv) repeated live-trapping of individually-marked squirrels can provide fecal samples for FGM (Fecal Glucocorticoid Metabolites) quantification and measures of parasites presence and shedding, information of phenotype and detailed reproductive data; (v) non-invasive methods to estimate physiological stress have already been developed; and (vi) reliable methods to measure personality have been documented and used on other sciurids (Boon et al. 2008; Boyer et al. 2010).

2 Hypotheses

In this project, I tested the following hypotheses using both mixed populations (red and grey squirrel) and populations with grey squirrels only:

1. Presence of grey squirrels increases stress levels in native red squirrels; removal of grey squirrels will reduce physiological stress in red squirrels.
2. Grey squirrels with higher levels of physiological stress will have a higher parasite load.
3. Grey squirrels with a high boldness score have higher parasite load because of higher encounter rate with the parasite's infective stages.
4. Increasing stress levels and/or higher parasite load are associated with lower reproductive success/investment in grey squirrels.
5. The relationship between parasite load, stress and reproductive success/investment is mediated by personality.

To test these hypotheses, the project aims to: (1) determine *Strongyloides robustus* (dominant helminth) abundance, intensity, prevalence and helminths richness, in Eastern grey squirrels; (2) determine personality of individually marked squirrels using trappability, trap-diversity indices, in both squirrel species, and arena tests, in Eastern grey squirrels; (3) measure levels of physiological stress with non-invasive techniques (Fecal Glucocorticoid Metabolites, FGM), in both squirrel species; and (4) measure reproductive output: success (number of uterine scars in females) or investment (testes mass in males) in Eastern grey squirrels.

The various hypotheses are treated in different chapters.

Interspecific competition may cause high physiological stress levels in Eurasian red squirrel in co-occurrence with the invasive squirrel species. Measuring FGM concentrations is a non-invasive technique that can help to identify physiological stress in free-ranging species, but the method needs to be validated for each species. In order to investigate this competition scenario, the first necessary step was to validate this method for the native species (**Chapter 1**). Afterwards, it was possible to evaluate physiological stress levels of Eurasian red squirrel in relation to Eastern grey squirrel presence and subsequent to their removal (**Chapter 2**).

As abovementioned, the interactions between parasites, physiological stress and personality could exacerbate the impact of invasive species. Personality can be measured through different tests: in particular, I applied indirect indices, already used on other sciurid species, to determine squirrels personality (**Chapter 3**) and I validated them looking at the correlation with direct personality measurements from arena tests. Then, I used the methods previously identified to explore the relationship between personality and parasites in Eastern grey squirrel (**Chapter 4**).

Reproductive success/investment could be influenced by the cumulative effect of factors indicated above, and determine invasive species population growth and speed of range expansion. Hence, I evaluated a staining technique of uterine scars count on invasive species populations (**Chapter 5**). Next, I applied this technique to determining Eastern grey squirrel fecundity. In a final step, on the invasive species, I investigated if the relationship between parasite load, physiological stress and reproductive success (uterine scars in females) and/or investment (testes mass in males) was mediated by direct effect of personality on physiological stress and parasite load, that in turn indirectly influence reproductive output (**Chapter 6**).

Chapter 1

Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season

1.1 Summary

This Chapter describes the development of an assay which can be used to quantify physiological stress using fecal samples of Eurasian red squirrel (*Sciurus vulgaris*). This assay can help to address questions regarding causes and consequences of physiological stress in wildlife and how environmental changes could affect it. Results, obtained from captures of free-living squirrels and subsequent held in captivity, showed that fecal glucocorticoid metabolite (FGM) concentrations had a lag time to peak excretion, after stressor induced by initial capture and handling, ranging from 24 to 36 h. Moreover, FGM concentrations, measured in wild caught free-living squirrels, were higher in lactating females respect to nonbreeding or pregnant squirrels and no differences were found in males with abdominal or scrotal testes. Also, FGM concentrations were higher in winter and lower in summer. Subsequent investigations on whether or not physiological stress is induced by the presence of invasive species, such as Eastern grey squirrel (*Sciurus carolinensis*), will be carried out using this assay to quantify physiological stress in Eurasian red squirrels.

1.2 Introduction

Physiological stress reactions are multifaceted mechanisms exhibited by organisms in response to environmental changes. The causes and consequences of physiological stress reactions are increasingly studied in a wide range of studies in population ecology, behavioral ecology, conservation physiology, and animal welfare (Romero 2004; Palme et al. 2005; Dantzer et al. 2014; Romero and Wingfield 2015). For example, ecological factors such as food availability (Kitaysky et al. 1999), predation risk (Clinchy et al. 2013), or competition (Creel et al. 2013; Dantzer et al. 2013) can induce physiological stress reactions and these changes can affect a range of behavioral (Koolhaas et al. 1999; Mateo 2007; Cockrem 2013) or life history traits (Breuner et al. 2008; Bonier et al. 2009; Crespi et al. 2013). Physiological stress reactions may play a role in determining levels of parasite infection (Chapman et al. 2006; St Juliana et al. 2014), because chronic stress may reduce an individual's immune response, increasing its susceptibility to infections (Romero 2004). Another field of growing interest is that of conservation physiology, which is an integrative discipline that aims to understand the effects of environmental perturbations and predict the fate (persistence) of wildlife populations or species using physiological measures such as stress hormones (Wikelski and Cooke 2006; Cooke et al. 2013; Dantzer et al. 2014).

Activation of the vertebrate neuroendocrine stress axis initiates a host of behavioral and physiological changes in response to both predictable and unpredictable environmental challenges

(Sapolsky et al. 2000; Romero 2004). A major part of the vertebrate neuroendocrine stress response is coordinated by the hypothalamic–pituitary–adrenal (or interrenal) axis and ultimately results in the secretion of glucocorticoid steroid (stress) hormones (cortisol, corticosterone) that can cause a number of pronounced behavioral and physiological responses (Sapolsky et al. 2000; Möstl and Palme 2002; Romero 2004). Although a variety of different techniques exist to assess whether an animal is "stressed" (Sheriff et al. 2011; Dickens and Romero 2013; Romero and Wingfield 2015), measuring the activity of the vertebrate neuroendocrine stress axis usually involves quantifying glucocorticoids. In all species, this can be challenging because the sampling method should not affect or bias the glucocorticoid levels themselves. In all species but especially those of conservation concern, the sampling method should also not in itself negatively influence the individuals sampled. Fortunately, glucocorticoids can also be quantified in biological samples such as feces, urine, or hair which, unlike blood sampling, can be obtained in a relatively noninvasive manner (Sheriff et al. 2011). The measurement of glucocorticoids in fecal samples collected from wild animals has proven to be particularly useful (Sheriff et al. 2011; Romero and Wingfield 2015). Steroid hormones in the blood are metabolized and conjugated by the liver and excreted into the gut via bile where they are additionally metabolized through the gut microbiome before defecation (Taylor 1971; Brownie 1992; Palme et al. 2005). Measures of glucocorticoids in fecal samples therefore represent measures of the metabolites of glucocorticoids but they are closely correlated with circulating glucocorticoid concentrations (Mateo and Cavigelli 2005; Sheriff et al. 2010).

Assays to measure fecal glucocorticoid metabolites (FGM) have now been developed for many vertebrate species over the past 25 years (comprehensive list available from Palme 2016). However, their application must be carefully validated in each species in which they are used, even in closely related species (Touma and Palme 2005; Sheriff et al. 2011; Fanson et al., in press). The benchmark of a biological validation is to show that an experimental increase in circulating glucocorticoid levels is reflected in FGM concentrations (Touma and Palme 2005). Assays to measure FGM are often validated through an application of some type of experimental stressor (e.g., temporary capture—Harper and Austad 2001; Bosson et al. 2013; Hämäläinen et al. 2014) or pharmaceutical challenge that increases adrenal glucocorticoid production (e.g., adrenocorticotropic hormone [ACTH] injection—Dantzer et al. 2010). The latter is sometimes impossible in species of conservation concern because the capture, restraint, and injections may have detrimental effects on free-living study animals. In some species, this is surmountable by conducting pharmaceutical validations (ACTH injections) in captive- or zoo-housed animals of the same species (e.g. Goymann et al. 1999; Dehnhard et al. 2001; Pribbenow et al. 2014). However, the selection and availability of wildlife species in zoos is limited and so biological validations that do not employ ACTH injections such as temporary immobilization or translocation are also commonly used (Touma and Palme 2005; Pribbenow et al. 2014; Munerato et al. 2015). Moreover, ACTH injections require considerably more expertise (injection procedure) and validation procedures to identify the dosage of ACTH that is required. Ideally, a study should conduct both physiological (with ACTH injection) and biological (such as temporary captivity or exposure to predator cues) validations but this is not always possible especially in species of conservation concern (Touma and Palme 2005).

In some parts of Europe, Eurasian red squirrels (*Sciurus vulgaris*) are threatened with local extinction by the introduction and subsequent spread of the Eastern gray squirrel (*Sciurus carolinensis*—Gurnell and Pepper 1993; Martinoli et al. 2010; Bertolino et al. 2014). Consequently, the Eurasian red squirrel has become a species of conservation concern in the British Isles and Italy. The introduction of an invasive species that increases competition for food and other resources may increase physiological stress in the native species. This could directly contribute to declines in reproduction or survival or increase susceptibility of the native species to infection from novel parasites from the invasive species. In Eurasian red squirrels, it is currently not possible to study these questions because of the lack of noninvasive methods to measure stress. The goal of this study was to validate an enzyme immunoassay (EIA) to measure FGM in the Eurasian red squirrel.

We performed a biological validation of this assay where we captured squirrels and temporarily held them in captivity for 48 h. We measured FGM concentrations in samples collected approximately every 4 h over the 48-h period to determine if and when FGM concentrations significantly increased compared to those measured in samples collected at initial capture. We did not perform a physiological validation (ACTH injection) because this species is of conservation concern and therefore ACTH injections were not possible. Biological validations such as temporary captivity that we used here should provide equivalent validation of an assay to measure FGM concentrations. We also measured FGM concentrations in natural populations of Eurasian red squirrels over a 3-year period. We used these samples to explore how sex, reproductive condition, and a measure of body condition (body mass) affected FGM concentrations. These samples were collected throughout the year (summer, autumn, and winter), allowing us to also investigate seasonal patterns of FGM concentrations.

1.3 Materials and methods

1.3.1 Study species

The Eurasian red squirrel has a social organization that differs between the sexes: adult females tend to defend exclusive core areas against other females while among neighboring males there exists a dominance hierarchy with older and heavier males occupying the largest home ranges and overlapping with more females than younger males of lower body mass (Wauters and Dhondt 1989, 1992; Di Pierro et al. 2008; Romeo et al. 2010). There is no sexual size dimorphism (Wauters et al. 2007). The mating system is promiscuous, although most females only mate with a dominant male of high body mass (Wauters et al. 1990). Reproduction is seasonal: mating activity peaks in January to February and April to May, with females giving birth to 1–2 litters per year (spring litters born in March to April, summer litters in June to July). These patterns of reproduction are strongly affected by food abundance and, in the mountains, by elevation (Wauters and Lens 1995; Wauters et al. 2008; Rodrigues et al. 2010). At higher elevations, females tend to have a single litter per year, with parturition dates occurring from April to early July based on conifer seed availability (Wauters et al. 2008; Rodrigues et al. 2010). Increased tree-seed availability improves red squirrel body mass, reproductive rate, and survival, resulting in an increase in population growth and density (Wauters et al. 2004; Boutin et al. 2006; Wauters et al. 2008). The species occupies a wide variety of forest types, occurring in both continuous forests and fragmented woodlands. Although red squirrels are well adapted to fragmented habitats and have good dispersal capacities (Wauters et al. 2010), populations inhabiting forest fragments have lower densities and genetic diversity and higher parasite loads than those in continuous forests (Wauters et al. 1994; Santicchia et al. 2015a).

1.3.2 Study sites

We trapped Eurasian red squirrels in 5 study sites in Lombardy, North Italy (Table 1.1). Two sites (Vanzago, Castelbarco) are lowland mixed deciduous woodlands in the Po plain while the other 3 (Bormio, Cancano, Valfurva) are subalpine conifer forests in the Central Italian Alps (1,620–2,150 m elevation). Detailed data on forest structure and composition of the alpine sites in the Alps are given elsewhere (Wauters et al. 2008; Salmaso et al. 2009; Rodrigues et al. 2010). The lowland sites (160–170 m elevation) are mature mixed deciduous woodlands dominated by oaks

(*Quercus robur*, *Q. petraea*, *Q. rubra*) and hornbeam (*Carpinus betulus*), with some birch (*Betula* sp.), bird cherry (*Prunus avium*), elm (*Ulmus minor*), sweet chestnut (*Castanea sativa*), and black locust (*Robinia pseudoacacia*).

Table 1.1 – Location of study sites in Lombardy, North Italy and periods in which Eurasian red squirrels were captured for collection of fecal samples (sample size: total fecal samples collected and per sex) to measure FGM concentrations. Vanzago is the study site where we trapped squirrels both for the captive experiment (Exp.) and to obtain FGM concentrations of animals trapped and released after handling (Wild). FGM = fecal glucocorticoid metabolite.

Study site	Coordinates	Elevation (m)	Sampling period	Sample size (males, females)
Vanzago (Exp.)	45°31′N, 8°58′E	160–170	2–5 Dec. 2014 19–23 Jan. 2015 17–20 Mar. 2015	17 (11, 6)
Vanzago (Wild)	45°31′N, 8°58′E	160–170	2–5 Dec. 2014 19–23 Jan. 2015 17–20 Mar. 2015	8 (3, 5)
Castelbarco	45°35′N, 9°31′E	155–165	3–6 Dec. 2013	6 (3, 3)
Valfurva	46°27′N, 10°31′E	1,620–1,870	26–29 May 2014 29 Sept. to 2 Oct. 2014 25–28 May 2015 1–4 Oct. 2015	73 (39, 34)
Bormio	46°27′N, 10°30′E	1,950–2,150	9–13 Sept. 2014 30 June to 3 July 2015	10 (7, 3)
Cancano	46°33′N, 10°15′E	1,940–1,970	14–18 Sept. 2014 15–18 June 2015 7–10 Sept. 2015	14 (8, 6)

1.3.3 Livetrapping, handling, and fecal samples collection from wild squirrels

We trapped squirrels in 5 sites during trapping sessions that lasted 4–5 days each (sites and trapping dates listed in Table 1.1). We used Tomahawk traps (model 202, Tomahawk Live Trap Co., Hazelhurst, Wisconsin) with a fine mesh added underneath traps to prevent contamination between urine and feces. We placed traps on the ground or against tree trunks at a height of about 1.50 m and prebaited with hazelnuts 3–4 times over a 1-month period. We checked traps 2–3 times a day. Each trapped squirrel was flushed into a light cotton handling bag with a zipper (Wauters et al. 2007) to minimize stress during handling and individually marked using numbered metal ear-tags (type 1003 S, 10 by 2 mm, National Band and Tag, Newport, Kentucky). We weighed squirrels to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland) and measured the length of the right hind foot (without nail: ± 0.5 mm) with a thin ruler (Wauters et al. 2007). We recorded sex, age, and reproductive condition following Wauters and Dhondt (1995). A female's reproductive status was defined as nonbreeding (anestrous, vulva small, no longitudinal opening, not lactating), post-estrous and pregnant (vulva partly or strongly swollen with longitudinal opening, enlarged abdomen during late pregnancy), or lactating (nipples large, milk excretion can be stimulated). We recorded reproductive condition of males (testes size and position) as nonbreeding (testes abdominal or semi-scrotal and scrotum small) or breeding (testes scrotal and scrotum large). After capture and handling, fecal samples were collected from underneath the traps using forceps and placed individually into 1.5-ml vials (Dantzer et al. 2010) and the fine mesh and ground under the traps were cleaned to remove any remaining fecal material. We only used fecal samples from squirrels that had not previously been trapped or handled within 72 h prior to capture. We placed fecal samples into an insulated bag with wet ice packs while in the field and then stored them at −20°C within 3–4 h after collection

(Dantzer et al. 2010). Trapping and handling squirrels complied with the current laws on animal research in Italy and were carried out under permission of the Region of Lombardy (Decree n. 11190 of 29/11/2013). All of these procedures abided by guidelines of the American Society of Mammalogists (Sikes et al. 2011).

1.3.4 Capture and husbandry of squirrels for laboratory validation experiments

We trapped 17 red squirrels (6 females, 11 males) between December 2014 and March 2015 at the Vanzago study site that were subsequently held in captivity for 48 h using the structures of the local wild animal rescue center (Centro Recupero Animali Selvatici, WWF Vanzago). Four individuals were used twice (because in the 1st trial they had defecated only a few times) for a total of 21 experiments. Frequent trap checking prevented any squirrel from spending more than 2 h in a trap. Handling (see above) ended by taking the animal completely out of the cotton handling bag and holding it with a glove, checking reproductive condition, and doing a tape-test to look for parasite eggs (see Romeo et al. 2015).

After capture and handling, we released each animal into its own cage, a Tomahawk single door cat/rabbit collapsible trap (66 × 25 × 25 cm, model 205, Tomahawk Live Trap Co.) with a polycarbonate panel covering the entrance to avoid injury. Cages were kept in a warm room (about 10°C), lighted to simulate the natural photoperiod of late winter to early spring. Each cage contained a nest box with bedding material (hay) and was provided with ad libitum food (hazelnuts and fresh apple, the latter providing hydration, for a total of 1,098 kJ for 48 h). Cages were raised from the floor and a mesh underneath each trap prevented feces and urine from contaminating each other. We collected feces from the mesh and only when clearly not mixed with urine also from the polycarbonate board underneath the cage.

We collected fecal samples using forceps approximately every 4 h for a total of 48 h (except from 2200 to 0900 h) and stored in a 1.5-ml vial at −20°C. At the last check of each day (2200 h), we collected all samples and only collected fresh feces the following morning (0900 h) to minimize collection of samples that had been left at room temperature for > 4 h. Because squirrels are diurnal, it is unlikely much defecation happened until the early morning hours. After collection, we cleaned the forceps and any urine and feces underneath the trap with ethanol to avoid contamination. During housing, we did not manipulate squirrels in any fashion to avoid causing any added stress. All female squirrels were reproductively quiescent upon capture, while males had scrotal testes in 9 out of 14 cases. Upon completion of this study (48 h), we weighed all squirrels and returned them to the site of capture.

1.3.5 Extraction of hormone metabolites

Samples were stored at −20°C until analysis and shipped to the University of Michigan (Ann Arbor, Michigan) on dry ice. Fecal samples were then lyophilized for 14–16 h to remove moisture and then the entire fecal sample was pulverized into a fine powder using a mortar and pestle along with liquid nitrogen. Approximately 0.05 g of dry feces was weighed on an analytical balance (precision to 0.001 g) and placed into a 2-ml microcentrifuge vial. One milliliter of 80% methanol was then added to these samples and they were vortexed on a multitube vortex for 30 min (Touma et al. 2003; Palme 2005; Palme et al. 2013). The samples were then centrifuged at 2,500 × g for 15 min. Next, the supernatant was aspirated and stored in individual 1.5-ml tubes at −20°C until analysis.

1.3.6 Enzyme immunoassay

We used a 5α-pregnane-3β, 11β, 21-triol-20-1 EIA to measure FGM levels (Touma et al. 2003; Dantzer et al. 2010). This EIA detects glucocorticoid metabolites with a 5α-3β, 11β-diol structure (for cross-reactivity, see Touma et al. 2003). The same EIA has been used to measure fecal cortisol metabolite levels in North American red squirrels (*Tamiasciurus* *hudsonicus*—Dantzer et al. 2010) and has been used successfully to measure FGM in a variety of rodent species (lab mice—Touma et al. 2004; rats—Lepschy et al. 2007, 2010; Columbian ground squirrels *Urocitellus columbianus*—Bosson et al. 2009; Eastern gray squirrels *S. carolinensis*—Bosson et al. 2013; and 3 different chipmunk species—Montiglio et al. 2012a, Hammond et al. 2015).

Details of the EIA procedure can be found in Palme and Möstl (1997), Möstl et al. (2005), and Dantzer et al. (2010). Samples were assayed in duplicate, and if the intra-assay coefficient of variation between duplicates was > 15%, these samples were analyzed again. The intra-assay coefficient of variation for all samples was 6.01%. High (~52% binding) and low (68% binding) fecal extract pools were used as quality controls to measure inter-assay precision (Möstl et al. 2005). The inter-assay coefficients of variation for the high and low pooled fecal extracts were 11% and 17%, respectively (n = 9 plates). FGM levels are expressed as ln ng/g dry feces.

1.3.7 Statistical analyses

We used linear mixed-effects models (LMM) with ln-transformed FGM concentrations to meet assumptions of normality and homeoscedasticity. We first assessed the effects of captivity on the 17 squirrels that were captured and temporarily held in captivity for 48 h. During this time, we collected fecal samples approximately every 4 h except during the night (from 2200 to 0900 h). We included hour of fecal sample collection as a factor and sex in this model to determine if FGM concentrations were higher 4–48 h after capture compared to those in samples collected at initial capture (0 h post capture).

Second, we used LMM to test if FGM concentrations at the time of capture (0 h after capture) from squirrels differed from those from 1) squirrels that were held in captivity for 4–48 h and 2) wild squirrels that were never held in captivity. In both models, we included a dichotomous (or indicator) predictor variable (captive or wild squirrels) and sex of the squirrel. In these models, there was no a priori reason to expect that males and females would react to the stress of captivity differently and initial analyses indicated no sex-specific interaction between sample type (collected at initial capture, collected 4–48 h into captivity, or wild caught) and sex. Therefore, we excluded interaction terms between sex and hour in these analyses.

We determined if FGM concentrations measured in wild squirrels differed between females and males, among different reproductive conditions, the different seasons they were collected in, and a measure of body condition (body mass at capture) in 3 separate models. In 1 model for females only, we investigated if FGM concentrations differed among females that were 1) estrous or pregnant; 2) estrous, lactating, or had recently weaned offspring; or 3) those that were anestrous and not lactating (called nonbreeding hereafter). In a 2nd model for males only, we determined if FGM concentrations differed between males with abdominal or scrotal testes. In a model including both females and males, we identified whether FGM concentrations differed between the sexes, varied among 3 distinct seasons (winter [December to March], summer [May to August], or autumn [September to October]) or were associated with body mass at capture (ln transformed). There was no evidence for sex differences or effects of reproductive condition in how season of fecal sample collection or body mass was associated with FGM concentrations so we did not include interaction terms for sex or reproductive condition and season of collection and body mass.

It should be noted that our analyses of season are confounded with differences in habitat as samples collected in the winter months (n = 14) were only collected from low-elevation deciduous forests and not high-elevation coniferous forests (Table 1.1). This is because the high-elevation sites are inaccessible during the winter months and so all of our samples from the autumn and summer months (n = 97) come from the high elevation sites. When we compared our model that contained the 3-level fixed effect for season versus a separate model with the same data that contained habitat type (low or high elevation) instead of season, the models did not differ from each other as assessed with the Akaike information criterion corrected for small sample sizes (ΔAICc = 0.44—Burnham and Anderson 2002). Unequal sample sizes between the 2 habitat types (low elevation, n = 14; high elevation, n = 97) also preclude analyses for habitat type at this point. Our analyses for season should provide an indication of how FGM concentrations vary with season though this confounding factor needs to be mentioned.

FGM concentrations might be affected by factors other than the characteristics of the individual squirrels. We conducted a separate model where we included fecal sample mass as a sole predictor variable to identify if FGM concentrations were affected by mass of the fecal sample we extracted.

All analyses were performed in R version 3.2.1 (R Core Team 2015) using the lme4 package (version 1.1-8—Bates et al. 2015). For each of the models described above, we conducted LMM with FGM concentrations (expressed as ln-transformed ng/g dry feces) as the dependent variable and squirrel identity as a random intercept term to account for repeated samples on the same individuals. We estimated the unadjusted repeatability (R) of FGM concentrations for captive and free-living squirrels using the estimates of within- and among-female variance from our LMMs that did not include any fixed effects (Nakagawa and Schielzeth 2010). We considered these repeatability estimates to be statistically significant if their 95% confidence intervals (CIs) did not include 0 (estimated using parametric bootstrapping with 1,000 permutations—Nakagawa and Schielzeth 2010). Finding significant repeatability in this context suggests that some squirrels consistently differ in their FGM concentrations in response to captivity (captive squirrels) or changes in reproductive condition and season (wild squirrels). In the models for the FGM concentrations from free-living squirrels, we also included a random intercept term for year of collection to account for repeated samples in the same years (2013, n = 6; 2014, n = 37; 2015, n = 68). We assessed significance of the pair-wise comparisons either using t-tests (using Satterthwaite approximations to degrees of freedom in R package lmerTest, version 2.0-29) or Tukey's post hoc comparisons test (R package multcomp version 1.4-1—Hothorn et al. 2008).

1.4 Results

1.4.1 *Effect of temporary captivity on FGM concentrations*

FGM concentrations were influenced by time since initial capture ($F_{8,67} = 2$, $p = 0.06$). Compared to fecal samples collected at initial capture (0 h after capture, n = 17 samples, 9.83 ± 0.29 ln ng/g dry feces; Figure 1.1), squirrels that were captured and temporarily held in captivity (6 females, 11 males) had FGM concentrations that were 52.8% higher 24 h after initial capture (n = 16 samples, 10.52 ± 0.18 ln ng/g dry feces, Tukey honest significant difference [HSD], $p = 0.048$) and 28.7% higher 32 h after initial capture (n = 10 samples, 10.45 ± 0.18 ln ng/g dry feces, Tukey HSD, $p = 0.042$). Of the 12 individuals for which we had repeated samples at 0 and 24 h post capture, 9 had higher FGM concentrations 24 h post capture (Figure 1.2a). All 9 individuals for which we had repeated samples at 0 and 32 h post capture had higher FGM concentrations 32 h post capture (Figure 1.2b). FGM concentrations were also 34.3% higher 48 h after initial capture (n = 12, 10.51 ± 0.14 ln ng/g dry feces), though this was not significant (Tukey HSD, $p = 0.088$). The mean and median lag time from initial capture to peak excretion tended to be from 24 to 36 h (Figure 1.1). For those individuals for which we collected a fecal sample at initial capture and thereafter, the time to peak excretion of FGM concentrations was 29.8 ± 11.7 h (mean ± SD). There were no significant differences among the other different collection periods (Figure 1.1) and there were no sex differences in FGM concentrations ($t_{30} = 1.1$, $p = 0.31$). We obtained an average of 5 estimates of FGM concentrations from each of 17 squirrels over the 48-h period of captivity. FGM concentrations were repeatable within individual squirrels while they were in captivity (R = 0.52, 95% CI = 0.25–0.69). This can be visualized in Figure 1.2 showing that individual squirrels with higher FGM concentrations than other squirrels at initial capture (0 h) also generally had higher FGM concentrations than these same squirrels 24 and 32 h after capture.

1.4.2 *Comparing FGM concentrations of captive and wild squirrels*

FGM concentrations from captive squirrels at 0 h after capture (n = 17 samples, 9.83 ± 0.29 ln ng/g dry feces) did not differ when compared to FGM concentrations of free-ranging squirrels (n = 111, 9.81 ± 0.07 ln ng/g dry feces, $t_{126} = 0.01$, $p = 0.92$; Figure 1.3) but increased significantly when squirrels remained in confinement for 4–48 h (n = 73, 10.4 ± 0.07 ln ng/g dry feces, $t_{86} = 27.2$, $p < 0.0001$; Figure 1.3) compared to those from free-ranging squirrels.

1.4.3 *Effect of sex, reproductive condition, body mass, and season on FGM concentrations in wild squirrels*

FGM concentrations were influenced by reproductive state in females ($F_{2,45} = 3.4$, $p = 0.044$). Pregnant females (n = 10 samples) had significantly lower FGM concentrations than females that were lactating or had recently weaned their offspring (n = 14, Tukey HSD, p = 0.027; Figure 1.4). FGM concentrations in nonbreeding females (n = 27) did not differ from those that were pregnant (n = 10, Tukey HSD, p = 0.45) or lactating/recently weaned (Tukey HSD, p = 0.22). Males with abdominal (n = 33) or scrotal (n = 27) testes had similar FGM concentrations ($t_{57} = -0.96$, $p = 0.34$; Figure 1.4).

Overall, FGM concentrations varied significantly among the 3 different seasons ($F_{2,76} = 4.95$, $p = 0.009$; Figure 1.5). FGM concentrations in samples collected from squirrels during the summer (May to August) were significantly lower than those collected in the winter (Tukey HSD, p = 0.015) and tended to be lower than those collected in the autumn (Tukey HSD, p = 0.062). FGM concentrations in winter tended to be higher than those in autumn but this was not statistically significant (Tukey HSD, p = 0.41; Figure 1.5). FGM concentrations did not differ between females (n = 51 samples, 9.85 ± 0.08 ln ng/g feces) and males (n = 60 samples, 9.78 ± 0.11 ln ng/g feces, $t_{96} = 0.06$, $p = 0.81$) and there was no association between body mass at capture and FGM concentrations ($t_{96} = 0.19$, $p = 0.66$), or interaction between body mass and sex ($t_{96} = 0.06$, $p = 0.81$). FGM concentrations were not repeatable within individual free-living squirrels (R = 0.12, 95% CI = 0–0.45).

1.4.4 *Effect of fecal sample mass on FGM concentrations*

The mass of most samples we extracted was around the target value of 0.05 g (n = 201, mean ± SD: 0.047 ± 0.011 g, range = 0.009–0.056 g). Some samples were substantially lighter than our target extraction mass (n = 34 samples, 0.009–0.04 g) because that was the maximum amount of sample that we were able to collect. Despite this variation in fecal sample mass, there was no association between mass extracted and FGM concentrations in samples collected from wild and captive squirrels ($t_{199} = -0.8$, $p = 0.42$).

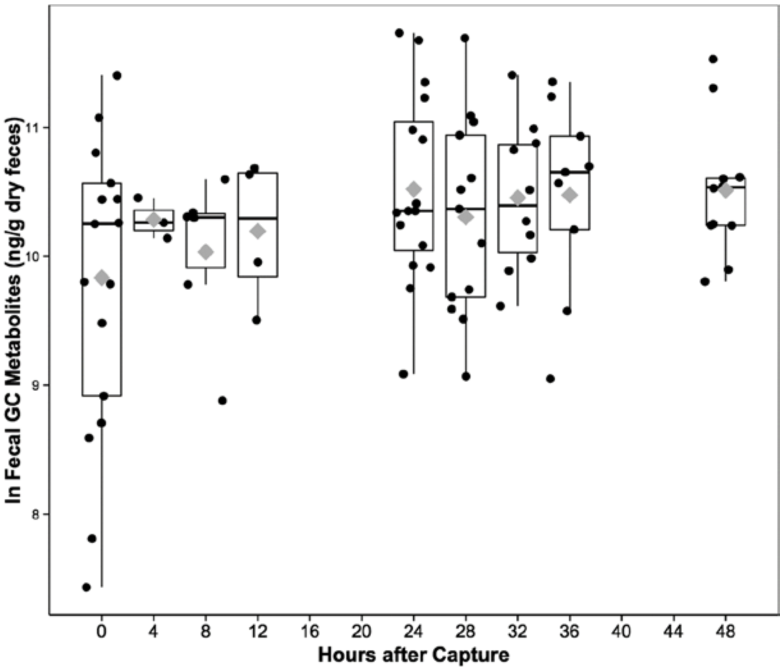


Figure 1.1 – Fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels (n = 11 males, 6 females) from initial capture (0 h after capture) to 48 h after initial capture. Fecal GC metabolite levels were significantly higher in samples collected 24 h (p = 0.047) and 32 h (p = 0.042) after initial capture. Boxplots show median (solid horizontal line), mean (gray diamond), and 1st (25%) and 3rd (75%) quartiles. Fecal samples were not collected overnight during captivity (2200–0900 h) so no data are shown for 16, 20, 40, and 44 h after capture.

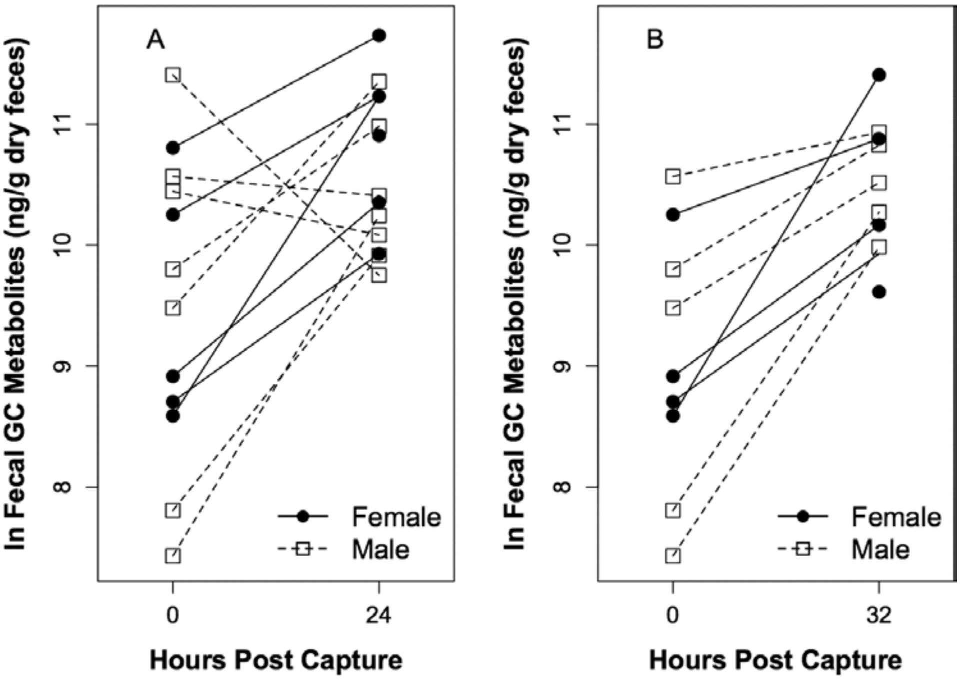


Figure 1.2 – Fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels from initial capture (0 h after capture) to A) 24 and B) 32 h after initial capture. Lines connect points of individual squirrels that were sampled at each time period.

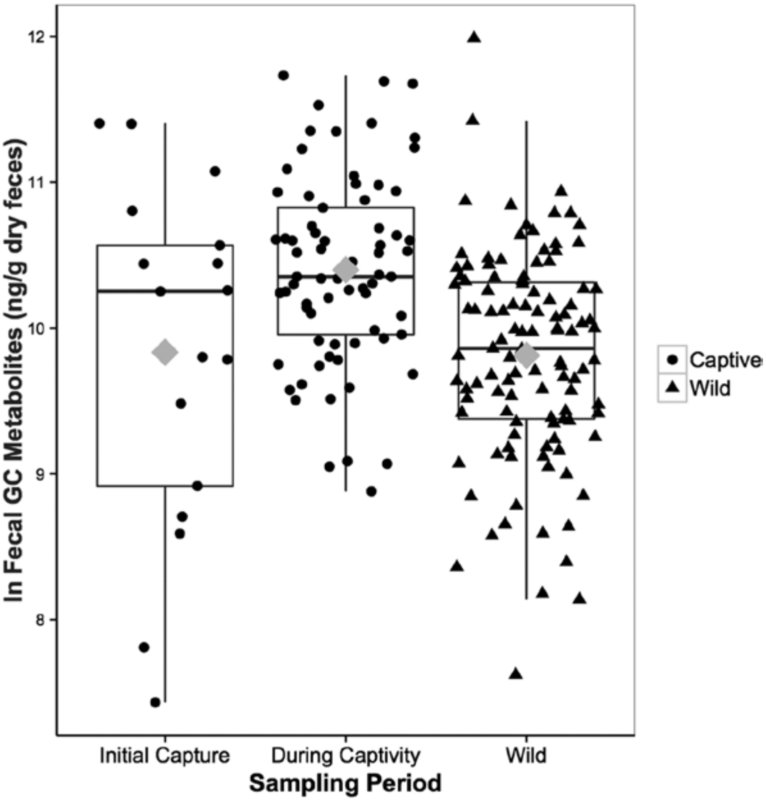


Figure 1.3 – Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels at initial capture (time 0 of capture, n = 17 samples), captive red squirrels collected 4–48 h after initial capture (n = 73 samples), and those collected from wild squirrels that were not maintained in captivity after initial capture (n = 111). Boxplots show median (solid horizontal line), mean (gray diamond), and 1st (25%) and 3rd (75%) quartiles. Some data points shown represent samples collected from the same individuals.

1.5 Discussion

We show that livetrapping and temporary capture elevated FGM concentrations in Eurasian red squirrels and that these changes in adrenocortical activity were detected through the use of our EIA using an antibody that detects glucocorticoid metabolites with 5α-3β, 11β-diol structure. FGM concentrations were significantly higher in samples collected 24 and 32 h after initial capture compared with FGM concentrations in samples collected 0 h after initial capture. Squirrels that were in captivity for 4–48 h had significantly higher FGM concentrations than those that were free-ranging and never placed into captivity. Our assay also detected significant repeatable differences in FGM concentrations among captive squirrels. In free-ranging squirrels, pregnant squirrels had lower FGM concentrations than those that were lactating or nonbreeding, whereas there were no differences in FGM concentrations between males with scrotal or abdominal testes. Using 3 years of fecal samples collected during the summer, autumn, and winter months, we show that FGM concentrations are lowest in summer and highest in the winter.

Assays to measure fecal hormone metabolites should be carefully validated (Buchanan and Goldsmith 2004; Touma and Palme 2005; Sheriff et al. 2011; Fanson et al., in press). Two caveats of this study are that we did not use an ACTH challenge and we did not test the sensitivity of our antibody to detect variation in FGM concentrations compared to other available antibodies. An ACTH challenge is often used as a pharmacological validation that a specific EIA to measure FGM concentrations can detect increases in endogenous glucocorticoid production from the adrenal glands (Touma and Palme 2005; Sheriff et al. 2011). However, biological validations, such as the use of temporary captivity in our study, are equally suited to validate an assay to measure FGM concentrations because they cause an acute stress response (Touma and Palme 2005). Capture, translocation, and temporary captivity induces profound changes in the hypothalamic–pituitary–adrenal axis (Romero and Wingfield 1999; Dickens et al. 2009, 2010). Within 2–3 min of initial capture, animals mount a physiological stress response where endogenous glucocorticoid production is increased (Kenagy and Place 2000; Romero and Reed 2005; Delehanty and Boonstra 2009) and this increase in plasma glucocorticoid levels can be sustained for the first few hours (Fletcher and Boonstra 2006; Dantzer et al. 2010) or days (Dickens et al. 2009) of captivity (an acute stressor). Previous studies have used a similar biological validation as ours showing that FGM concentrations were elevated after 4 h of captivity (Harper and Austad 2001; Bosson et al. 2013; Hämäläinen et al. 2014) compared to pre capture samples and remained elevated for the next 96 h (Hämäläinen et al. 2014). In our case, we show that capture and confinement (an acute stressor) significantly increased FGM concentrations, which indicates that our EIA can detect biologically relevant information about endogenous glucocorticoid production. Ideally both physiological (ACTH) and biological (e.g. temporary captivity) validations should be conducted in the same study but this was not possible here because the species is of conservation concern and not readily available in zoos.

We used a group-specific antibody that detects glucocorticoid metabolites with a 5α-3β, 11β-diol structure. We show that this antibody was effective in detecting significant increases in FGM concentrations in response to temporary capture. Thus, this antibody has now proven to be useful in detecting variation in FGM concentrations in a variety of rodent species (Touma et al. 2003; Lepschy et al. 2007; Bosson et al. 2009; Dantzer et al. 2010; Lepschy et al. 2010; Montiglio et al. 2012a; Bosson et al. 2013; Hammond et al. 2015). Furthermore, we found that fecal sample mass was not associated with FGM levels, and that low mass fecal samples (0.009–0.04 g) could be used to assess FGM levels. This is important as some authors report inaccurate results from samples of low mass (< 0.02 g dry weight), and in species where fecal mass may be limited it is important to validate the use of low mass samples (see Millspaugh and Washburn 2004, and references therein).

Recently, some studies have tested the efficacy of multiple antibodies to detect variation in the concentrations of some specific FGM (e.g., Heistermann et al. 2006; Montiglio et al. 2012a; Shutt et al. 2012; Hämäläinen et al. 2014; Fanson et al., in press). While valuable, in most cases, these studies conclude that each of the multiple antibodies that they have tested is useful in detecting changes in the endogenous glucocorticoid production though some antibodies are more sensitive (show stronger increases in FGM following adrenocortical activation) than others within a species (e.g. Montiglio et al. 2012a). Interestingly, studies in diverse taxa suggest that the acute stress response to capture and restraint (Manogue et al. 1975; Narayan et al. 2013; Pakkala et al. 2013) or ACTH injections (Sheriff et al. 2012) may not be as severe as exposure to natural stressor such as a predator (but see Harris et al. 2012). This suggests that the methods that are often used to physiologically (ACTH injection) or biologically (capture, restraint, translocation) validate assays to measure FGM concentrations (Touma and Palme 2005; Sheriff et al. 2011) may produce subtler increases in FGM concentrations than variation in the factors (food and predator abundance, competition, climate, infection status) most workers are interested in. Because our assay is detecting changes in glucocorticoids in response to capture, this suggests that our assay should detect changes in FGM concentrations of Eurasian red squirrels in response to variation in these ecological factors.

We were not able to directly measure gut passage time in this study but we did find that peak FGM concentrations occurred 24–36 h after initial capture. In most individuals, FGM concentrations were significantly higher 24 h after initial capture. This differs from some previous studies in different squirrel species showing that the time until peak excretion of FGM was 8–12 h following an ACTH challenge (Dantzer et al. 2010; Montiglio et al. 2012a; Sheriff et al. 2012) or handling stressor (Dantzer et al. 2010). However, several recent studies in other squirrel species also found that FGM concentrations peaked 24 h (Hammond et al. 2015) or 16–40 h (Bosson et al. 2013) after ACTH injection. It is also notable that in our study, FGM concentrations were higher 4, 8, and 12 h after initial capture but these were not statistically significant. This suggests that an acute stress response that elevates endogenous glucocorticoid production in Eurasian red squirrels should be evident in the feces at least 24 h later if not earlier. This also indicates that FGM concentrations measured in fecal samples collected within 4–48 h after capture will be biased by the previous capture and handling event. Because squirrels were continuously held in captivity for 48 h (likely a more chronic form of stress), whereas wild-caught squirrels are only captured and then released, it is likely that FGM concentrations in wild-caught squirrels that are released after capture return to normal more quickly than 48 h after initial capture.

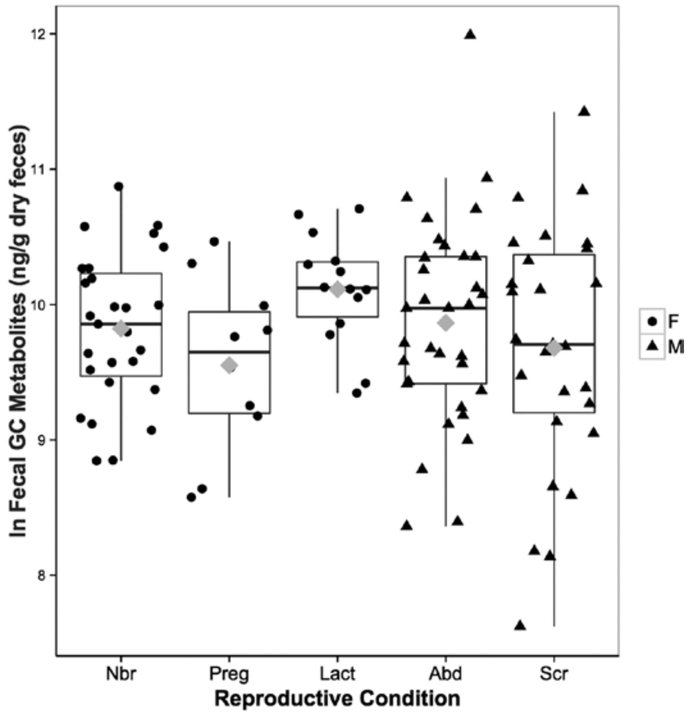


Figure 1.4 – Effects of reproductive condition on fecal glucocorticoid (GC) metabolite concentrations in wild-caught Eurasian red squirrels. Fecal GC metabolite concentrations were measured in samples from pregnant females or those in estrous (n = 10, “Preg”), females who were lactating or had recently weaned their offspring (n = 14, “Lact”), nonbreeding females (n = 27, “Nbr”), and males with abdominal (n = 28, “Abd”) or scrotal (n = 32, “Scr”) testes. Symbols show females (“F”) and males (“M”). Boxplots show median (solid horizontal line), mean (gray diamonds), and 1st (25%) and 3rd (75%) quartiles. Some data points shown represent samples collected from the same individuals.

We found no sex differences in FGM concentrations in captive or wild squirrels and no sex difference in the effects of temporary captivity on FGM concentrations. Differences in plasma glucocorticoid levels can be caused by a variety of factors including the effects of gonadal steroid hormones and sex hormone binding globulins (Handa et al. 1994; Cavigelli et al. 2005; Lepschy et al. 2007; Weiser and Handa 2009). Sex differences in FGM concentrations could be caused by a variety of factors such as sex differences in the metabolism and excretion of hormone metabolites (Touma et al. 2003; Millspaugh and Washburn 2004; Cavigelli et al. 2005; Goymann 2005; Palme et al. 2005; Goymann 2012). While sex differences in FGM concentrations have been shown in some species, they have not been found in others and the results are far from consistent (Touma and Palme 2005; Goymann 2012; Harris et al. 2012). We note that the lack of sex differences in FGM concentrations in our study and in others could likely be due to not having enough samples around the period of time when sex differences in plasma glucocorticoid levels are most obvious such as when females are pregnant or lactating compared to nonbreeding males or females (Kenagy and Place 2000; Boonstra et al. 2001; Reeder et al. 2004; Edwards and Boonstra, 2016). Similarly, the lack of resolution on pregnancy stages in wild animals may obscure any general differences between females and males. For example, measurement of FGM concentrations in females in the early and middle stages of pregnancy may be quite similar to those from nonbreeding males, whereas there may be vast differences between the sexes if the samples were collected from females in the late stages of gestation (Dantzer et al. 2010).

FGM concentrations were repeatable among captive but not free-ranging squirrels. Repeatability in the context of our method of measurement suggests that some squirrels in captivity consistently had higher FGM concentrations across the different sampling periods than others. Wild squirrels did not exhibit significant repeatability of FGM concentrations across changes in reproductive conditions or seasons though the latter may be due to low statistical power (Wolak et al. 2011; but see Bell et al. 2009) given that we obtained < 2 fecal samples for 82 different free-living squirrels (111 total samples). Our results from captive squirrels adds to the growing number of studies showing significant repeatability of either plasma or fecal glucocorticoid levels (Cockrem et al. 2009; Dantzer et al. 2010; Ouyang et al. 2011; Fletcher et al. 2015).

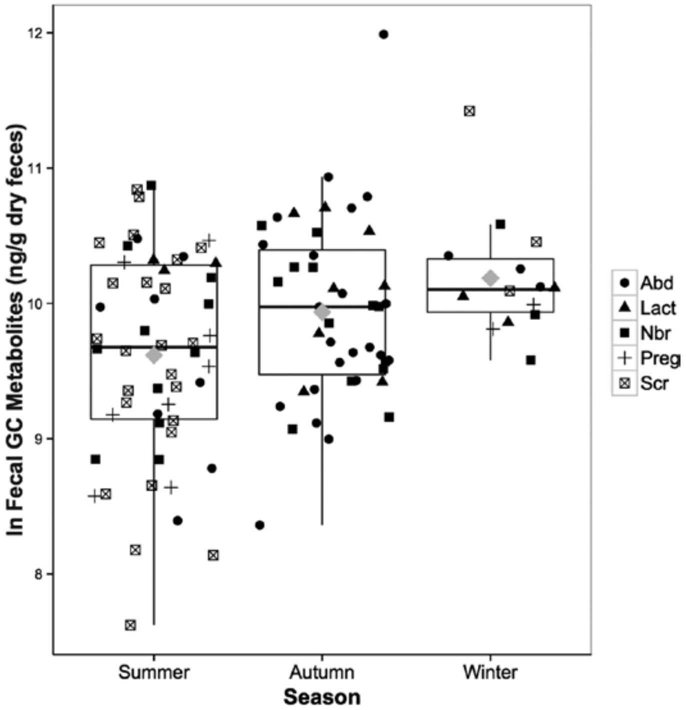


Figure 1.5 – Fecal glucocorticoid (GC) metabolite concentrations in female and male Eurasian red squirrels captured in the summer (n = 54, May– August), autumn (n = 43, September–October), and winter (n = 14, December–March). Boxplots show median (solid horizontal line), mean (gray diamonds), and 1st (25%) and 3rd (75%) quartiles. Symbols show reproductive condition for females that were pregnant ("Preg"), lactating ("Lact"), or nonbreeding ("Nbr") and for males that had abdominal ("Abd") or scrotal ("Scr") testes. Some data points shown represent samples collected from the same individuals.

FGM concentrations were highest in females that were lactating or recently finished lactating, whereas they were lowest in pregnant females. Lactation is one of the most energetically demanding activities exhibited by mammals (Naya et al. 2008; Fletcher et al. 2012) and increased production of glucocorticoids may help to mobilize energy stores to facilitate lactation (Kenagy and Place 2000; Romero 2002; Romero and Wingfield 2015). Thus, our results suggest that biologically relevant increases in energetic-expenditure (lactation) can be detected in Eurasian red squirrels by measuring FGM concentrations. Why FGM concentrations were lowest in pregnant females is not clear but other studies in different squirrel species have also found that females have higher FGM concentrations when they are lactating compared to when they are pregnant (Aschauer et al. 2006). This is also possibly related to the low sample sizes (n = 10) we had compared to those from nonbreeding or lactating/postlactating females. We also found no difference in FGM concentrations between males with scrotal or abdominal testes. Although we assume that males with scrotal testes are in breeding condition, it is possible that we would recover some difference in FGM concentrations between breeding and nonbreeding males if we had measured these in samples collected during the peak breeding season (when males were actively mating) and nonbreeding season. Of course, in all these cases, it is important to mention that changes in concentrations of fecal hormone metabolites with reproductive condition could reflect a real biological phenomenon (i.e., matching a change in circulating hormone levels). Alternatively, these could reflect a change in metabolism or gut passage time that occurs with differences in reproductive condition (Goymann 2012).

We observed substantial seasonal changes in FGM concentrations where they increased from the spring/summer (May to August) until the winter months (December to March). Autumn trapping coincided with the autumn dispersal peak of both subadult and adult red squirrels, while during winter months animals were engaged in mating chases and/or, for some females, had started lactating (Wauters and Lens 1995; Wauters et al. 2008, 2010). Thus, winter FGM concentrations may have been elevated because the squirrels were breeding (Romero 2002). However, there is not a general pattern in mammals as glucocorticoid levels are elevated during the breeding season compared to the pre- or post-breeding seasons in some species (Vera et al. 2013; Corlatti et al. 2014; Jachowski et al. 2015) but not in others (Place and Kenagy 2000; Romero et al. 2008; Delehanty and Boonstra 2011; Bauer et al. 2014; Fletcher et al. 2015). Although speculative, increased FGM levels in autumn and winter could thus be related to more frequent intraspecific interactions linked to dispersal, higher predation risk when foraging on the ground and reduced food quality and/or more extreme weather conditions (cold, snow cover) in winter. In autumn and winter, Eurasian red squirrels tend to forage more on the ground to cache tree seeds (September to October—Wauters and Casale 1996) which could conceivably increase their risk of predation. Also, late winter may be a period of food shortage, at least in some years, with squirrels feeding more often on poor-quality (low energy and higher fiber content) resources, such as buds and fungi growing under the bark of dying or injured branches (Lurz et al. 2005). In contrast, during summer, Eurasian red squirrels forage mainly in the tree canopy on flowers and maturing seeds. The seasonal changes we observed in FGM concentrations may also be caused by changes in diet (Dantzer et al. 2011). An alternative explanation is presented by a confounding factor in our study. Specifically, 2 of our sites were in low-elevation deciduous forest, whereas 3 of our sites were in high-elevation coniferous forests (Table 1.1). Because the high-elevation sites were inaccessible during the winter months, we were unable to collect fecal samples from squirrels in these areas during the winter. As such, the seasonal changes we observed could be due to differences between squirrels inhabiting these different types of habitats, though at present we cannot address this without further study where samples are collected from the 2 different types of habitats during the autumn, winter, and summer months.

In conclusion, we validate an EIA to measure FGM concentrations in Eurasian red squirrels by showing that capture and temporary captivity significantly elevate FGM concentrations and that this assay can detect changes in FGM concentrations in response to changes in reproductive condition or seasonal/ habitat factors. As in other species, this assay will be useful to detect how ecological factors induce physiological stress in wildlife and associations between measures of physiological stress and survival and reproduction (Bonier et al. 2009). We expect this assay will prove to be particularly useful in understanding whether the presence of an invasive species (gray squirrels, *S. carolinensis*) are causing stress in Eurasian red squirrels that are contributing to their decline and local extinction throughout parts of Europe (Gurnell and Pepper 1993; Martinoli et al. 2010; Bertolino et al. 2014).

Chapter 2

Stress in biological invasions: alien grey squirrels increase physiological stress in native Eurasian red squirrels

2.1 Summary

This Chapter investigates possible increases in physiological stress in a native species (Eurasian red squirrel, *Sciurus vulgaris*) in relation to the presence of an invasive species (Eastern grey squirrel, *Sciurus carolinensis*). Extraction of glucocorticoid metabolites from fecal samples, and their subsequent measurements with assay described in Chapter 1, showed that native species glucocorticoid concentrations were three times higher in sites where they co-occurred with invasive grey squirrels compared to sites without the invasive species. Moreover, stress hormones in native species increased after colonization by grey squirrels and decreased in relation to experimental reduction of grey squirrels abundance. Overall, total squirrel density (both red and grey) or differences in the intensity of intraspecific competition (red squirrel density) did not influence FGM concentrations, allowing to exclude density of arboreal squirrels (independently from the species they belong to) as source of variation in native species FGM concentrations. This Chapter shows that the invasive species acts as a stressor for native red squirrels, but the mechanisms by which its presence influences native species variation in FGM concentrations are still unknown.

2.2 Introduction

The introduction of non-native species that subsequently become invasive (invasive alien species, IAS) can cause large economic losses and affect human activities and health (Simberloff et al. 2013). Moreover, IAS represent a threat to native species worldwide through different ecological processes (Keller et al. 2011), such as interspecific competition (e.g. Cadi and Joly 2003; Gurnell et al. 2004), predation (Berger et al. 2007; Banks and Dickman 2007), transmission of infectious diseases (Daszak et al. 2000; Strauss et al. 2012), and even changes in ecosystem functioning (Ehrenfeld 2010; Strayer 2012). Such detrimental effects can lead to extinction of native species and consequent loss of biodiversity (Clavero and García-Berthou 2005; Keller et al. 2011). For example, the introduction of alien species in a new environment can produce high levels of niche overlap with ecologically similar native species, and competition for key resources (Glen and Dickman 2008; Mazzamuto et al. 2017), that can ultimately result in exclusion competition and extinction of the native species (Mooney and Cleland 2001; Broennimann et al. 2007).

Interspecific interactions may also impact populations through sublethal individual-level effects (Boronow and Langkilde 2010; Anson et al. 2013), that cause an increase in physiological stress (acute or baseline stress). In fact, animals have a suite of behavioral, hormonal and physiological mechanisms to cope with harmful environmental stimuli (stressors, Romero 2004). The two most important physiological responses are the stimulation of the sympathetic nervous system (resulting in the release of catecholamines) and the activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in the secretion of glucocorticoids (GCs), lasting several minutes to hours, which helps to restore homeostasis (Sapolsky et al. 2000). However, while the occurrence of short-term elevated GC concentrations (acute stress) can help an individual to escape from life-threatening situations (Wingfield et al. 1998), chronic activation of the HPA axis and elevated GC concentrations over a longer period of time (several weeks-months) may have negative effects on fitness (body condition, survival, and/or reproductive output; e.g. Sapolsky et al. 2000; Narayan et al. 2015; Jessop et al. 2015). If the stressor persists and GCs remain elevated, alterations of behavior and/or energy balance, inhibition of growth and/or reproduction (Cabezas et al. 2007; Sheriff et al. 2009), increase in blood glucose levels, suppression of digestion (Caso et al. 2008) and suppression of immunity and the inflammatory response (Romero 2004; Raouf et al. 2006; St. Juliana et al. 2014) can be observed. Competitive food resource exploitation (Chase et al. 2016), parasite-mediated competition (Raouf et al. 2006; St. Juliana et al. 2014), introduction of (alien) predators (Berger et al. 2007; Anson et al. 2013), and/or invasive competitors (Boronow and Langkilde 2010; Narayan et al. 2015) are all documented cases of direct and/or indirect interspecific interactions, that show, through different mechanisms, how negative stimuli lead to increased physiological stress in at least one of the species involved.

In many vertebrate species, the amplitude and duration of the chronic stress response correlates with the overall health of an animal (Boonstra et al. 1998; Martin 2009). Consequently, the so-called baseline GC concentrations, which in wild mammals are measured primarily using concentrations of fecal glucocorticoid metabolites (FGM levels; e.g. Möstl and Palme 2002; Millspaugh and Washburn 2004; Sheriff, Dantzer et al. 2011) are increasingly used in ecological and conservation studies as indices of animal well-being. Hence, measuring these hormones (stress response) in individuals of a native species in situations with and without the alien species (the environmental stressor) can help us understand if and how the stressor affects animals in native species' populations (Dantzer et al. 2014).

Although there are many studies on the impact of IAS on native species, only a few have used glucocorticoid metabolites (before and after the stressor) to investigate the degree of physiological impact of invasive predators on native species (frogs, Narayan et al. 2013; Narayan et al. 2015; lizards or iguanas, Berger et al. 2007; Graham et al. 2012; Anson et al. 2013; Jessop et al. 2015).

Here, we examined whether the presence of an invasive species causes an increase in physiological stress in individuals of a native species. We use the well-known case of competitive replacement of the Eurasian red squirrel (*Sciurus vulgaris*) by the introduced invasive Eastern grey squirrel (*Sciurus carolinensis*) in Europe to explore effects of stress at the individual level using fecal glucocorticoid metabolites (FGM; Sheriff et al. 2011; Dantzer et al. 2014; Dantzer et al. 2016; Haigh et al. 2017). Tree squirrels are often successful invaders (Bertolino 2009; Di Febbraro et al. 2016), and the rapid spread of the invasive grey squirrel and subsequent decline and widespread extinction of the native red squirrel on the British Isles and in parts of Italy has been documented in many studies. The two species have similar space use and activity patterns, and compete for food resources, resulting in smaller body size, and reduced female reproduction and juvenile recruitment in red squirrels (Wauters et al. 2002a; Wauters et al. 2002b; Gurnell et al. 2004), ultimately causing a decrease in population size and (local) extinction (Bertolino et al. 2014). On the British Isles, a squirrel pox virus determines disease-mediated competition, with grey squirrels acting as a reservoir and transmitting the virus to red squirrels, for which, in most cases, the infection is lethal (Mc Innes et al. 2013; White et al. 2016).

We first compared FGM concentrations in individual red squirrels co-occurring for more than two years with grey squirrels (red-grey sites), with FGM concentrations of squirrels in sites not colonized by the alien species (red-only sites). If native red squirrels did not become sensitized or habituated to this threat, we predicted that FGM concentrations in red squirrels would be higher in red-grey than in red-only sites. However, since the correlative nature of this approach makes it difficult to isolate the invader as the causal factor driving observed differences (Graham et al. 2017), we also tested the direct impact of interactions between invasive and native species. We did this in two ways: (1) monitoring changes in FGM concentrations of red squirrels in two study sites that were colonized by the invader during this study (measuring FGM concentrations before and after colonization) and comparing them with variation in FGM that occurred over the same time-period in non-colonized red squirrel populations; and (2) removing grey squirrels in red-grey sites over a period of six months, and concomitantly monitoring changes in FGM concentrations in native red squirrels. If the invader causes an increase in FGM concentrations in the native species (i.e. the presence of grey squirrels is the driving factor), we predicted that: FGM concentrations will be higher in red squirrels after the two study sites were colonized than before the colonization by the alien species (experiment 1); and FGM concentrations in syntopic red squirrels (red-grey sites) will decrease after the removal of the grey squirrels (experiment 2).

2.3 Materials and methods

2.3.1 Study sites

We trapped Eurasian red squirrels in five study sites without grey squirrels (historically red-only sites) in Lombardy, North Italy (Table S1). Two of these sites (Vanzago, Castelbarco) were colonized by the alien species during the study; hence they were used as red-only sites before colonization (respectively before March 2015 and November 2014), and as red-grey sites afterwards. We trapped both squirrel species (red-grey sites) in seven study sites in Lombardy and Piedmont, Northern Italy (Table S1). All red-grey sites and the two red-only sites mentioned above (Vanzago, Castelbarco) are lowland mixed deciduous woodlands in the Po plain, while the other three red-only sites (Bormio, Cancano, Valfurva) are subalpine conifer forests in the Central Italian Alps (1620-2150 m elevation).

2.3.2 Live-trapping, handling and fecal sample collection

We trapped squirrels in all sites during trapping sessions that lasted four or five days each (sites and trapping dates listed in Table S1). We used Tomahawk traps (model 202, Tomahawk Live Trap, WI, USA) with a fine mesh added underneath traps to prevent contamination between urine and feces. We checked traps two to three times a day. Each trapped squirrel was individually marked using numbered metal ear-tags (type 1003 S, National Band and Tag, Newport, KY, USA). We weighed squirrels to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland) and measured the length of the right hind foot (without nail, 0.5 mm) with a ruler (Wauters et al. 2007). A female’s reproductive status was defined as non-breeding (anestrous, vulva small, no longitudinal opening, not lactating), post-estrous, and pregnant (vulva partly or strongly swollen with longitudinal opening, enlarged belly during late pregnancy), or lactating (nipples large, milk excretion can be stimulated). We recorded reproductive condition of males (testes size and position) as non-breeding (testes abdominal or semi-scrotal and scrotum small) or breeding (testes scrotal and scrotum large). We used the minimum number of animals known to be alive from trapping and observations (MNA, see also Wauters et al. 2008) during each trapping session as an estimate of population size and squirrel density. Previous studies showed that MNA estimates were strongly correlated with estimates from capture-mark-recapture models (e.g. MARK, Wauters et al. 2008).

To test effects of removal of the alien species on FGM concentrations in co-occurring native red squirrels we analyzed samples from four study sites where all grey squirrels that were trapped over three subsequent periods (one period every six to ten weeks between November 2015 and May 2016) were removed. If the number of grey squirrels removed in period i is mi then m1 + m2 + m3 = mtot the total number of grey squirrels removed over the entire experiment. Red squirrel fecal samples collected in period 1 corresponded with high grey squirrel densities (mtot), those collected in period 2 with intermediate densities of grey squirrels (m2 + m3), and those collected in period 3 with the lowest grey squirrel densities (m3) (see Table 2.1). We used mtot to estimate the minimum number of animals known to be alive (MNA) of the alien species, considering that no immigration or recruitment of juveniles occurred during the removal period. Removal of grey squirrels was part of the LIFE09 NAT/IT/000095 EC-SQUARE project: animals were euthanized by CO2 inhalation, following the EC and AVMA guidelines (Close et al. 1996, 1997; Leary et al. 2013).

After capture and handling, fecal samples were collected from underneath the traps using forceps, and placed individually into 1.5 mL vials (Dantzer et al. 2010) and the fine mesh and ground under the traps were cleaned to remove any remaining fecal material. We only used fecal samples from squirrels that had not previously been trapped or handled within 72 h prior to capture to minimize effects of capture stress on FGM concentrations (Dantzer et al. 2016). We obtained multiple samples in different seasons/periods from most squirrels, but not all captured individuals produced feces within a given trapping session. Each fecal sample was classified as being taken in the morning (10.00 – 13.00h) or in the afternoon (15.00 – 18.00h) to account for potential variation in FGM concentration over the 24 h cycle (Millspaugh and Washburn 2004). We placed fecal samples into an insulated bag with ice packs while in the field, and samples were stored at -20°C within 3-4 h after collection, which should not cause temperature-induced changes in fecal hormone metabolite concentrations (Dantzer et al. 2010). Trapping and handling of squirrels complied with the current laws on animal research in Italy, and was carried out with permission of the Region of Lombardy (Decree n. 11190 of 29/11/2013). All of these procedures abided by ASM guidelines (Sikes and Gannon 2011).

2.3.3 Extraction of hormone metabolites and enzyme immunoassay

Methods of extraction of FGM and enzyme immunoassay validation for red squirrels and grey squirrels are described in detail elsewhere (Bosson et al. 2013; Dantzer et al. 2016). Briefly, samples were lyophilized overnight, ground up under liquid nitrogen and weighed to 0.05 g (± 0.004 g), and extracted using 80% methanol, shaking at 1500 r.p.m. on a multivortex for 30 min, centrifuging at 2500 g for 15 min, and aspirating the supernatant. Supernatants were diluted in assay buffer and assayed using a 5α-pregnane-3β, 11β, 21-triol-20-one enzyme-immunoassay (EIA) which detects glucocorticoid metabolites with a 5α-3β, 11β-diol structure (for cross-reactivity see Touma et al. 2003) to measure FGM concentrations (ng/g dry feces, Dantzer et al. 2010, 2016). Samples were analyzed in duplicate. We used data already published by Dantzer et al. (2016) (n = 125), and 193 new fecal samples were assayed on a total of 22 EIA plates. Pools of grey squirrel fecal extracts were used as intra-assay controls at dilutions of 1:50 (~30% binding) and 1:400 (~70% binding). Average intra-assay coefficients of variation (CVs) were 9.5% and 9.4% respectively for pools diluted 1:50 and 1:400. Inter-assay CVs were estimated from standards of known concentration with a high (n = 22 plates, 12.3% binding) and low (n = 22 plates, 81.6% binding) concentration that had inter-assay CVs of 16.1% and 9.3%, respectively.

2.3.4 Statistical analyses

All analyses were performed in R version 3.3.3 (R Development Core Team, 2017) using the lme4 package (version 1.1-12, Bates et al. 2015). For each of the models described, we conducted linear mixed-effects models with FGM concentrations (transformed using the natural logarithm, ln of ng/g dry feces) as the dependent variable and squirrel identity (ID) nested in study site as a random intercept term to account for repeated samples on the same individuals. Sex and reproductive condition nested in sex were added as fixed effects to account for potential changes in FGM concentrations with reproductive activity in males and females (Goymann 2012; Dantzer et al. 2016). Residuals were visually inspected to verify the assumptions of normality and homoscedasticity (Zuur et al. 2010). Where necessary, we assessed significance of pair-wise comparisons using differences of least square means (DLSM) with Satterthwaite approximations to degrees of freedom in R package lmerTest (version 2.0-33, Kuznetsova et al. 2016). All full models (see below) contained interactions. When interactions were not significant and including them did not improve model fit (ΔBIC between model with and without interactions > 2.0; Schwarz and Gideon 1978), they were removed from the models to obtain reliable parameter estimates for the fixed effects.

2.3.5 Comparisons between red-only vs. red-grey sites (Observational data)

We first assessed the direct effects of invasion status (sites with only red squirrels vs. sites with both red and grey squirrels) including also season (winter [December to March], spring-summer [April to August], or autumn [September to November]) and daytime (animal sampled in morning or afternoon) as factors, and body mass as a continuous variable. We also tested the interaction between sex and invasion status to explore whether the effect of the presence of grey squirrels on FGM concentrations in red squirrels differed between the sexes. Study site nested in invasion status was added as random intercept to account for potential differences in FGM concentrations between sites. Since sites with only red squirrels occurred in coniferous as well as in deciduous forests, we also explored the effect of habitat type on FGM concentrations (models and results in Supplementary material).

Table 2.1 – Data of the grey squirrel removal experiment in four study sites with both squirrel species. Period = capture period with removal (see methods); Mean Number Alive (MNA) of red squirrels (density/ha between brackets); Estimated number of grey squirrels present at the start of each removal period (density/ha between brackets); Number of grey squirrels removed during each capture period, mi, (the cumulative% grey squirrels removed at the start of the capture period, hence this is 0% for Period 1). ln FGM (ng/g dry feces) = changes in the concentration of FGM (ln FGM, mean ± SD, sample size between brackets) over time (period) in red squirrels, following removal of alien grey squirrels.

Period	Red squirrels MNA (N/ha)	Grey squirrels present (N/ha)	Grey squirrels removed (m_i) (%)	ln FGM (ng/g dry feces) red squirrels (n)
Vanzago (74.73 ha)				
Period 1 (January 2016)	16 (0.21)	18 (0.24)	12 (0)	(13) 10.890 ± 0.996
Period 2 (March 2016)	19 (0.25)	6 (0.08)	5 (67)	(19) 10.284 ± 1.051
Period 3 (May 2016)	16 (0.21)	1 (0.01)	1 (94)	(12) 10.372 ± 1.176
Lambro (18.43 ha)				
Period 1 (November 2015)	6 (0.33)	54 (2.93)	24 (0)	(5) 10.441 ± 0.467
Period 2 (December 2015)	5 (0.27)	30 (1.63)	11 (44)	(5) 10.170 ± 0.942
Period 3 (March 2016)	6 (0.33)	19 (1.03)	19 (65)	(6) 9.321 ± 0.887
Passatempo (18.33 ha)				
Period 1 (November 2015)	9 (0.49)	22 (1.20)	9 (0)	(8) 10.958 ± 1.234
Period 2 (March 2016)	10 (0.55)	13 (0.71)	12 (41)	(9) 10.642 ± 1.417
Period 3 (May 2016)	11 (0.60)	1 (0.05)	1 (95)	(11) 10.352 ± 0.918
Castelbarco (65.86 ha)				
Period 1 (December 2015)	21 (0.32)	44 (0.67)	10 (0)	(12) 10.361 ± 0.715
Period 2 (January 2016)	19 (0.29)	34 (0.52)	17 (23)	(14) 9.922 ± 0.964
Period 3 (March 2016)	25 (0.38)	17 (0.26)	17 (61)	(17) 10.392 ± 0.919

2.3.6 Effect of colonization by grey squirrels

We had two study sites (45 km apart) that were colonized by the alien species during the study, and we explored whether FGM concentrations in red squirrels changed after the colonization (samples collected between 6-10 months after the pre-grey sample collection, see also Table S1). We also used data of three non-colonized study sites (Valfurva, Bormio and Cancano, see Table S1) that were monitored over the same time-period as control data to test if differences in FGM were simply time-related. We investigated the effects of colonization (yes or no), time-period (before vs. after, the dates of colonization), and their interaction, and included sex, reproductive condition nested in sex, daytime and body mass in the full model. Study site nested in colonization status was added as a random intercept to account for potential differences in FGM concentrations between sites.

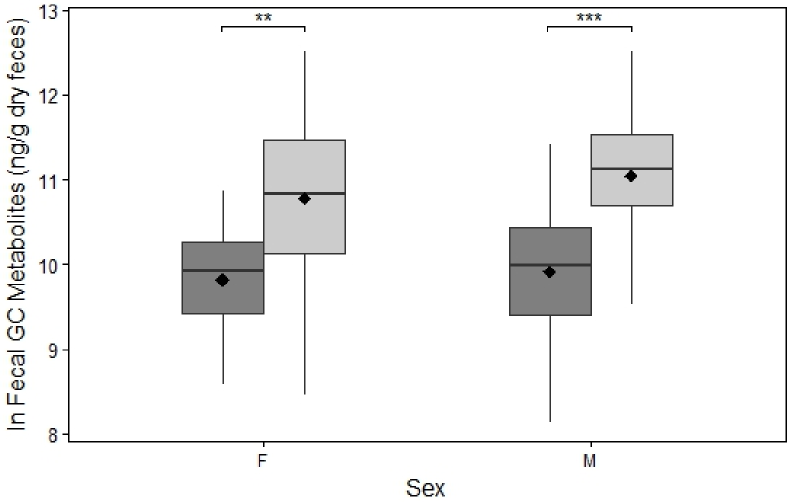


Figure 2.1 – Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels between red-only (dark-grey color; n = 125, 57 females, 68 males) and red-grey (light-grey color; n = 135, 57 females, 78 males) study sites per sex (F = females; M = males). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. ** p < 0.01, *** p < 0.001.

2.3.7 Removal experiment

We assessed the effects of grey squirrel removal considering treatment period (initial sampling when no grey squirrels had been removed yet = period 1; intermediate sampling, with a low proportion of grey squirrels removed = period 2; final sampling with a higher proportion of grey squirrels removed = period 3; see methods and Table 2.1), study site, sex, reproductive condition nested in sex and daytime as factors, and body mass as an explanatory variable, and two factor interactions between the period by study site, and period by sex, to explore whether the removal of grey squirrels would result in a significant decrease in FGM concentrations in sympatric red squirrels. In order to account for the fact that the observed differences in FGM concentrations between removal periods were influenced by seasonal variation, we also explored a model in which we used season as a fixed effect instead of removal period (both factors could not be fitted in a single model because of problems with collinearity, Zuur et al. 2010). We used the difference in BIC value to test which model best fitted the data (Schwarz and Gideon 1978).

2.3.8 Testing effect of density

In order to account for possible effect of squirrel density (individuals/hectares) on the average FGM concentration of red squirrels, we tested the correlation between native species mean FGM concentration (expressed as ln transformed ng/g dry feces) per study site (see Table S1), for both red-only and red-grey sites, and density of red squirrel, grey squirrel and both species together. For the removal experiment, we also checked the correlations between mean FGM concentration and red squirrel, grey squirrel and total squirrel density (both species) for the three periods of grey squirrel removal per study site (Table 2.1).

2.4 Results

2.4.1 FGM concentrations of red squirrels in red-only vs. red-grey sites (Observational data)

Although statistical analyses were based on ln transformed values of FGM concentrations, we here reported also the not transformed values to show the order of magnitude of the differences between sites. FGM concentrations in native red squirrels (260 samples from 166 different animals) were three times higher in sites that contained invasive grey squirrels (n samples = 135, mean ± SD = 78133 ± 61074 ng/g dry feces) than in sites that only contained native red squirrels (n = 125, 24890 ± 20566 ng/g dry feces; estimate of the invasion status effect on ln FGM 0.97 ± 0.20; $t_8 = 4.85$; $p = 0.0012$). There was no evidence that the presence of invasive grey squirrels impacted the FGM concentrations of the two sexes differently (sex by invasion status interaction $t_{165} = 0.98$; $p = 0.33$; Figure 2.1). FGM concentrations did not differ between the sexes (estimate males against females on ln FGM -0.04 ± 0.25; $t_{210} = 0.18$; $p = 0.86$), or with reproductive condition (males: breeding compared to non-breeding 0.06 ± 0.16; $t_{247} = 0.40$; $p = 0.69$; females pregnant compared to lactating -0.12 ± 0.23; $t_{249} = 0.52$; $p = 0.60$; pregnant compared to non-breeding -0.34 ± 0.23; $t_{226} = 1.50$; $p = 0.14$). Native red squirrels had lower FGM concentrations in spring-summer than in autumn (spring-summer against autumn estimate -0.33 ± 0.14; $t_{220} = 2.39$; $p = 0.018$; all other comparisons $p > 0.05$). FGM concentrations did not vary significantly with daytime (hour of fecal sample collection, $t_{223} = 0.76$; $p = 0.45$) or squirrel body mass ($t_{226} = 0.30$; $p = 0.76$).

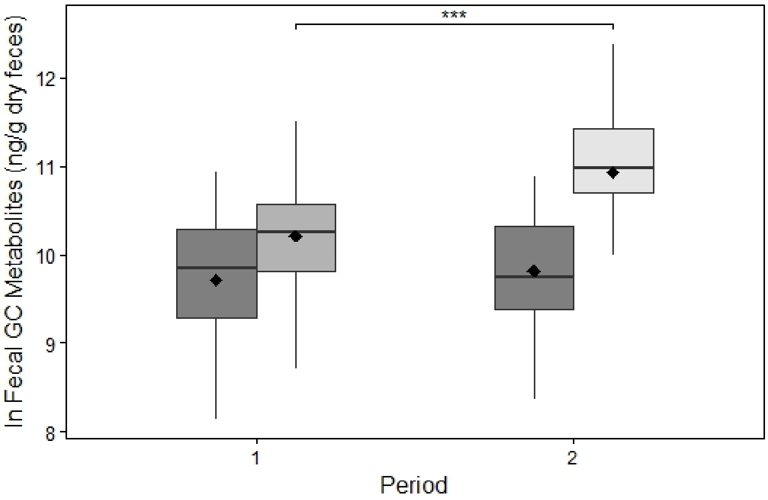


Figure 2.2 – Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels between pre-colonization (period 1, grey color, n = 29) and post- colonization (period 2, light-grey color, n = 58) of sites invaded by the grey squirrels, and over the same time-period in areas not colonized by the invasive species (period 1, dark-grey color, n = 34; period 2, dark-grey color, n = 62). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. *** p < 0.001.

2.4.2 Effect of colonization by grey squirrels

FGM concentrations in red squirrels increased with time-period and with colonization status, and there was a significant time by colonization status interaction (Table S2). Red squirrels in the two sites colonized by the alien species during our study (29 samples before, 58 samples after colonization) had a significant increase in FGM from the pre- to post-colonization period (difference in ln FGM estimate 0.74 ± 0.18; $t_{173} = 3.93$; $p = 0.0004$; Figure 2.2). In contrast, red squirrels from the three sites without colonization did not show a significant increase in FGM concentrations over the same time-period (34 samples in first period, 62 samples in second period, difference in ln FGM estimate 0.21 ± 0.15; $t_{152} = 1.27$; $p = 0.21$; Figure 2.2). In this dataset there was no statistically significant effect of sex, reproductive condition nested in sex, daytime, or body mass on FGM concentrations (Table S2).

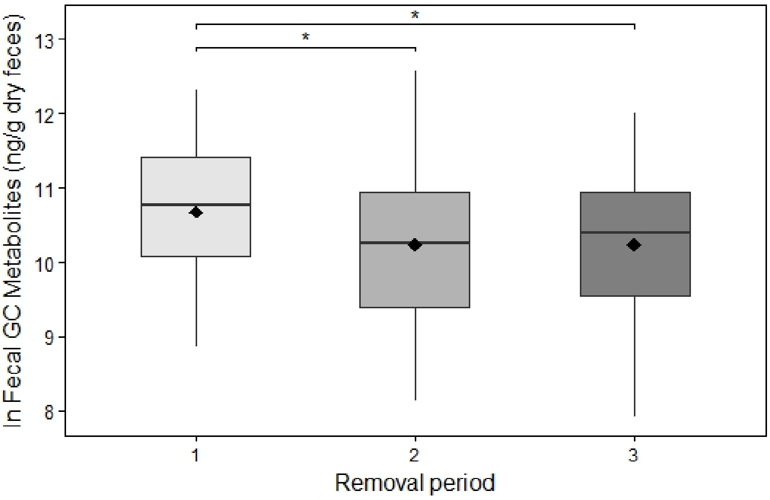


Figure 2.3 – Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels in trapping period 1 (high grey squirrel density, light-grey color), period 2 (with lower numbers of grey squirrels, grey color) and period 3 (low grey squirrel density, dark-grey color). During the removal experiment 131 samples of 67 different red squirrels were collected: 38 at the start of removal (period 1), 47 during period 2 and 46 samples during period 3. Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. * $p < 0.05$.

2.4.3 Removal experiment

After the removal of alien squirrels, FGM concentrations in co-occurring red squirrels decreased significantly in periods 2 and 3 compared to FGM concentrations at the start of the experiment (period 1, Figure 2.3 and Table 2.1). Interactions between period and study site and between period and sex were not significant, and were removed from the model (Table S3). Daytime and body mass also did not influence FGM concentrations and were removed during stepwise model selection (Table S3). FGM concentrations in red squirrels decreased significantly after the first removal session, but there was no further, significant, decrease after the second removal session (period 2-period 1 estimate -0.58 ± 0.23, $t_{121} = 2.53$; $p = 0.012$; period 3 – period 1 estimate -0.50 ± 0.23, $t_{121} = 2.18$; $p = 0.031$; period 2 – period 3 estimate 0.08 ± 0.21; $t_{121} = 0.35$; $p = 0.73$).

There was no difference between males and females in FGM levels, but in this dataset breeding males had higher FGM levels than non-breeding males (Table S3). Variation between study sites was nearly significant only for two sites, with overall lower FGM levels in Lambro than in Passatempo (Table 2.1; difference in ln FGM estimate -0.63 ± 0.32; $t_{121} = 1.89$; $p = 0.06$).

The selected model which included 'period' had a lower BIC value than the model which included 'season' (ΔBIC = 4.53), and differences in FGM concentrations between seasons were not statistically significant (all p > 0.10).

2.4.4 Density effect

There was no correlation between mean FGM concentration of red squirrels per study site and squirrel density (red, grey or both species; Table 2.2 and Figure 2.4), neither using the observational data nor the removal experiment data.

Table 2.2 – Correlations between mean FGM concentration (ln ng/g dry feces) of red squirrels and density (ind/ha) of both species (singularly or together) for a) observational data and b) removal experiment data.

	Range	r	df	p
a) observational data				
Red squirrel density	0.13 – 1.11	0.19	10	0.55
Grey squirrel density*	0.11 – 2.28	-0.17	5	0.71
Total squirrel density	0.13 – 2.66	0.46	10	0.13
b) removal experiment				
Red squirrel density		0.30	10	0.34
Grey squirrel density		-0.20	10	0.54
Total squirrel density		-0.15	10	0.64

* Calculated only in red-grey study sites.

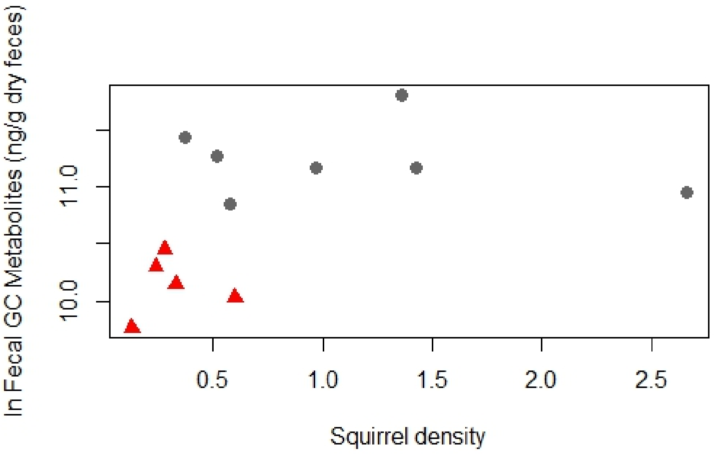


Figure 2.4 – Relationship between squirrel density (individuals/hectares) and mean FGM (ln ng/g dry feces) of red squirrels in red-only (red triangles) and red-grey study site (grey dots) (only observational data).

2.5 Discussion

We explored whether the presence of an invasive alien competitor affected fecal glucocorticoid metabolite concentrations in individuals of a native species. We predicted that the threat posed by invasive grey squirrels would increase FGM concentrations in co-occurring native red squirrels. FGM concentrations were significantly higher in sites invaded by grey squirrels than in red-only sites, and in the two areas colonized by grey squirrels during our study, FGM concentrations in the native red squirrels increased with respect to pre-colonization concentrations. Both results support our hypothesis and suggest that the invader is the causal factor driving observed differences in FGM concentrations (see also Graham et al. 2017). This was further supported by our grey squirrel removal experiment. Removal of grey squirrels resulted in a significant decrease in glucocorticoids in co-occurring red squirrels over a two month interval, taking into account seasonal variation in glucocorticoid concentrations and changes in reproductive condition in both males and females (see also Boonstra et al. 2001; Dantzer et al. 2010). The reduction in glucocorticoids was more pronounced in two sites: these were the two smallest woodlands that, at the start of the experiment, had the highest grey squirrel densities (Passatempo and Lambro, Table 2.1) and where red squirrel home ranges were more strongly overlapped by individuals of the alien species (Wauters unpubl. data).

2.5.1 Comparisons between red-only vs. red-grey sites (Observational data)

Native red squirrels had higher FGM concentrations in areas where they co-occurred with the alien grey squirrels than did those in areas without the introduced competitor. This pattern is supported by previous studies. Stress levels of native lizards (*Sceloporus undulatus*) were higher in sites with a long history of co-occurrence with invasive fire ants (*Solenopsis invicta*) than in sites without the invader (Graham et al. 2012). Similar results were found in a system of a native lizard (the lace monitor, *Varanus varius*) and an introduced alien predator (red fox, *Vulpes vulpes*) in Australia. In habitats with high fox densities, lizards produced a significantly greater basal and capture stress-induced corticosterone response compared to individuals in low–fox density habitat, suggesting competition with red foxes, perhaps via nutritional stress and increased hypersensitivity of the adrenocortical axis in lizards (Jessop et al. 2015). So far, it is not clear whether such responses mediate lizard fitness. Another study examined whether introduced foxes caused elevated glucocorticoid (GC) hormone concentrations (predator stress hypothesis) on a native predator (*Varanus varius*) and a prey species (ringtail possum, *Pseudocheirus peregrinus*). No differences were found in glucocorticoid concentrations or in hemoparasite loads between areas with and without fox control in either of the two native species (Anson et al. 2013). The different effects of foxes found by these two studies suggest that interspecific interactions have a greater role in eliciting a corticosterone response than a fear effect caused by the predator’s presence. These types of observational studies reveal the need for experimental manipulations to investigate the potential causality of the relationship between the presence of invasive species and increased stress in native species.

2.5.2 Pre – post colonization

The colonization by grey squirrels in two of our study sites can be considered as a natural experiment of introducing an alien competitor. The strong increase in FGM concentrations we detected following colonization of grey squirrels suggests that grey squirrels are causing the higher levels of physiological stress detected in field samples from the native species. Extra support to this interpretation is given by the fact that no such increase occurred in red squirrels monitored over the same time-period in sites that were not colonized. A similar pattern was documented in the only study we found which used experimental approaches to investigate changes in stress levels in the native and endangered Fijian ground frog (*Platymantis vitiana*) caused by the presence of introduced cane toads (*Rhinella marina*) that can prey on the native frogs (Narayan et al. 2013, 2015). Artificial exposure or introduction of the alien predator resulted in an increase in urinary corticosterone metabolites (Narayan et al. 2013), and subsequently reduced reproductive success (fewer eggs laid in enclosures which also contained alien predators than in no-predator enclosures) in the endemic species (Narayan et al. 2015).

2.5.3 The removal experiment

A next step is manipulation of the presence/density of the alien species that acts as stressor. Previous studies used an approach where they compared areas without predator manipulation to areas with predator manipulation (e.g. Anson et al. 2013; Jessop et al. 2015). We used a new approach: removal over time comparing the observed changes in fecal glucocorticoid metabolites within populations using sites as independent replicates. In other words the FGM concentrations in individual red squirrels within a given population/study site are measured under high pressure of grey squirrels (no removal) and compared with lower pressure of the alien species (after removal). This experiment may be confounded by temporal effects on glucocorticoids, such as the season effect found in the data comparing red-only with red-grey sites, and the time-span from the beginning of the removal and the measurements. However, our models showed that seasonal variation in FGM measures was not statistically significant in this dataset and that models with removal period better fitted the data than models including a season effect. Moreover, the strongest decrease in FGM concentrations of red squirrels after removal of grey squirrels occurred between period 1 and period 2. FGM data for period 1 were gathered in autumn and winter, and all data for period 2 were taken in winter, and in the large dataset comparing red-only with red-grey sites there was no significant difference between winter and autumn levels of glucocorticoids. In a dataset of red squirrels in areas without the invasive species, glucocorticoids were even higher in winter than in autumn (Dantzer et al. 2016), hence the opposite trend. Hence, these data support our conclusion that the observed decrease in fecal glucocorticoid metabolites in red squirrels was indeed caused by a reduction of the density of alien grey squirrels.

We found that FGM concentrations in native red squirrels decreased significantly (between the first and second period) in relation to invasive species removal. FGM levels also remained lower in the third period (see Figure 2.3), but a further decrease with respect to period 2 was observed only in the two smallest study sites, where, as mentioned above, grey squirrels occurred at the highest densities when the experiment started (see Table 2.1). This suggests that the impact of the alien competitor on individual red squirrels' FGM concentrations is probably related to the degree of home range (or core-area) overlap, and the resulting differences in food competition and pilfering of red squirrel caches by overlapping greys (Wauters and Gurnell 1999; Wauters et al. 2002a).

2.5.4 Are population level FGM concentrations determined by squirrel density?

Theoretically, the observed increase in physiological stress in red squirrels in sites shared with grey squirrels could be simply due to a larger amount of food competitors, independent of whether they belong to the alien or the native species. In other words, higher FGM concentrations in red-grey than in red-only sites might be simply influenced by crowding of competitors and an increase in red squirrel densities might produce the same effect as a large number of co-occurring grey squirrels. Unfortunately, this hypothesis can not be tested experimentally, since it is impossible to artificially increase red squirrel densities. The reason is that the red squirrel is a semi-territorial and asocial species which occurs at relatively low densities ranging from 0.2 – 1.2 ind/ha (Wauters et al. 2004, 2008). Space use differs between the sexes: females defend a core-area of high quality habitat from other females (intra-sexual territoriality; Wauters and Dhondt 1992) while males use home ranges that overlap considerably both with those of other males and with female core-areas, facilitating mating opportunities (Wauters and Dhondt 1992; Wauters et al. 2001; Di Pierro et al. 2008). Food abundance, space use and density are strongly linked, and density dependent emigration and/or reduction of local recruitment occur, regulating red squirrel densities at levels much below those expected based purely on resource-energy availability (e.g. Wauters et al.

2004, 2008; Boutin et al. 2006). Grey squirrels, in contrast, present a semi-social system, characterized by kin group formation and extensive home range overlap both between females and males and between animals of the same sex (Gurnell et al. 2001; Koprowksi 1994). This species occurs at higher densities respect to red squirrels (1.0 to 6.0 ind/ha in natural deciduous woodlands, up to 10-15 squirrels/ha in parks with artificial feeding; Koprowski 1994; Gurnell 1996; Chapter 4 Table 4.5).

However, if overall squirrel density or differences in the intensity of intraspecific competition would be the driving factors behind the increased stress levels in red squirrels co-occurring with grey squirrels, we would expect a correlation between average (population level) FGM concentrations and, respectively, total or red squirrel density in the various study sites. Our results showed that this was not the case: FGM concentrations in red squirrels were not correlated with total squirrel density, grey squirrel density or with intraspecific (red squirrel) density, allowing us to exclude the possibility that variation in FGM concentration is simply related to red squirrel density or to crowding of arboreal squirrels, independent of the species they belong to. This was further confirmed by the removal experiment, where variation in the experimentally reduced grey squirrels densities, total densities, or the resulting fluctuations in red squirrel densities were not correlated with spatio-temporal variation in FGM concentrations.

In summary, these results showed that squirrels density (both red and grey) did not influenced native species stress levels, suggesting that other ecological mechanisms related to the presence of the alien species are causing the measured variation in red squirrels FGM concentrations.

2.5.5 *Physiological relevance and fitness consequences*

In wild animals, it is difficult to assess the direct physiological relevance of increased FGM. However, we documented a 3-fold increase in FGMs in red squirrels in red-grey sites compared to red-only sites, a 2-fold increase in sites that were colonized by the alien species and a 1.5-fold decrease when grey squirrels were removed. Placing these results in the context of other studies from the literature is difficult, because changes in FGMs reported in our and other studies are highly influenced by the assay used to quantify the change in GCs (e.g. Fanson et al. 2017). Nonetheless, a few studies suggest that the changes in FGMs that we observed are comparable to chronic stress paradigms in the laboratory, and that they may have other effects on life history traits. For example, in laboratory rats, exposure to a variable chronic stress paradigm caused a ~1.5 fold increase in plasma corticosterone levels (Herman et al. 1995). In a communally breeding rodent (*Ctenomys sociabilis*) the difference in baseline glucocorticoid levels between animals kept in less or more stressful situations was about 1.5 to 2-fold (Woodruff et al. 2013). A previous study in North American red squirrels (*Tamiasciurus hudsonicus*) showed that pregnant females have 30% higher FGMs when high population density conditions were simulated, and that this was associated with a change in offspring postnatal growth rates (Dantzer et al. 2013). Therefore, we conclude that alien grey squirrels increase FGM concentrations in native red squirrels, supporting the physiological impact of elevated levels of glucocorticoids.

To date, few studies have been able to demonstrate the consequences of prolonged elevations of glucocorticoids caused by IAS on native species' fitness components (Narayan et al. 2015). Our study was too short to reveal fitness consequences of the increase in glucocorticoids levels in red squirrels in areas invaded by the congener. However, our previous studies clearly demonstrated negative effects of grey squirrels, exacerbated at high densities of the alien species, on red squirrel body size and reproductive rate (Wauters et al. 2001; Gurnell et al. 2004). Both these effects are in agreement with alterations of the hormonal functions of glucocorticoids in regulating of body growth and reproduction (Cabezas et al. 2007; Sheriff et al. 2009).

Moreover, studies on animal models indicate that chronic stress can reduce immune-efficiency (Sapolsky et al. 2000), and further research will explore whether this mechanism is involved in the probability that naïve red squirrels acquire an alien parasite, *Strongyloides robustus*, typical of grey squirrels (Romeo et al. 2015).

The interaction between invasive and native species is a phenomenon that occurs through many different mechanisms (e.g. direct competition, parasite-mediated competition, disease transmission, predation, habitat destruction or alteration) in a wide variety of ecosystems. This study shows that direct and/or indirect effects of increases in physiological stress in native species caused by the presence of an invasive alien species should be considered as one of these mechanisms. We suggest that future studies should assess if and how changes in stress levels are involved in species interactions and invasion processes and to what extent it has direct or indirect (e.g. by increasing susceptibility to parasite infections) fitness consequences for the native species.

Chapter 3

Habitat-dependent effects of personality on survival and reproduction in red squirrels

3.1 Summary

This Chapter examines relationships between personality and fitness components and their variation with changes in population density and/or habitat quality in Eurasian red squirrels (*Sciurus vulgaris*). Indices of personality (boldness and pure exploration), expressed as PCA scores, derived from the number of captures (trappability) and number of different traps where an individual was captured (trap diversity), were analyzed in relation to local survival in either of the sexes and a measure of reproductive success in females (total number of litters produced during lifetime), under different conditions of food availability. The relationships between boldness and local survival differed between habitats in both sexes. Bold squirrels survived better than shy ones in Norway spruce forest. Pure exploration behavior was negatively correlated with local survival in all habitats and in both sexes. Female reproductive success increased with body mass and decreased with the tendency to explore, a relationship consistent across habitat types. Bolder females did not have a reproductive advantage in years of poor food availability. This Chapter shows that costs and benefits of boldness vary with spatio-temporal differences in availability of high-quality food resources which may help to maintain variation in personality in red squirrels in heterogeneous landscapes. The high repeatability of our personality-trait estimates shows that the indirect indices developed here are reliable estimates of squirrel personality and can be used for further studies.

3.2 Introduction

According to evolutionary theory, the way an individual behaves under local conditions should be adaptive because of natural selection favoring animals whose behavior increases fitness, given that this behavior is heritable. Variation in behaviors is maintained because of spatio-temporal changes in intrinsic and/or extrinsic environmental conditions, that cause variation in selective pressures (e.g. evolutionarily stable strategies) (Carter et al. 2013; Weiss and Adams 2013). However, from the perspective of adaptive behavior, the fact that animals display variation in individual behaviors that are consistent over time (within or across generations) and/or under different contexts is more difficult to explain (Dall et al. 2004; Réale et al. 2007; Biro and Stamps 2008). Animal personality refers to between-individual differences in behavior that persist through time (Biro and Stamps 2008; Carter et al. 2013). These differences in individual personality, also called temperament or ‘coping style’ (Bergmüller and Taborsky 2010), are often measured through traits such as reaction to potential risks or handling novelty, aggressiveness and sociability (e.g. Réale et al. 2007). If differences in personality are adaptive, they may affect how populations respond to environmental change and how species are able to persist (Dall et al. 2004; Le Coeur et al. 2015).

An individual’s willingness to take risks, referred to as boldness, has been investigated in many wild populations showing that this personality trait is related to several ecological aspects, such as propensity to explore and acquire information about the environment, dispersal distance and/or speed (Fraser et al. 2001; Minderman et al. 2010; Korsten et al. 2010; van Overveld et al. 2015). Although recent studies have shown that personality can affect several components of fitness (Dingemanse and Réale 2005; Patterson and Shulte-Hostedde 2011; Mutzel et al. 2013; Zhao et al. 2016), it remains unclear whether bold phenotypes have a fitness advantage over shy ones. Several studies on birds and mammals found that bold and/or fast exploring individuals are often the most dominant ones during intraspecific encounters (Dingemanse and de Goede 2004; Rödel et al. 2015). For example, fast-exploring males of the great tit (*Parus major*) occupied better quality territories and fast-fast pairs produced offspring in best condition (Both et al. 2005). In Norway rats (*Rattus norvegicus*) explorative individuals survived longer than less explorative ones (Cavigelli and McClintock 2003). However, in an across species meta-analysis Smith and Blumstein (2008), exploring the effects of exploration and boldness on fitness components (survival and/or reproductive success), reported contrasting findings. Some studies showed that bolder animals encounter more risks due to their exploring tendency, which resulted in higher susceptibility to predation and higher mortality risk (e.g. Bremner-Harrison et al. 2004; Carter et al. 2010; Hall et al. 2015). In the European rabbit (*Oryctolagus cuniculus*), explorers had significant survival costs during the juvenile life-stage (Rödel et al. 2015). Still a different pattern was revealed in Eastern chipmunks (*Tamias striatus*) where animals with average exploration levels suffered higher mortality than extreme phenotypes (Bergeron et al. 2013).

Variation in personality may be maintained by fitness trade-offs of different behaviors. For example, bold (active and aggressive) female North American red squirrels (*Tamiasciurus hudsonicus*) were more likely to bequeath their territory, which increases overwinter survival of their offspring, thus their reproductive success, but they were less likely to survive to the following spring (Boon et al. 2008). In addition, spatio-temporal variation in ecological selection pressures can play a role in maintaining animal personalities in populations in heterogeneous environments, both in short- and long-lived species (Réale et al. 2000; Le Coeur et al. 2015; Vetter et al. 2016).

So far, most studies on personality-fitness relationships have been carried out in a single population and we know only few studies on mammals that have investigated effects of personality on fitness in different habitats (e.g. Boon et al. 2007; Martin and Réale 2008; Monestier et al. 2015).

Here we measured personality traits in the Eurasian red squirrel (*Sciurus vulgaris*) in three different habitats, and investigated to what extent boldness and exploration tendency of adults were correlated with phenotypic traits (body size and body mass) and with fitness components. The red squirrel is a good model species to investigate relationships between personality and fitness components under different habitat conditions for several reasons. 1) previous studies on other tree squirrels have shown that personality traits can affect habitat and/or space use, survival and reproductive success (e.g. Boon et al. 2008; Boyer et al. 2010; Le Coeur et al. 2015); 2) reliable methods and indices to study personality have already been developed on other sciurids (Boon et al. 2008; Boyer et al. 2010); 3) long-term capture-mark-recapture data of individually-marked squirrels combined with radio-tracking show high capture probabilities for the majority of resident adults and provide detailed information of phenotype, local survival and reproductive success (of females) (Wauters et al. 2004, 2007, 2008).

Our main hypothesis is that costs and benefits of personality types vary according to environmental conditions. Hence, we predict that in different habitats (with spatio-temporal variation in food availability) different personality types will perform better; in other words, bold squirrels may perform better than shy ones in one habitat type, but not in other habitat(s). To test this hypothesis we first checked for correlations of personality indices with body size and body mass, since in this species heavier males tend to be more aggressive and have higher mating success (Wauters and Dhondt 1989, 1992; Wauters et al. 1990), while in females body mass positively affects the probability to enter estrus and lifetime reproductive success (Wauters and Dhondt 1989, 1995). Next, we explored potential advantages or disadvantages of being bold by studying whether our indices of boldness and exploration were correlated with local survival in both sexes and with a measure of reproductive success in females, and whether these relationships varied among habitats. Finally, we also tested the hypothesis that being bold might be advantageous for reproduction only in years of poor food availability. We used capture-mark-recapture data of individually marked squirrels to obtain data of sex, body size and body mass, and estimates of local survival and female reproductive success. The total number of captures (trappability) and number of different traps where an individual was captured (trap diversity), over a maximum of two years, were used as indices of the personality traits boldness and exploration, respectively (Boon et al. 2008; see methods for details).

3.3 Materials and methods

3.3.1 Study species

Eurasian red squirrels have overlapping home ranges, with more intensively used core- areas, whose size differs between the sexes, and in relation to season and habitat quality (e.g. Wauters and Dhondt 1992; Lurz et al. 2000; Wauters et al. 2001, 2005). The mating system is promiscuous, although most females only mate with a dominant male of high body mass (Wauters et al. 1990). Reproduction is seasonal, with 1 to 2 litters per year, and is strongly affected by the female's body condition, food abundance and, in the mountains, elevation (Wauters and Dhondt 1995; Wauters et al. 2008; Rodrigues et al. 2010). In our study areas, females can be lactating between April and October and may produce one or two litters/year (Boutin et al. 2006; Wauters et al. 2008). Most animals disperse as juveniles and subadults (from 4 to 10 months old, Wauters and Dhondt 1993; Wauters et al. 1993, 2010). Once settled, adult squirrels tend to have a high site-fidelity. In the three study areas, the capture probabilities at population level (including also animals not used in this study because of too short trapping histories) were high and stable over time (mean capture probability ± SE: CED 0.81 ± 0.09; OGA 0.89 ± 0.05; RHE 0.81 ± 0.08). There is no sexual size dimorphism (Wauters et al. 2007).

3.3.2 Study areas and food availability

We studied red squirrels in three study areas in the Italian Alps, two in Lombardy and one in Valle d'Aosta, N. Italy. Distances between the areas are CED-OGA 58 km, CED-RHE 217 km, OGA-RHE 271 km (Trizio et al. 2005). Cedrasco (CED, 46°07' N, 9°48' E, elevation 1100 – 1600 m a.s.l.) is a mixed montane conifer forests on the northern slope of the Orobic Alps, N. Italy. The study area extends over 76 ha of mature secondary forest (mean tree density ± SD = 449 ± 163 trees ha-1), with large areas of even-age stands dominated by silver fir (*Abies alba*, tree cover 56%) and Norway spruce (*Picea abies*, 25%), with sparse Scots pine (*Pinus sylvestris*, 5%), larch (*Larix decidua*, 3%) and dead trees (5%). Spruce and fir were interspersed at a fine-grained level and not clumped. At lower elevations, spruce and fir were mixed with some beech (*Fagus sylvatica*, 7% tree cover).

The second study area, Oga (OGA, 46º28´N, 10º22´E, elevation from 1280 m to 1450 m a.s.l.), is part of a mature mixed montane conifer forest (47 ha, mean tree density ± SD = 765 ± 251 trees ha-1), dominated by Scots pine (*Pinus sylvestris*, 88.7% tree cover) with some Norway spruce (*Picea abies*, 8.8%) and larch (*Larix decidua*, 2.5%). The third study area, in Val di Rhêmes (RHE, 45°39'N, 7°12'E, elevation 1740 – 1890 m a.s.l.) is a mature Norway spruce forest (85%) with some mixed patches of larch (11%) and spruce (69 ha, mean tree density ± SD = 773 ± 333 trees ha-1). Snags (dead trees) are common (4%), occurring throughout the study area.

Each year between 26 July – 9 August, the new (current year) maturing cones were counted in the canopy of sample trees of all conifer species to estimate food availability. Methods are described in detail elsewhere (Salmaso et al. 2009; Di Pierro et al. 2011). Based on species-specific energy values per cone (kJ/cone) and data on tree species composition and density (Salmaso et al. 2009), these counts allowed us to estimate the average yearly tree seed-energy production in each study area (Table 3.1).

The three populations had similar sex-ratio's and age structure (Wauters et al. 2007, 2008). There were no differences in allelic richness between areas, but squirrels at RHE had slightly lower observed heterozygosity than at CED and OGA (10 microsatellites, Trizio et al. 2005). All had goshawk, red fox and marten as potential squirrel predators.

Table 3.1 – Food abundance estimates (conifer seed-crop in 103 kJ/ha, mean ± SE) and number of red squirrels (males, females) trapped and used to measure personality per year in the three study areas. Total number of squirrels refers to sample size of different individuals used in the analyses.

	Study areas					
Year	**CED**		**OGA**		**RHE**	
	Food	Squirrels	Food	Squirrels*	Food	Squirrels*
1999	4357 ± 570		1732 ± 419			
2000	159 ± 63*	4 (3, 1)	391 ± 82*	16 (10, 6)	497 ± 252*	11 (8, 3)
2001	3087 ± 440	10 (7, 3)	588 ± 83	20 (10, 10)	913 ± 109	18 (11, 7)
2002	1867 ± 296	14 (8, 6)	735 ± 153	23 (14,9)	1773 ± 154	21 (12, 9)
2003	846 ± 226*	20 (11, 9)	552 ± 81	21 (13, 8)	1010 ± 130	20 (11, 9)
2004	6142 ± 728	18 (11, 7)	863 ± 111	21 (12, 9)	2402 ± 233	19 (11, 8)
2005	4022 ± 828	16 (10, 6)	352 ± 73*	14 (8, 6)	22 ± 5*	21 (10, 11)
2006	6249 ± 907	14 (9, 5)		5 (3, 2)	1363 ± 179	13 (8, 5)
2007	2547 ± 528	9 (7, 2)			1171 ± 133	8 (4, 4)
2008		1 (1, 0)			96 ± 27*	4 (2, 2)
Mean/Total	3253 ± 849	45 (27, 18)	745 ± 192	40 (21, 19)	1027 ± 273	56 (30, 26)
% CV	66%		63%		75%	

*years of poor seed-crop

3.3.3 Trapping and handling

Trapping was carried out in three periods per year (April-May, June-July, September-October) between April 2000 and October 2006 in OGA and between April 2000 and April 2009 in CED and RHE (Table 3.1). A trapping session involved the use of 25 (CED), 23 (OGA) or 30 (RHE) ground-placed Tomahawk "squirrel" traps (models 201 and 202, Tomahawk Live Trap, WI, USA). Steep slopes and mountain orography made it impossible to set traps in a perfect grid, hence traps were more or less homogeneously distributed over the study area, with distances of 100-130 m between traps and average trap density of 0.7-0.8 traps ha^{-1}. In CED and RHE the forest continued beyond the boundaries of the study area. In contrast, the study area of OGA was entirely surrounded by hard edges (road and parking area, meadows, river). Traps were pre-baited with sunflower seeds and hazelnuts 4 to 6 times over a 30 day period, then baited and set for 6-10 days, until no new, unmarked squirrels were trapped for at least 2 consecutive days (Wauters et al. 2008). Traps were checked two-three times per day. Each trapped squirrel was flushed into a light cotton handling bag with a zipper or a wire-mesh "handling cone" to minimize stress during handling, and individually marked using numbered metal ear-tags (type 1003 S, National Band and Tag, Newport, KY, USA). It was weighed to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland) and the length of right hind foot (without nail) was measured (0.5 mm) with a thin ruler. Foot length is a measure of structural size and remains fixed once the animal has reached adult size (12-18 month old). In contrast, body mass can further vary with season, age or between years with poor or high food availability (Wauters et al. 2007). Therefore, in our models (see "Effects of personality on fitness components") foot length as adult and average body mass over the three trapping sessions in the first year the squirrel was classified as adult (12-18 months old, see also Wauters and Dhondt 1989) were used as measures of phenotypic parameters. Sex and age class were determined on the basis of external genitalia and body mass, with juveniles weighing less than 250 g (Wauters and Dhondt 1995). Female reproductive condition was determined based on external genitalia and condition of the nipples: pregnant females have enlarged vulva and small nipples, lactating females have swollen nipples and milk excretion can be stimulated, post-lactation females have still large nipples but no longer produce milk (Wauters and Dhondt 1995).

3.3.4 Personality: trappability and trap diversity indices

We calculated for each individual two indices of personality: a trappability and trap diversity index. The first is a measure of tendency to take risks (boldness), the second of the animal's propensity to explore novel environments. In sciurid rodents, these two indices have been shown to be repeatable measures of personality, but they also tend to be correlated (Boon et al. 2008; Boyer et al. 2010). The indices were estimated using the standardized values, per study area, of the ratio of number of captures on the length of capture period (i.e. the number of capture days from the first to the last trapping session for a given animal; boldness); or the ratio of the number of different traps in which an animal was captured on the number of available traps (exploration). Since recent studies have shown that personality traits to some degree can change over time (flexible component of personality, e.g. Dingemanse et al. 2012), we only used capture data of the first two years an individual was present (using only subadults and adults). Since we only had three sessions/year, using only first year data would strongly reduce the variation between individuals in trappability and trap diversity. Standardization was done for each study area separately because of differences in capture histories and number of available traps. Since the two standardized indices were strongly and positively correlated ($r = 0.76$; $n = 141$; $p < 0.0001$), they could not be used together as explanatory variables in multivariate models because of lack of independence when estimating their partial effects. Therefore, we used a principal component analysis (PCA) to derive two new variables, the principal components PC1 and PC2 (orthogonally rotated), maximizing the variance of these among individual squirrels. PC1 (loadings: 0.707 trappability index + 0.707 trap diversity index, eigenvalue 1.33) explained 88% of total variance, PC2 12% (loadings: -0.707 trappability index + 0.707 trap diversity index, eigenvalue 0.49). For males, PC1 and PC2 were not correlated ($r = -0.08$; $n = 78$; $p = 0.46$). However, for females there was a significant but weak correlation ($r = 0.28$; $n = 63$; $p = 0.025$). PC1 has a high score for those animals that are often captured and in many different traps, so it can be considered as a measure of boldness which includes both risk taking and exploration. PC2 has a high score for those animals that are captured few times but nearly always in a different trap, hence indicating a specific exploration component of the animal's personality. Recent data on arena tests (open field test and mirror image stimulation test) with red squirrels showed that trappability and trap diversity indices of personality are confirmed by behavior in the arena test, as in other sciurids (Boon et al. 2008; Boyer et al. 2010; Le Coeur et al. 2015; our unpubl. data).

Our data could potentially contain a bias in estimates of trappability, trap diversity and survival, due to a lower capture probability for individuals living at the edge of the study areas (e.g. Boyer et al. 2010). However, position and size of home ranges of 91 radio-tracked squirrels (for details see Wauters et al. 2005; Romeo et al. 2010; Di Pierro et al. 2011) showed that their large home range overlapped with several traps (5 to 10) both at study area periphery and more central ones. Hence, to eliminate the risk of a potential bias we removed from our dataset those animals only caught in edge traps. Radio-tracking data also confirmed the reliability of our survival estimates, with only two (2%) radio-tagged squirrels still present (alive) after the last capture event.

3.3.5 Repeatability of trapping indices

To analyse the link between trapping characteristics and individual personality we needed to verify that trapping characteristics were consistent across years for the same individual (i.e. trapping characteristics as an expression of personality, e.g. Boyer et al. 2010). Using a restricted sample of 48 individuals that were caught in two successive years during the study period, we estimated the repeatability, also called Intra-class Correlation Coefficient (ICC), in the number of captures per year and in trap diversity per year with a Linear Mixed Model (LMM) (Nakagawa and Schielzeth 2010). Repeatability was estimated using the R package rptR v0.9.2 (CI=95%, number of parametric bootstraps for interval estimation=5000, number of permutations used when calculating asymptotic p-values=1000; Stoffel et al. 2017). In this case, number of captures was log transformed (using natural logarithm) and trap diversity (number of different traps) square root transformed to meet assumptions of normality (Shapiro-Wilk's test on transformed data, both $W > 0.95$) (see also Boyer et al. 2010). We included sex, study area and year and second order interactions as fixed effects and squirrel identity as random factor. Length of capture period was constant between the two years.

3.3.6 Effects of personality on fitness components

To explore relationships between personality and fitness parameters we estimated local survival of squirrels of both sexes and reproductive success of females. Length of residency was used as a proxy for local survival and was estimated as the number of months between first and last capture, for those squirrels that remained on the study area for at least 6 months (see also Wauters et al. 2007). To avoid potential bias in survival estimates due to selective disappearance or between population differences in age structure, we only used squirrels from extinct cohorts (n=132). Reproductive success was measured as the number of litters produced per individual female throughout its lifetime, using only females that were trapped in at least two out of three sessions/year (for models on number of litters). In April-May females could be scored as pregnant or lactating if they had a spring litter, in June-July as pregnant-lactating in case of a single intermediate litter or an autumn litter or as post-lactating in case of spring litter, in September-October as lactating or post-lactating (autumn or intermediate litter respectively). Hence, females that were trapped in at least two out of three sessions allowed to distinguish between production of no, a single or two litters/year. Since both parameters are counts, we used them as dependent variables in GLM models with Poisson error distribution and investigated the effects of study area, body mass, foot length (as co-variate of body mass, see Wauters et al. 2007), and the boldness and exploration scores (PC1 and PC2) from the PCA. Because of relatively low sample size, we tested the second order interactions of these variables with study area separately, by adding and removing one interaction per model. When none of these interactions were statistically significant (all partial $p > 0.05$) we presented models only with main effects. In contrast, if one of the interactions was significant we used pairwise comparisons between study areas to explore how patterns differed between them. In some models, significant interactions were found: they always indicated a different relationship for study area RHE in comparison to study areas CED and OGA (see results). Therefore, in these cases separate models for (a) CED and OGA, and (b) RHE were further tested using only the main effects. Since patterns of persistency in the study areas were sex-biased (higher local survival in males than in females) the models were tested for males and females separately (see also Wauters et al. 2004).

We also tested the hypothesis that being bold might be advantageous for reproduction only in years of poor food availability (e.g. Le Coeur et al. 2015). We classified seed-crops in two categories: poor vs. medium-good seed crop (Table 3.1). In each reproductive period (spring and summer-autumn) we determined if a female produced a litter or not (binary dependent variable, 190 observations of 57 different females) and investigated relationships with study area, food availability (poor vs medium-good) and PC1 score along with the interaction of PC1 with food availability, using a mixed effects logistic regression model which included squirrel identity as a random effect. Spring litters were related to the previous year's seed-crop, summer-autumn litters to the same year's seed-crop (Boutin et al. 2006; Wauters et al. 2008).

Distributions of data on local survival and reproductive success were skewed. Therefore, we repeated the above models also using a negative binomial distribution of the dependent variable. Since results were similar as for models with a Poisson distribution we only showed results of the latter.

All tests of significance are two-tailed and the significance level was set at 0.05. Statistical analyses were done using SAS/STAT 9.4 software (Copyright © 2011, SAS Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Trappability and trap diversity indices

Within a period of maximum two years (see methods), the number of captures per individual red squirrel varied between 2 and 41 times (median 7, mean ± SE = 9.4 ± 0.6 captures), and number of different traps from 1 to 13 (median 5, mean ± SE = 5.2 ± 0.2 traps).

On average, males were trapped more often than females (males, n = 78, 11.2 ± 1.0 captures; females, n = 63, 7.2 ± 0.7 captures; $F_{1,135} = 11.5$, p = 0.0009), and in more different traps (males, n = 78, 5.8 ± 0.4 traps; females, n = 63, 4.4 ± 0.3 traps; $F_{1,135} = 9.90$, p = 0.002). The number of times a squirrel was captured did not differ significantly between study areas (p = 0.25); however, on average squirrels were trapped in more different traps in OGA (n = 40, 5.3 ± 0.5 traps) and RHE (n = 56, 5.8 ± 0.4 traps) than in CED (n = 45, 4.2 ± 0.4 traps; area effect $F_{2,135} = 4.59$; p = 0.012; Figure 3.1). These differences between areas did not influence our results since trapping indices were standardized within study areas.

We observed consistent individual variation across years in number of captures (n = 48; repeatability = 65%, 95% CI = 46 – 81%, Likelihood ratio test LRT = 25.9; df= 1; p < 0.001) and in trap diversity (repeatability = 69%, 95% CI = 52 – 83%, Likelihood ratio test LRT = 29.9; df= 1; p < 0.001). Thus, trappability and trap diversity indices are consistent through time suggesting they are reliable estimates of a red squirrel's personality.

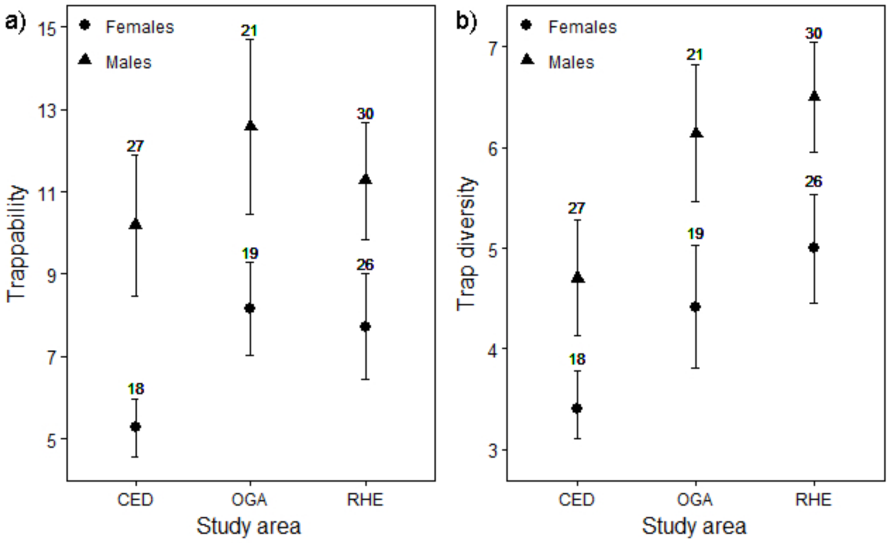


Figure 3.1 – a) Trappability and b) trap diversity (mean ± SE) of male (triangles) and female (circles) red squirrels in three study areas in the Italian Alps. Sample size per study area and sex above error flags.

3.4.2 Phenotypic variation and personality

Neither foot length, nor body mass were correlated with the personality scores (Table 3.2). These patterns were consistent when analyzed per sex (Table 3.2).

3.4.3 Fitness components of males

Local survival of male red squirrels was higher in OGA (n = 21, mean ± SD = 27 ± 19 months) than in CED (n = 24, 19 ± 10 months) and RHE (n = 29, 18 ± 15 months) and relationships between PC scores and survival differed between study areas (Comparison RHE against OGA and CED, all partial $p < 0.05$). In study areas OGA and CED, local survival increased with body mass but not with body size (Table 3.3a). Both PC scores for personality were negatively related with local survival (Table 3.3a).

In study area RHE patterns were different. Larger males had a higher local survival and boldness score (PC1) was positively related to survival. The effect of body size was much stronger than of body mass. The specific exploration score (PC2) was negatively related to local survival as in the other study areas (Table 3.3b). In the full model, the partial effect of PC1 was not significant, but considered as a single factor there was a positive relationship between boldness score and local survival (estimate 0.13 ± 0.04, $\chi^2 = 12.9$; $p = 0.0003$).

Table 3.2 – Pearson's correlation coefficient with significance level for correlations between foot length, body mass and personality scores of red squirrels: a) all animals; b) and c) per sex. Sample size between brackets.

Both sexes (n = 141)	**Foot length**	**Boldness score (PC1)**	**Exploration score (PC2)**
Body mass	r = 0.49, p < 0.0001	r = 0.001, p = 0.98	r = -0.007, p = 0.93
Foot length		r = 0.06, p = 0.49	r = 0.04, p = 0.60
Males (n = 78)			
Body mass	r = 0.63, p < 0.0001	r = 0.09, p = 0.44	r = -0.04, p = 0.73
Foot length		r = 0.08, p = 0.46	r = 0.01, p = 0.90
Females (n = 63)			
Body mass	r = 0.38, p = 0.002	r = -0.04, p = 0.74	r = -0.03, p = 0.02
Foot length		r = -0.001, p = 0.99	r = 0.11, p = 0.39

3.4.4 Fitness components of females

Also among female red squirrels, local survival was higher in OGA (n = 19, mean ± SD = 20 ± 17 months) than in CED (n = 15, 14 ± 8 months) and RHE (n = 24, 15 ± 8 months) and the relationship between survival and the two PC scores differed between study areas (Comparison RHE against OGA and CED, all partial $p < 0.01$). In study areas OGA and CED the GLM model gave the same results as for males: there was no significant effect of body size on female survival, while heavier females survived longer than those of lower body mass (Table 3.4a). Local survival was negatively related to both PC scores, but only PC2 was significant (Table 3.4a). In contrast, in RHE the coefficient of foot length was negative, while that of body mass positive, indicating a strong positive effect of body condition (mass rather than size) on local survival of female red squirrels (Table 3.4b). As for males in this study area, the boldness score (PC1) was positively related, and the specific exploration score (PC2) negatively related to female survival (Table 3.4b).

The number of litters that females weaned while present on the study area, varied between 0 and 7 (n = 57, mean ± SD = 1.67 ± 1.54). Heavier females produced more litters than those of poorer body mass (body mass parameter estimate 0.019 ± 0.005; $\chi^2 = 13.7$; df = 1; $p = 0.0002$). Tendency to explore was negatively related to variation in number of litters produced (partial effect PC2 score -0.86 ± 0.24; $\chi^2 = 12.3$; df = 1; $p = 0.0005$). On average, females produced more litters in OGA (n = 17, mean ± SE = 2.29 ± 0.54 litters) than in RHE (n = 23, 1.52 ± 0.23 litters) and CED (n = 17, 1.24 ± 0.31 litters; area effect $\chi^2 = 5.91$; df = 2; $p = 0.052$), but pairwise differences were significant only between OGA and CED ($p < 0.05$). Partial effects of foot length or boldness score (PC1) were not significant (both $p = 0.10$).

In periods of poor seed-crops (46 observations), there were 16 cases (35%) of offspring production and 30 cases (65%) of females not reproducing. In contrast, in years of medium-good seed-crops (144 observations) females were more likely to produce offspring (81 cases, 56% against 63 cases of females not reproducing; food availability estimate 0.96 ± 0.38; $\chi^2 = 6.42$; df = 1; $p = 0.011$). Bolder females were not more likely to produce offspring than shy ones (PC1 $\chi^2 = 0.10$; df = 1; $p = 0.75$), and this did not change between periods of poor against medium-good seed-crops (PC1 by food availability interaction $\chi^2 = 0.69$; df = 1; $p = 0.40$). There was no effect of study area ($\chi^2 = 1.94$; df = 2; $p = 0.38$).

Table 3.3 – General Linear Models with Poisson distribution of parameters affecting variation in local survival of male red squirrels. After significant interactions of PC scores and foot length with study area, models were tested separately for CED and OGA (interactions, all partial $p > 0.05$) and for RHE (interactions with both CED and OGA, all partial $p < 0.05$).

Fixed effects	Estimates	χ^2 (df = 1)	p-value
(a) **CED & OGA** (n = 45)			
Study area		32.6	< 0.0001
Body mass	0.014 ± 0.002	42.7	< 0.0001
Foot length	-0.058 ± 0.048	1.43	0.23
PC1 score	-0.047 ± 0.022	4.15	0.042
PC2 score	-0.376 ± 0.071	28.4	< 0.0001
(b) **RHE** (n = 30)			
Body mass	-0.0019 ± 0.0031	0.39	0.53
Foot length	0.221 ± 0.041	29.6	< 0.0001
PC1 score	0.037 ± 0.035	1.06	0.30
PC2 score	-0.478 ± 0.061	60.9	< 0.0001

Table 3.4 – General Linear Models with Poisson distribution of parameters affecting variation in local survival of female red squirrels. After significant interactions of PC scores with study area, models were tested separately for CED and OGA (interactions, all partial $p > 0.05$) and for RHE (interactions with both CED and OGA, all partial $p < 0.05$).

Fixed effects	Estimates	χ^2 (df = 1)	p-value
(a) **CED & OGA** (n = 34)			
Study area		10.9	0.001
Body mass	0.0092 ± 0.0024	14.8	< 0.001
Foot length	-0.005 ± 0.037	0.02	0.89
PC1 score	-0.099 ± 0.056	3.11	0.078
PC2 score	-1.057 ± 0.120	77.6	< 0.0001
(b) **RHE** (n = 26)			
Body mass	0.0090 ± 0.0025	13.0	0.0003
Foot length	-0.124 ± 0.035	12.5	0.0004
PC1 score	0.158 ± 0.038	17.4	< 0.0001
PC2 score	-0.494 ± 0.115	18.6	< 0.0001

3.5 Discussion

Our analyses of capture-mark-recapture data of individual Eurasian red squirrels showed that scores derived from the number of times a squirrel is trapped (trappability) and from the number of different traps visited (trap diversity), during the first one or two years of presence, are repeatable across years and therefore can be used as indices of an individual's personality. There was no correlation between boldness or exploration scores and body size or body mass of the squirrels, suggesting that personality was not related to body condition, a good proxy of an individual's quality. We found that the relationships between boldness score and local survival/residency of individual squirrels differed between habitats, in males as well as in females. Bold individuals survived better than shy ones in the habitat dominated by Norway spruce (RHE), while in the other two study areas, a Scots pine dominated forest (OGA) and a mixed spruce-fir forest (CED) bolder squirrels survived less well. The second PCA score, related to specific exploration behavior, was negatively correlated with local survival in all habitats and in both sexes. For females, the relationships between personality and body mass on one hand, and reproductive output (number of litters in lifetime) on the other hand, were consistent across habitat types. Reproductive success increased with female body mass, but decreased with the tendency to explore. Bolder females did not have a reproductive advantage in years of poor food availability.

3.5.1 *Reliability of trappability and trap diversity as indices of personality*

In this study, we used two indirect indices of personality: trappability which is generally linked to risk taking behavior, and the tendency to visit more different traps (trap diversity) which is considered as a measure of exploration (Boon et al. 2008; Boyer et al. 2010). Similarly to studies on other Sciuridae we found consistent individual differences across years in both indices suggesting they are reliable estimates to assess personality of Eurasian red squirrels (Boon et al. 2008; Boyer et al. 2010; Montiglio et al. 2012b; Le Coeur et al. 2015). Moreover, capture probabilities of red squirrels tend to be high and this was the case also in the study areas used here with high average capture probability per session which varied little over seasons or years (see section "Study species", see also Wauters et al. 2008), allowing us to use and compare data over several years.

Males were trapped on average more often and in more different traps than females, which is consistent with space use differences between the sexes with males using larger home ranges than females (Wauters and Dhondt 1992; Romeo et al. 2010; Di Pierro et al. 2011). Both sexes were captured in more different traps in OGA and RHE than in CED, which may have been due to different numbers of available traps. This potential bias among study areas was eliminated by standardizing trappability and trap diversity within each study area. The first component of the PCA (PC1), measuring both risk taking and exploration tendency and thus considered as a general boldness score, explained 88% of total variance and therefore was the most relevant component. Nevertheless, we feel that also PC2 is biologically relevant since it allowed to measure a pure exploration tendency. We admit that care is needed in using this component because of the low variance explained, but where models show a significant contribution of PC2, it is not more prone to bias or type I error compared to PC1.

3.5.2 Personality and phenotypic characteristics

Since personality and body growth can be related, body size/mass should be taken into account when testing for effects of personality on reproductive parameters (Rödel and Meyer 2011; Guenther and Trillmich 2015). Several studies on mammals showed that heavier animals are bolder and more explorative than conspecifics of lower body mass, especially among pups (Rödel and Meyer 2011; Guenther and Trillmich 2015; Vetter et al. 2016). Bolder animals also often tend to be the more aggressive ones (proactive-reactive syndrome) (Huntingford 1976; Koolhaas et al. 1999; Bell and Stamps 2004; Sih et al. 2004; Groothuis and Carere 2005) and in our study species, heavier animals were more aggressive than squirrels in poorer condition (Wauters and Dhondt 1989). However, here we found that personality scores of red squirrels were not correlated with body mass or size (foot length). In high-density populations of red squirrels, where encounters between individuals are frequent, body mass tends to increase with age and heavier, older males are more aggressive than lighter, younger ones and these heavier males are more likely to mate (Wauters and Dhondt 1989; Wauters et al. 1990). Heavier, older females occupy higher quality (exclusive) core areas and have higher reproductive success than animals of lower body mass (Wauters and Dhondt 1989, 1992, 1995). We must underline however that in the alpine population studied here, densities were much lower (0.2 to 0.5 squirrels/ha against 0.8 to 1.5/ha in the lowland high-density populations) with less frequent encounters (our unpubl. data) which may lead to animals being less aggressive independent of their body size/mass. This will be tested in future research using Mirror Image Simulation (MIS) in arena tests (e.g. Réale 2007; Boon et al. 2008).

3.5.3 Body condition, personality and survival

In agreement with previous studies, also in the dataset used here, individual variation in body mass is a major factor affecting survival: heavier squirrels of both sexes had a higher local survival than animals of poorer body mass (see also Wauters and Dhondt 1989; Wauters et al. 2007). This is a general pattern found in many mammals for which there is a direct relationship between body mass and survival (Festa-Bianchet et al. 1997) and in relation to food availability (Boutin and Larsen 1993). In fact, average survival was higher in the Scots pine dominated forest of OGA where spatio-temporal variation in tree-seed availability was lower than in the other two habitats (Table 3.1; see also Wauters et al. 2008; Salmaso et al. 2009). In our study, personality was correlated to local survival of both male and female squirrels in different ways according to the study area. In Norway spruce dominated forest (RHE), bolder squirrels of both sexes had a higher survival than shy ones, although the pattern was more evident in females. Home range sizes of male and female red squirrels in this habitat were much larger than in the Scots pine wood of OGA and the mixed conifer forest of CED (Wauters et al. 2005; Romeo et al. 2010; Di Pierro et al. 2011). When spatio-temporal variation in food availability is high, having larger home ranges may allow better access to limited food resources, and bolder/exploring animals may use larger ranges than shy ones (Bell 2007; van

Overveld and Matthysen 2010; Minderman et al. 2010). Alternatively, bold animals may have better resource acquisition abilities when resources are scarce or more widely distributed (Biro and Stamps 2008), since their high activity or exploration (compared to shy individuals) may bring more opportunities to discover essential resources in a heterogeneous environment (Wolf et al. 2007). In contrast, in our other two study areas (OGA and CED), bold animals had lower local survival or residency than shy ones, which is in accordance with Boon et al. (2008) who found that bolder (higher trappability) female American red squirrels (*Tamiasciurus hudsonicus*) were less likely to survive until the spring of the following year. These habitat related differences could be explained by a higher cost of risk taking behavior in the latter two study areas, potentially due to higher risk of predation (Sih et al. 2004; Boon et al. 2008) or parasitism (Morand et al. 2006; Boyer et al. 2010; Patterson and Schulte-Hostedde 2011). Although Eurasian red squirrels have a poor macroparasite fauna (only one gastrointestinal helminth and two ectoparasites that are common, with prevalence > 10%), there is marked variation in parasite abundance and/or prevalence between individuals, at least partly related to variation in host body mass and habitat type (Romeo et al. 2013; Santicchia et al. 2015a). It should be further investigated whether bolder red squirrels indeed have higher parasite infections in some habitats, as was the case in Siberian chipmunks (*Eutamias sibiricus*) (Boyer et al. 2010).

Another possible trade-off in costs/benefits of being bold was found in introduced Siberian chipmunks in France: bolder animals had a higher survival and reproductive success than shy ones in low resource years, but this was not the case when resources were abundant (Le Coeur et al. 2015). In great tits (*Parus major*), faster explorers were favored under lower densities but slower explorers under higher densities and temporal variation in local density represented the primary factor explaining personality-related variation in viability selection (Nicolaus et al. 2016). Our multi-year data on survival in different habitats produced rather similar patterns, with an advantage for bolder squirrels only in the habitat with higher variability in food abundance and squirrel density (RHE, Table 3.1; see also Wauters et al. 2008; Bisi et al. 2016).

In this study we separated a general boldness-exploration component of personality from a second pure exploration component (see also Boon et al. 2008). Increased predation risk, encountering more parasite infective stages and higher energy demands may all be involved in explaining why we always found a negative effect of our pure exploration score (PC2) on local survival (both sexes) and on reproduction (females). However, two points must be made when considering the effects of our exploration score. First, as stated above, it explains only a limited amount of the variation in personality among individual squirrels (12%); hence, its relationships should be interpreted with care. Second, we are measuring local survival of squirrels in the study area: this measure might include animals that disappeared from our study areas due to emigration, especially among those at the lower tail of the survival distribution (those with 6-12 months of local survival) (e.g. Ergon and Gardner 2014). Although most dispersal in red squirrels occurs during the juvenile-subadult phase (Wauters and Dhondt 1993; Wauters et al. 2010, 2011), some adults do undertake dispersal movements (Wauters and Dhondt 1993; Lurz et al. 1997). However, in general site fidelity of adult red squirrels is high and in our study areas 91 out of 107 (85%) radio-tracked adults remained within established home ranges, with most cases of dispersal occurring in RHE in response to a poor seed-crop (e.g. Wauters et al. 2005; Romeo et al. 2010; Di Pierro et al. 2011). We tried to remove dispersers as much as possible from our dataset by using only adults that remained at least for 6 months on the study area and, among radio-tracked animals, using only those that settled. Apart from this methodological point, our pure exploration score (PC2) was negatively related to the fitness components studied (survival and reproduction). Only a few studies found a small positive effect of exploration tendency on survival (and no relationship between exploration and reproductive success, e.g. Smith and Blumstein 2008), whereas others reported negative effects as in this study (Carter et al. 2010; Hall et al. 2015), although often related to early life-stages (Rödel et al. 2015). We only used subadults and adults in our dataset, but still wider movements by pure explorers might increase predation risk and/or levels of chronic stress compared to less explorative animals. How stress levels may be linked to personality under differential conditions of habitat or population density are challenges for future research (e.g. Dantzer et al. 2013, 2016).

3.5.4 Body condition, personality and female reproduction

Boldness and exploration behaviors could influence also female reproduction by different mechanisms. We found that heavier females produced more litters than those of poorer body mass, in agreement with previous studies on red squirrels (Wauters and Dhondt 1989, 1995; Wauters et al. 2007). Because of such strong effects of a female's body condition, variation in personality might have only weak relationships with reproductive success. In this study we found no effect of boldness on reproductive success but a negative effect of exploration. Female red squirrels that explore a lot could be less efficient in obtaining and defending exclusive core-areas (intrasexual territoriality, Wauters and Dhondt 1992), and/or spend less time nursing their offspring. This hypothesis could be tested by radio-tracking squirrels, investigating the relationships of personality with degree of core-area overlap and time spent away from the nest during lactation. Another point to consider is that maybe we need more detailed data of reproductive success: not only number of litters produced, but also data on number of young produced or weaned/litter (Wauters and Dhondt 1995; Santicchia et al. 2015b) to reveal (weak) relationships between personality and reproductive success. Negative effects of exploration on reproductive success were also found in other studies. For example, Vetter et al. (2016) demonstrated that, under high food availability, being less aggressive and less explorative positively influenced the post-weaning litter size in wild boar (*Sus scrofa*).

We believe that our study provides some insight in how personality traits can produce fitness trade-offs under different environmental conditions. So far, our sample size was too small to explore if and how personality might relate to trade-offs among different fitness components, for example differential investment in survival or reproduction. Hence, our results suggest the need for long-term studies measuring individual variation in personality traits, phenotypic factors, space use (home range size, degree of overlap, home range quality) and different fitness components combining data from populations living under different abiotic conditions (habitat types, food supplies) to better assess the impacts of personality on an animal's "performance" both before and during its period of reproductive activity.

Chapter 4

The price of being bold: personality affects endoparasite infection in introduced Eastern grey squirrels

4.1 Summary

This Chapter investigates the role of grey squirrel (*Sciurus carolinensis*) personality (boldness/exploration tendency) in the probability of being infected by their dominant gastro-intestinal helminth *Strongyloides robustus*. Indirect personality indices (trappability as measure of boldness and trap-diversity as a measure of exploration), derived from capture-mark-recapture data and subsequently expressed using the scores of the first Principal Component, were correlated with direct measurement obtained from arena tests. In particular, capture-mark-recapture data (PC scores) and arena test produced consistent measures of the personality trait activity-exploration. Results showed that bolder animals were more likely to be infected by *S. robustus*. The method to determine parasite load described here will be used in Chapter 6. Host personality had no effect on parasite intensity in already infected grey squirrels, which was correlated with host body mass, suggesting a fitness-related energy trade-off that could lead to a less effective immune response. This Chapter describes as differences in personality-related host behavior can result in a non-random distribution of parasites among hosts. Moreover, indirect indices of personality, firstly described in Chapter 3, are here validated and supported by direct measurements. This finding strengthens that trappability and trap-diversity are a reliable method to assess personality in squirrels.

4.2 Introduction

Animal personality has been defined as consistent between-individual differences in behavior maintained over time and/or across ecological contexts (Réale et al. 2007; Dingemanse et al. 2012). Differences in individual personality are measured through traits such as reaction to potential risks or handling novelty, aggressiveness and sociability (e.g. Gosling 2001; Réale et al. 2007).

Among vertebrates, individual variation in personality has been demonstrated to affect variation in fitness among animals within a population (Smith and Blumstein 2008; Biro and Stamps 2008). Discovering important resources (Wolf et al. 2007), acquiring intraspecific dominance (Dingemanse and de Goede 2004; Rödel et al. 2015) and increasing survival (Cavigelli and McClintock 2003) are benefits that have been associated with animal boldness (risk-taking behavior) and/or exploration tendency. However, other studies described contrasting patterns with high costs related to boldness/exploration, such as increased chance to encounter predators (Biro et al. 2004; Sih et al. 2004; Boon et al. 2008), or reduced survival or reproductive success (Réale et al. 2000, 2009; Réale and Festa-Bianchet 2003; Sih et al. 2003).

Another potential ecological effect of personality is its influence on the dynamics of parasite infections (Moore 2002, Hawley et al.

2011, Ezenwa et al. 2017). Risk-taking behavior, exploration tendency, activity and sociality are all traits that may affect hosts' exposure to parasites by altering contact rates among individuals and/or increasing chances of encountering infective stages in the environment (e.g., Moller et al. 1993; Cote and Poulin 1995; Able 1996; Loehle 1997; Altizer et al. 2003; Ezenwa 2004; Hawley et al. 2011; Ezenwa et al. 2017). Bold and explorative animals might not only act as "super-receivers" (individuals that are more likely to acquire parasites), but also as "super-spreaders" by sustaining the spread and transmission of infections (Ezenwa et al. 2017).

The link between personality and parasitism is particularly relevant in the context of invasive alien species (IAS) and their impact on native communities. Parasites may mediate the spread and impact of IAS in multiple ways: on one hand IAS may introduce novel parasites in ecosystems (Tompkins et al. 2011; White et al. 2016), on the other hand they may affect the dynamics of already existing parasites by adding a novel host to the community (Roy and Handley 2012; Telfer and Brown 2012). Therefore, recently, the potential role of behavioural (personality) traits in shaping processes of invasion ecology has received increased attention (Chapple et al. 2012). Some empirical studies have documented that variation in personality traits can affect status and/or intensity of infection by parasites in populations of IAS, suggesting that these interactions between personality traits and parasitism may affect the establishment and/or dispersal success of an invasive species and even its impact on the communities it invades (invasiveness, e.g. Cote et al. 2010; Réale et al. 2010; Sih et al. 2012).

Here we use one of the most successful and impacting invasive alien mammal species, the Eastern grey squirrel (*Sciurus carolinensis*) introduced to N. Italy, to investigate the hypothesis that differences in personality affect individual variation in the occurrence and abundance of endoparasites.

We first present direct (arena test) and indirect methods (capture-mark-recapture indices) that produce reliable and repeatable measures of a grey squirrel's personality and illustrate variation in personality traits. In particular, to calculate indirect indices, we measured boldness and tendency to explore using standardized indices based on the number of times an individual was captured (trappability), and the number of different traps in which it was captured (trap diversity), respectively. These indirect indices have been used in many studies of personality in sciurid rodents (e.g. Boon et al. 2008; Boyer et al. 2010; Le Coeur et al. 2015; Santicchia et al. submitted). If bold animals are also the more active ones, we predict that bolder and/or more explorative grey squirrels will be more likely to encounter free-living infective stages (larvae) of their dominant helminth parasite and consequently will have higher parasite loads than shy individuals. However, we expect a stronger effect of these personality traits on squirrels' infection status rather than intensity of infection (i.e. number of helminths per infected host). This because, by influencing hosts' exposure, boldness and exploration tendency will mostly affect the probability of becoming infected by endoparasites. However, once the host becomes infected, we expect that other, immunity-related, factors may become more important in determining individual parasite load. Indeed, nematodes within the genus *Strongyloides* elicit complex, density-dependent immune responses (Paterson and Viney 2002; Romeo et al. 2014b), and it is thus likely that personality (in term of exposure to parasite infective stages) will have a negligible impact on their numbers, which are instead mainly regulated by immune-mediated processes.

4.2.1 Study species

Eastern grey squirrels have been introduced in the British Isles and in parts of Italy (Teangana et al. 2000; Gurnell et al. 2015; Bertolino et al. 2014), where they determined the decline of native Eurasian red squirrels (*Sciurus vulgaris*) through competition for food resources (Wauters et al. 2002a, b; Gurnell et al. 2004, 2015), and, exclusively on the British Isles, through disease-mediated competition (Sainsbury 2000; Tompkins et al. 2002; Stritch et al. 2015). In Italy, the grey squirrel is an invasive species subject to control for the conservation of native Eurasian red squirrels and Calabrian black squirrels (*Sciurus meridionalis*, Wauters et al. 2017). Consequently, large numbers of carcasses are available to obtain quantitative data on endoparasitic infections. Alien grey squirrels in Italy have fewer macroparasites (both ecto- and endoparasites) than in their native range and only one dominant gastro-intestinal helminth, *Strongyloides robustus*, which they carried along from N. America (prevalence 57% Romeo et al. 2014a). In its native range, this parasitic nematode commonly infects several squirrel species (Chandler 1942) and in Italy was found to spill over to Eurasian red squirrels (Romeo et al. 2015).

4.3 Materials and methods

4.3.1 Study areas, trapping and handling squirrels

We trapped grey squirrels in 5 study areas (Piobesi, RS, Berroni, Commande, MOR) located in Piedmont, Northern Italy. All areas are private woodlands or parks with mature mixed broadleaf forest vegetation and few ornamental conifers, surrounded by agricultural landscapes. Trapping areas corresponded with total woodland/park areas so there was no issue of edge-trap effects (e.g. Boyer et al. 2010). Grey squirrel populations in these high-quality fragments are considered part of a metapopulation which originated from a release that occurred in 1948 (Bertolino et al. 2014).

In each site, we first carried out at least 2 Capture-Mark-Recapture (CMR) sessions (one every two months) to collect data for the estimation of indirect personality indices. In 4 areas (Piobesi, RS, Berroni and Commande) CMR sessions were then followed by a final removal session in which alien squirrels were culled for the subsequent collection of quantitative parasitological data.

The number of traps used varied slightly between sessions and/or study areas. A trapping session involved the use of 16 (Piobesi), 16 (RS), 17 (Berroni), 30 (Commande), 35-48 (MOR) ground-placed Tomahawk “squirrel” traps (models 201 and 202, Tomahawk Live Trap Co., WI, USA). Trapping, handling and the determination of sex and age were carried out as described in detail elsewhere (Wauters and Dhondt 1995; Wauters and Gurnell 1999; Gurnell et al. 2001). During removal sessions, alien squirrels were culled by CO2 inhalation following EC and AVMA guidelines (Close et al. 1996; Leary et al. 2013) and each carcass was immediately placed in a sealed plastic bag and stored at -20°C for later examination. Trapping and handling squirrels complied with current laws on animal research in Italy and were carried out with permit of the authorities for wildlife research and management of Turin and Cuneo Provinces (Respectively, D.D. 294-34626 of 2014 and Prot. n. 0002624 of 13/01/2014) and of the Italian Institute for Environmental Protection and Research (ISPRA).

To obtain density of hosts in each study area (population size divided by trapping area), we estimated the population size using mark recapture model with Schumacher Eschmeyer estimator (Krebs 1989) as implemented in the R (R Development Core Team 2017) package *fishmethods* (Nelson 2017). This model estimates population abundance through capture, recapture and newly marked animal data over all capture sessions, assuming a closed population during the entire trapping period (Krebs 1989). Thus, we calculate population sizes only considering capture sessions without grey squirrel removal to meet the assumption of closed populations.

4.3.2 Personality indirect indices

We calculated two indirect indices of personality: a trappability and trap diversity index. The first is a measure of tendency to take risks (boldness), the second of the animal’s propensity to explore novel environments. Trappability was estimated using the ratio of number of captures on the length of capture period (i.e. the number of trap checks, from now on occasions, from the first to the last trapping session for a given animal). Trap diversity was estimated using the ratio of number of different traps in which an animal was caught on the average number of available traps from the first to the last session. Subsequently, both ratios were standardized per study area because of differences in capture histories and number of available traps. Recent studies have shown that personality traits to some degree can change over time and with age (flexible component of personality, e.g. Dingemanse et al. 2012), therefore we excluded juvenile squirrels from the analysis. In addition, to avoid any potential bias due to squirrel dispersal during late summer-autumn (Koprowski 1994), and any possible effect of removal, we calculated trappability and trap diversity considering only capture sessions from late October to May, hence without using the removal session.

Before analysis, we checked for independence between the two standardized personality indices (see also Chapter 3 and Santicchia et al. submitted). Since trappability and trap diversity were strongly and positively correlated ($r = 0.85$; $n = 207$; $p < 0.0001$), they could not be added together as explanatory variables in multivariable models because of lack of independence when estimating their partial effects. Therefore, we used a principal component analysis (PCA) to derive new variables, the principal components which maximize the variance of these among individual squirrels. However, since the first component (PC1) explained 92% of total variance, we only retained this variable as a single measure of an individual's personality. PC1 had a high score for those animals that are often captured and in many different traps, so it can be considered as a measure of personality which includes both risk taking and exploration.

Repeatability of the two indirect indices was calculated on a subsample of 127 individuals that were caught in at least two capture sessions. We applied the function *rpt*, using bootstrap and permutation, as implemented in the R (R Development Core Team 2017) package *rptR* (Stoffel et al. 2017) on a Linear Mixed Model (LMM) to estimate confidence intervals and standard errors (Nakagawa and Schielzeth 2010). We included study area, sex, session, number of occasions, number of active traps as fixed effects and squirrel identity as random intercept. The likelihood ratio test (LRT) was used to test for significance of the random effect of each model (Pinheiro and Bates 2000; Martin and Réale 2008). Since in several species, personality traits can be related to body size or body mass (e.g. Martin and Réale 2008; Rödel and Meyer 2011), we also checked for correlations between squirrel's body size (foot length) and mass with its PC1 score.

4.3.3 Personality: direct measurements

On a different subset of 83 individuals, from Commande and MOR study areas, we performed arena test experiments which consisted of two parts (Table 4.1 ; S1). First an Open Field Test (OFT) to estimate activity and exploration levels in a novel environment (Walsh and Cummins 1976; Martin and Réale 2008), followed by Mirror Image Stimulation (MIS) to test aggressiveness, sociability or avoidance towards conspecifics (Svendsen and Armitage 1973). We performed OFT (6 min) and MIS (4 min) for each individual only once per capture-session to reduce stress and habituation in animals (minimum time between tests for the same individual: 26 days). In addition to check the assumptions of repeatability of personality-linked behavior we repeated both experiments (OFT and MIS) in different capture-sessions to have at least two arena tests for part of the individuals ($n = 37$). For each experiment we calculated the time that individuals spent in each behavioral state using the CowLog 3.0.2 software (Hänninen and Pastell 2009). Ethogram described in Table S1.

To reduce the number of behaviors observed into few personality-linked variables, we run a Factor Analysis (FA) with *varimax* rotation, on OFT and MIS separately. To select the number of factors to retain, we used *fa.parallel* function estimating communalities with the first factor using SMC (Squared Multiple Correlation), as implemented in the R (R Core Team, http://www.r-project.org) package *psych* (Revelle 2017). We also tested FA retaining different numbers of factors, exploring how behaviors were classified in one or more factors: number of factors retained with this biological criterion matched the previous approach. We derived 2 factors (FA) for OFT and 3 factors for MIS. Furthermore, we also defined expert-based behavior groups (each group corresponding to a personality-trait) to reduce the number of variables summing the percentages of those behaviors considered related to a personality-trait, relying on ethological knowledge (Wauters and Dhondt 1987, 1989; Wauters et al. 2001) and operator observation of videos (Table S2).

Aggressiveness was considered as the number of attacks towards the mirror during MIS. We did not include grooming in any of the expert-based groups since it is considered a behavior not related to squirrel's personality. To explore whether expert-based groups gave similar results as Factor Analysis, we calculated Pearson correlation coefficients between expert-based groups and each factor derived from Factor Analysis (for both OFT and MIS experiments) using square root transformed proportions to meet the assumption of normality.

Using a restricted sample of 37 individuals for which two arena tests were done we estimated repeatability, as described above. For each personality variable we run a LMM including study area, sex, experiment order (as indication of first experiment or subsequent ones) and squirrel identity as random intercept.

Table 4.1 – Structure of the different datasets used: sample size and the study areas for the type of data available and used in the different models.

Type of data used	**Study area**	**Sample size**
TRAPPABILITY AND TRAP DIVERSITY ESTIMATES	Berroni Commande MOR Piobesi RS	207 individuals
ARENA TEST	Commande MOR	128 experiments of 83 individuals
PARASITOLOGICAL EXAMINATION	Berroni Commande Piobesi RS	77 individuals

4.3.4 Parasitological examination

On a total of 95 culled grey squirrels, 77 also had personality measured and were examined for gastro-intestinal helminths following standard parasitological procedures (Romeo et al. 2013, 2014a; Table 4.1). *S. robustus* individuals were identified morphologically (Chandler 1942; Sato et al. 2007) and counted as described in Romeo et al. (2014a).

4.3.5 Statistical analyses

Reliability of indirect indices of personality is often explored by testing the degree of support from direct measurements (Boon et al. 2008; Boyer et al. 2010). To check for correlation between PC1 scores and direct measurements (OFT and MIS) we selected only the first experiment to avoid any potential problem of habituation (n = 83). Correlation between indirect indices and direct measurements were analyzed using Pearson's correlation.

We explored the effect of personality on four different descriptors/parameters of parasite infection, each one used as response variable in a specific Generalized Linear Mixed Model (GLMM) with random intercept. We first explored variation in *S. robustus* abundance (number of helminths per host, including uninfected hosts), which can be considered as a combined measure of both parasite prevalence (number of infected hosts/number of examined hosts) and parasite intensity (number of helminths per infected host) (Bush et al. 1997; Roszà et al. 2000). Then we ran two specific models on *S. robustus* intensity and infection status (infected/not infected) to disclose whether personality is equally related to different aspects of parasite infection. Finally, we investigated variation in helminth richness (number of different helminth species per examined host).

Variation in infection status (n = 77) was explored through logistic regression whereas variation in helminth richness was investigated through GLM with Poisson error distribution, whilst variation in *S. robustus* abundance (n = 77) and intensity (n = 56) was analyzed through GLMMs with negative binomial error distribution, in order to account for the aggregate distribution of parasites within the host population (Shaw et al. 1998). In all models, we examined the effect on infection of host personality (PC1 score), body mass and sex, and included study area as a random factor to account for extrinsic heterogeneities among sites (mainly density of hosts, see Table 4.1). Second order interactions between sex and personality and sex and body mass were also included in full models but were eliminated when not significant. Seasonal variation in infection parameters was not considered because all parasitological data were obtained from squirrels culled from the end of October to early January. Before model building, all the explanatory variables were examined for covariance and no collinearity issues were detected. Interpretation of final models was based on pair-wise t-test contrast of Least-Square Means (LSM), applying sequential Bonferroni correction (Holm 1979) for multiple comparisons. Unless otherwise specified, all values and parameter estimates are reported as mean (± SE). All the statistical analyses were carried out using the software R 3.3.3 (R Development Core Team 2017).

4.4 Results

4.4.1 Personality: direct measurements

We performed 128 arena tests on 83 different grey squirrels (36 males, 47 females); 37 animals were tested at least twice. We captured 48 individuals in MOR study area (22 males, 26 females) and 35 individuals in Commande (14 males, 21 females); 74 animals were newly captured at the time of the first experiment (41 MOR, 33 Commande) and 9 were recaptures (7 MOR, 2 Commande).

Individual differences in behavior among grey squirrels in the arena were large, with some single behaviors varying between 0 and 99% of time spent in the arena (Table 4.2). The most common behaviors were Locomotion, Scan, Immobile, Rise and Sniff during the OFT; and Back, Locomotion and Scan, during MIS (Table 4.2). We recorded only 1 aggressive interaction towards the mirror; hence MIS could not be used to estimate aggressiveness in this species. Also when considering the expert-based behavioral groups linked to personality traits, the variability between individual squirrels remained high. Overall, in OFT, activity and shyness were more common than exploration, and in MIS avoidance and “other” (behaviors related to activity or exploration) occurred most frequently (Table 4.2).

Factor Analysis (FA) produced two factors for OFT (cumulative variance explained 62%) and three for MIS (cumulative variance explained 53%) (Table 4.3).

In OFT the first factor had high scores for active and explorative behaviors, while the second factor had high scores (positive for hang and negative for immobile) for non-active, shy animals. Also in MIS, the first factor had high scores for active-exploration behaviors. The second factor was associated with social behaviors (sitting close to mirror and slow approach or no aggressive contact with mirror), while the third factor included those behaviors related to immobility (back of arena or hanging) and being alert, that highlight the propensity to avoid interaction with the mirror. Hence, FA3 high scores measured avoidance behavior.

Table 4.2 – Proportion time spent in different behaviors and expert-based behavior groups (hereinafter personality traits) in OFT and MIS experiment. Min and max: minimum and maximum value of proportion time, Mean (± SE): mean value of proportion time ± standard error. Sample size between brackets.

	OFT (n = 128)				**MIS (n = 128)**		
Behavior	**Min**	**max**	**mean (± SE)**	**Behavior**	**min**	**max**	**mean (± SE)**
Head dip	0	0.25	0.03 ± 0.004	Front	0	0.99	0.14 ± 0.03
Hang	0	0.99	0.15 ± 0.03	Locomotion	0	0.60	0.11 ± 0.01
Immobile	0	0.99	0.75 ± 0.03	Scan	0	0.59	0.18 ± 0.02
Locomotion	0	0.49	0.15 ± 0.01	Watch	0	0.98	0.17 ± 0.02
Rise	0	0.35	0.13 ± 0.01	Back	0	0.99	0.61 ± 0.03
Scan	0	0.63	0.28 ± 0.01	Rise	0	0.52	0.08 ± 0.01
Sniff	0	0.47	0.17 ± 0.01	Sniff	0	0.42	0.07 ± 0.01
Scratch	0	0.83	0.08 ± 0.01	Hang	0	0.99	0.14 ± 0.03
				Head dip	0	0.15	0.01 ± 0.003
				No-aggressive	0	0.38	0.04 ± 0.01
				Scratch	0	0.57	0.03 ± 0.01
				Slow	0	0.38	0.03 ± 0.01
Behavior group/Personality trait							
SHYNESS	0.31	0.99	0.86 ± 0.01	SOCIABILITY	0	0.99	0.17 ± 0.03
EXPLORATION	0	0.93	0.21 ± 0.02	AVOIDANCE	0	0.99	0.73 ± 0.03
ACTIVITY	0	0.71	0.36 ± 0.01	OTHER	0	0.91	0.28 ± 0.02
				ALERT	0	0.98	0.17 ± 0.02

Our expert based grouping into personality traits was supported by significant correlations between FA scores and the personality traits. For OFT (all n = 128), FA1 scores were positively correlated with exploration (r = 0.82, p < 0.001) and activity (r = 0.78, n = 128, p < 0.001), but negatively with shyness (r = -0.89, p < 0.001). FA2 scores were positively correlated with exploration (r = 0.27, p = 0.002) and negatively with shyness (r = -0.19, n = 128, p = 0.03). Thus, squirrels that are more active and explorative had a high FA1 score, shy animals a low FA1 score. For MIS (n = 128), FA1 scores were strongly and positively correlated with the group of other behaviors (active and exploration, r = 0.87). FA1 was also positively but weakly correlated with sociability (r = 0.24, p = 0.007) and alert (r = 0.21, p = 0.02) and negatively with avoidance (r = - 0.33, p < 0.001). FA2 scores were strongly and positively correlated with sociability (r = 0.97, p < 0.001) and strongly but negatively with avoidance (r = -0.79, p < 0.001). Hence, FA2 high scores are related to social squirrels and low scores to individuals avoiding the mirror image. FA3 scores were negatively correlated with alert behavior (r = -0.22, p = 0.01). In summary, active-exploring grey squirrels had high FA1 scores also in MIS. Social animals had high, and avoiding-shy animals low FA2 scores. Taking into account that MIS is performed to measure sociability-aggressiveness in relation to a conspecific, FA1 was, biologically, not the most important factor, since it measured those personality traits already explained by FA1 in OFT (active-exploration behavior). It must also be underlined that we recorded only one event of attack towards the mirror during MIS experiment.

The repeatability (n = 37) of the factors from FA and of the expert-based personality traits varied considerably (Table 4.4). In OFT, repeatability was significant for FA1 and also for the activity personality trait. Lower, and not significant for FA2 and shyness. Similarly, in MIS FA1 and other (active-exploration trait) had both high repeatabilities, and so had sociability (Table 4.4).

Table 4.3 – Factor Analysis for OFT and MIS.

Behavior	FA1 OFT	FA2 OFT	Behavior	FA1 MIS	FA2 MIS	FA3 MIS
Head dip	0.75		Front	0.11	0.99	
Hang	- 0.21	0.93	Locomotion	0.57		
Immobile	- 0.13	- 0.99	Scan	0.67	0.10	- 0.18
Locomotion	0.60		Watch	0.19		- 0.22
Rise	0.80		Back		- 0.52	- 0.72
Scan	0.52	- 0.28	Rise	0.74		
Sniff	0.83		Sniff	0.84	0.15	
Scratch	0.45	0.44	Hang	- 0.20	- 0.20	0.96
			Head dip	0.53		
			No-aggressive	0.49	0.52	
			Scratch	0.22		0.21
			Slow	0.69	0.26	
% Total variance	35%	27%		26%	14%	13%
% Cumulative variance	35%	62%		26%	40%	53%

4.4.2 Personality: indirect indices

We captured a total of 207 individuals (Table 4.1) Number of captures per individual grey squirrel varied between 1 and 10 (median 2, mean ± SE = 2.61 ± 0.12 captures), and number of different traps from 1 to 7 (median 2, mean ± SE = 1.98 ± 0.08 traps). There was no difference between the sexes in either mean number of captures ($F_{1, 201}$= 1.24, p = 0.27) or mean number of different traps between the sexes ($F_{1, 201}$= 1.79, p = 0.18).

Number of captures differed significantly between study areas ($F_{4, 201}$= 4.58, p = 0.001). It was higher in Piobesi and Berroni than in the other three study areas (DLSM, all $p < 0.05$; Berroni n = 33, 3.19 ± 0.32 captures; Piobesi n = 32, 3.44 ± 0.30 captures; Commande n = 40, 2.00 ± 0.20 captures; RS n = 47, 2.43 ± 0.22 captures; MOR n = 55, 2.4 ± 0.26 captures). The average number of different traps in which a squirrel was caught also differed between study areas ($F_{4, 201}$= 5.41, p = 0.0004). It was significantly higher in Piobesi than in Commande, MOR and RS (DLSM, all $p < 0.05$; Piobesi n = 32, 2.53 ± 0.22 traps; Commande n = 40, 1.8 ± 0.17 traps; MOR n = 55, 1.67 ± 0.12 traps; RS n = 47, 1.83 ± 0.13 traps), and also higher in Berroni than in MOR (DLSM, $p < 0.05$; Berroni n = 33, 2.39 ± 0.20 traps). These differences between areas did not influence our results since trapping indices were standardized within study areas.

We observed consistent individual variation, across trapping session, in number of captures (n = 127; repeatability ± SE = 0.25 ± 0.07, 95% CI = 0.14 – 0.41, Likelihood ratio test LRT = 13.9; df= 1; $p < 0.0001$) and in trap diversity (n = 127; repeatability ± SE = 0.29 ± 0.07, 95% CI = 0.18 – 0.45, Likelihood ratio test LRT = 18.9; df= 1; $p < 0.0001$). Thus, trappability and trap diversity show some degree of consistency through time supporting that they are reliable estimates of a grey squirrel's personality.

4.4.3 Relationship between indirect indices and direct measurements of personality

Among those grey squirrels for which we had both the indirect PC1 personality score from trapping data and the arena-test based direct measures of personality (n = 83), we found a positive and significant correlation of PC1 scores with behavior group "Other" from MIS (r = 0.27, p = 0.02) and nearly significant positive correlation with FA1 from MIS (r = 0.22, p = 0.05). "Other" included behaviors related to activity and exploration, and FA1 had high scores for active-exploring individuals. Hence arena-test and capture-mark-recapture data (PC1 score) produced consistent measures of the personality trait activity-exploration.

Table 4.4 – Personality variables details and repeatability on 37 arena tests. R: repeatability value, SE: standard error, 95% CI: confidence interval, LRT: likelihood ratio test value, df: degree of freedom, p: p value.

Personality variable	R	SE	95% CI	LRT	df	p
Factor Analysis						
FA1 OFT	0.38	0.14	0.11 – 0.65	4.97	1	0.01
FA2 OFT	0.22	0.14	0 – 0.52	1.31	1	0.13
FA1 MIS	0.40	0.13	0.14 – 0.67	6.78	1	0.01
FA2 MIS	0.24	0.14	0 – 0.55	1.72	1	0.10
FA3 MIS	0	0.10	0 – 0.34	0	1	1
Expert-based group						
ACTIVITY OFT	0.45	0.13	0.21 – 0.70	8.6	1	0.002
EXPLORATION OFT	0.18	0.14	0 – 0.49	0.83	1	0.18
SHYNESS OFT	0.13	0.14	0 – 0.47	0.33	1	0.28
OTHER MIS	0.49	0.12	0.25 – 0.72	10.3	1	0.001
AVOIDANCE MIS	0.20	0.14	0 – 0.51	1.46	1	0.11
SOCIABILITY MIS	0.39	0.13	0.13 – 0.65	5.69	1	0.01
ALERT MIS	0.06	0.12	0 – 0.39	0.08	1	0.39

4.4.4 Parasitological examination

Overall 56 out of 77 grey squirrels were infected by *Strongyloides robustus* (prevalence 73% ± 5.1%). Parasite abundance varied from 0 to 86 helminths per host (mean ± SE = 7.8 ± 1.6) and mean intensity (mI) of *S. robustus* was 10.7 ± 2.1 helminths per host. Only 12 grey squirrels hosted also other helminth species: *Trichostrongylus spp.* was found in 10 squirrels (13%, mean Intensity ± SE: 2.9 ± 0.6) and in two hosts we found 6 oxyurid nematodes and 1 specimen of cestode, respectively, but these parasites were not included in the analyses. Helminth richness varied from 0 to 3 different species per host. Results of parasitological analysis by study area are given in Table 4.5.

4.4.5 Relationship between body mass, personality and parasite infection

Body mass of grey squirrels was positively correlated with both foot length and PC1 score. When analyzed per sex, this pattern was consistent in females, but not in males where body mass was not correlated with PC1 score (Table 4.6).

The first, general model on *S. robustus* infection showed that endoparasite abundance in grey squirrels increased with both the host's PC1 score and body mass (Chapter 6 Figure S1). However, further models showed that infection status is affected only by PC1 score, with bolder, more explorative individuals having a higher probability of being infected by *S. robustus*. Conversely, intensity of infection did not vary with PC1 score, but was positively affected by host body mass. There was no significant effect on helminth richness, neither significant difference between males and females in either of the infection parameters and interactions of host sex with either PC1 score or body mass were not significant and were thus excluded from all the final models (all p > 0.22). Detailed results of the three models and estimates of parameters are presented in Table 4.7.

Table 4.5 – Data on *Strongyloides robustus* infection in grey squirrel hosts and on grey squirrel population size and density per study area. N = number of host examined; n = number of infected hosts; p = prevalence; mean abundance of *S. robustus*; mI = mean intensity (n. parasites infected/hosts); number of squirrels present on the study areas and squirrel density.

Study area	**n (p)**	**mean abundance ± SE**	**mI ± SE**	**n. of grey squirrels***	**Squirrel density (ind/ha**)**
Berroni (N = 8)	1 (12.5%)	0.25 ± 0.25	2.0 ± 0.0	30	6.1
Commande (N = 27)	18 (67%)	2.26 ± 0.61	3.39 ± 0.79	49	15.2
Piobesi (N = 18)	15 (83%)	19.0 ± 5.9	22.8 ± 6.62	29	11.3
RS (N = 24)	22 (92%)	8.17 ± 1.57	8.91 ± 1.62	46	7.8

* Schumacher-Eschmeyer (1943) estimator in Krebs (1989)

** Berroni (4.9 ha); Commande (3.2 ha); Piobesi (2.6 ha); RS (5.9 ha)

Table 4.6 – Pearson's correlation coefficient with significance level for correlations between foot length, body mass and PC1 of grey squirrels: all animals; and results per sex. Sample size between brackets.

Both sexes (n = 77)	**Foot length**	**PC1 (Boldness and exploration)**
Body mass	r = 0.41, p = 0.001	r = 0.28, p = 0.01
Foot length		r = 0.07, p = 0.58
Males (n = 29)		
Body mass	r = 0.42, p = 0.03	r = 0.25, p = 0.20
Foot length		r = 0.21, p = 0.30
Females (n= 48)		
Body mass	r = 0.43, p = 0.008	r = 0.32, p = 0.03
Foot length		r = -0.05, p = 0.77

Table 4.7 – Minimum selected models explaining observed variation in probability and intensity of infection by *Strongyloides robustus* and its abundance in grey squirrel hosts.

Response variable	**Predictor**	**Parameter estimate**	**df**	**χ^2**	**p**
Abundance	sex†	-0.30 ± 0.29	1	1.07	0.30
	bm	**0.008 ± 0.003**	**1**	**5.86**	**0.016**
	PC1 score	**0.24 ± 0.12**	**1**	**4.08**	**0.04**
Infection status	sex†	0.76 ± 0.68	1	1.24	0.27
	bm	0.01 ± 0.01	1	1.66	0.20
	PC1 score	**0.69 ± 0.31**	**1**	**4.85**	**0.03**
Intensity	sex†	0.12 ± 0.24	1	0.24	0.63
	bm	**0.007 ± 0.003**	**1**	**5.89**	**0.02**
	PC1 score	0.10 ± 0.10	1	0.97	0.32
Helminth richness	sex†	0.10 ± 0.09	1	1.08	0.30
	bm	0.0002 ± 0.003	1	0.01	0.94
	PC1 score	-0.01 ± 0.25	1	0.01	0.96

†males held as reference level

4.5 Discussion

We showed that trappability and trap-diversity indices were repeatable between trapping sessions and that the derived score from the first principal component (PC1 score) can be considered as a measure of personality which includes both risk taking and exploration behaviors. Moreover, it was correlated with a direct measurement (arena test) of the personality trait activity-exploration (FA1 score and "Other" in MIS). Hence, both methods produced consistent measures of the personality trait activity-exploration.

Exploring whether Eastern grey squirrel

personality differences affected individual variation in endoparasite infection, our results supported the first, general prediction that bolder, more explorative animals are more heavily infected by *S. robustus* than shy grey squirrels. However, we also found that host personality mainly influences the probability of acquiring *S. robustus*, whereas it has no effect on parasite intensity in already infected hosts, which is instead affected by host body mass. There was no significant effect on helminth richness. The latter is not a surprise since the populations of this invasive species in Italy are characterized by poor macroparasite fauna, linked to a marked parasite-release in comparison with populations in the native range (e.g. Romeo et al. 2014a).

4.5.1 Direct measurements of personality

Personality can be measured using behavioral tests, such as open field test (Réale et al. 2007; Martin and Réale 2008) and a mirror-image stimulation test (Svendsen and Armitage 1973), performed in a closed arena: a novel open space from which escape is prevented by a surrounding wall (Walsh and Cummins 1976). Open-field tests have been performed since early nineties on a variety of laboratory and domestic animals (rats: Hall 1934; Bronstein 1972; Archer 1973; mice: Nagy and Holm 1970; Wilson et al. 1976; mongolian gerbils: Oldham and Morlock 1970; domestic fowl chicks: Heiblum et al. 1998 and rabbits: Daniewski and Jezierski 2003). More recently this behavioral test has been used to measure personality in free-ranging animals, especially in rodents (Martin and Réale 2008, Lantova et al. 2011, Montiglio et al. 2012, Malange et al. 2016). Also mirror image stimulation test has been used to measure personality: in fish (Baenninger 1966), birds (Gallup and Capper 1970), mammals (Svendsen and Armitage 1973; Dochtermann and Jenkins 2007) and in free-ranging rodents (Blumstein et al. 2006; Boon et al. 2008; Shonfield et al. 2012; Kelley et al. 2015; Krause et al. 2015).

Here we used two different methods to score behaviors observed during arena test (OFT and MIS). Firstly, we applied a factor analysis to derive independent axes of correlated personality traits, secondly we used an expert-based approach to obtain a subjective assessment of personality traits. Carter et al. (2013), in a review on how animal personality is measured by behavioral ecologists, recommended factor analytic approach, despite the fact that several studies discourage the usage of that data-reduction techniques (Gosling 2001; Bell 2007; Carter et al. 2013). Personality traits identification should be supported by the usage of multi-test approach that allows to assess the test's appropriateness (Campbell and Fiske 1959; Carter et al. 2013). Therefore we used an expert-based trait selection, based on knowledge of *S. carolinensis* behavior and ecology that allowed to validate the purely statistical factor analysis approach, providing further aid for a biological interpretation of the behaviors defined in the ethogram (Burns 2008; Réale et al. 2007; Carter et al. 2013). The first factor derived from OFT and MIS had high scores for active and explorative grey squirrels, the second factor (MIS) had high scores for social animals and low scores for avoiding-shy animals. Both OFT and MIS gave similar results for individual squirrels and highly significant correlations between the factor analysis scores and the expert-based values indicated that arena test is a suitable method to define and measure personality traits in grey squirrels.

As described above several studies since nineties used arena test (e.g. open field and mirror image stimulation test) to assess animal personalities. Nevertheless, some limitations are still rising, especially regarding: (i) habituation to subsequent tests, (ii) measuring fear or anxiety instead than target behaviors, and (iii) capability of the tests to distinguish between activity and exploration (Martin and Réale 2008; Carter et al. 2013). Our observations suggest that at least part of these limitations also hold for our study species. We tried to reduce habituation by performing only one test per trapping session for a given animal. However, Carter et al. (2013) suggested that the context of the open-field test (free vs. forced test context) may create bias in behaviors: free open-field tests are more likely to measure voluntary exploration/curiosity and information gathering behavior, while forced open-field tests might also be measuring fear or anxiety (or both) (Misslin and Cigrang 1986). Hence, performing open-field test could lead to measuring different personality traits simultaneously determining an 'overlap' (Réale et al. 2007; Carter et al. 2013). Such overlap problem means that one test can be simultaneously influenced two different personality traits, hence measuring a mixture of them (Réale et al. 2007; Carter et al. 2013). This could be a problem related to mirror image stimulation test. Furthermore, a problem with MIS is that the animal completely controls the image that cannot respond independently. Svendsen and Armitage (1973), in a study on marmots (*Marmota flaviventris*), explained how during MIS the reflected animal is as active as the interacting animal and the image can never present a submissive or aggressive gesture unless it is initiated by the latter. This could raise questions if overall we were really measuring reaction to a conspecific or simply curiosity, and can also explain why in our case we had only one case of aggressive interaction with the mirror.

Our study species, the grey squirrel, is a semi-social animal, and does not defend an individual territory as for example North American red squirrels, whose aggressiveness was successfully measured by mirror image stimulation test (Boon et al. 2008; Shonfield et al. 2012; Kelley et al. 2015). Invasive grey squirrels, occur at high densities respect to native squirrel species (Shuttleworth et al. 2016), and among resident adults exists a dominance hierarchy related to environmental cues (important resources inside the animal's home range, Koprowski 1993a). Since inside the arena these environmental cues are lacking, MIS might be still sufficient to measure an individual's general tendency to behave sociable or to avoid staying near a conspecific (avoidance), but not to record aggressiveness. Our results from factor analysis support this assertion, indeed FA2 correlated positively with sociability and negatively with avoidance.

4.5.2 Indirect indices of personality

In several studies on free-ranging rodents in natural environments, animal personality has been estimated through capture-mark-recapture data (Boyer et al. 2010; Patterson and Shulte-Hostedde 2011; Montiglio et al. 2012; Le Coeur et al. 2015). Trappability is generally linked to risk taking behavior and the tendency to visit more different traps (trap diversity) is considered as a measure of exploration (Boon et al. 2008; Boyer et al. 2010; Le Coeur et al. 2015). In sciurid rodents, these two indices have been shown to be repeatable measures of personality, but they also tend to be correlated (Boon et al. 2008; Boyer et al. 2010). Our data agree with these previous findings and showed that the number of captures and the number of different traps are repeatable measures to estimate personality traits in grey squirrels (number of captures: r= 0.25; trap diversity: r=0.29). Moreover, they are within the range of repeatability values reported in literature on different taxa (Bell et al. 2009). Our multi-test approach to assess the validity of different (direct and indirect) measures was recommended by Carter et al. (2013) in their review, who suggested that ecologists should choose the appropriate behavioral tests in relation to study species. For instance, in sciurid rodents, trappability and trap diversity have been used in many studies and allow to assess voluntary boldness-exploration behavior instead of the more "forced" contexts measurements in arena tests (Misslin and Cigrang 1986). If our indices of personality traits are biologically reliable, both the unforced (capture data) and forced (arena test) measures should give comparable results. For example, in a population of wild lemurs, boldness measured in an experimental context (novel object test) was correlated with risk taking in a foraging task in nature, suggesting that both types of methods can be reliably used to estimated personality traits in wild animals (Dammhahn and Almeling 2012). Similarly, we found that indirect (PC1 score) and direct/experimental measures of a grey squirrel's personality trait boldness-exploration were consistent, although the significant correlation between the two indices was rather weak (r = 0.27).

4.5.3 Personality-parasite relationship

Several aspects of an animal's behavior are associated with exposure to some type of parasites. Ezenwa et al. (2017), in a review on host behavior-parasite relationships, highlighted how behavior and parasitism "are so tightly intertwined that we often cannot understand one without considering the other".

Different parasite groups or species will have different transmission strategies and variation in host behaviors are likely to influence exposure to different parasite infective stages depending on how they are transmitted (Barber et al. 2016; VanderWaal and Ezenwa 2016). Hammond-Tooke et al. (2012), in a study on the common bully (*Gobiomorphus cotidianus*), showed that parasitism is linked to specific behavioral traits and different parasite species are related in different ways to the consistency of behavioral responses displayed by individual fish. Their findings suggested that parasitism can modify animal personality (Barber and Dingemanse 2010), but the causality may be also reversed, such that more active and exploratory fish might have acquired more infections by high probability of encountering parasites (Wilson et al. 1993; Barber and Dingemanse 2010; Kortet et al. 2010; Poulin 2013).

Our findings show a positive relationship between PC1 (boldness/exploration) and *S. robustus* abundance and prevalence. After *S. robustus* eggs are shed with host feces, they hatch in the environment and worms undergo two moults until third-stage, infective larvae infect a new host by penetrating its skin (Bartlett 1995). *S. robustus* infective stages will thus likely be aggregated on the ground beneath tree canopies, and in general in those areas used by arboreal squirrels for foraging, seed-caching activities. Additionally, squirrels' dens and dreys have been suggested as a microenvironment that might favor eggs hatching and larvae development and survival (Wetzel and Weigl 1994; Bartlett 1995; Romeo et al. 2015). Hence, other than on the ground, infection by free-living, skin-penetrating larvae could occur inside the dens, and transmission might be facilitated by nest-sharing behaviors (Romeo et al. 2015). If bolder, more explorative squirrels forage more widely, interact more frequently with other squirrels and/or use more different nest sites (e.g. red squirrels, Wauters and Dhondt 1990) they are more likely to encounter free-living infective stages and become infected by *S. robustus* (e.g. Boyer et al. 2010 for ectoparasites).

Our data also confirmed earlier results, from study on a larger scale, that *S. robustus* abundance varied positively with host body mass (Romeo et al. 2014a). However, when the two components that determine parasite abundance (infection status and intensity of infection) were analyzed separately, we found that body mass did not explain probability of infection but correlated positively with intensity. In other words, once infected, heavier grey squirrels tended to host more *S. robustus* worms than animals of lower body mass.

Our results suggest that number of helminths per infected host was not influenced, as would be expected, by probability of encounter and re-encounter parasites infective stages, but, instead, determined by a predominant role of host body mass. This could be explained by heavier, often older and more dominant squirrels, investing more energy in spacing behavior (larger home ranges, Don 1983) survival and reproduction than in the immune system (Wauters and Dhondt 1989, 1992; Sheldon and Verhulst 1996; Boyer et al. 2010; Romeo et al. 2014b). Thus, such energy trade-offs could lead to an ineffective immune response against parasite infection (Pelletier et al. 2005). Indeed, immune response in case of infections with helminths of the genus *Strongyloides* is complex, and high intensity of infection could stimulate the host immune response and act as infection regulator (Paterson and Viney 2002; Romeo et al. 2014b).

Similar findings on relationship between parasites and personality or body mass (or body condition index) were found in other sciurid species. Ecoparasite infestation (abundance) has been reported to be positively influenced by exploration tendency and body condition index in least chipmunk (*Tamias minimus*: Bohn et al. 2017). Moreover, activity-exploration (related to space use) personality traits can positively influence tick load (ectoparasite abundance) in Siberian chipmunk (*Tamias sibiricus*: Boyer et al. 2010), and bold and dominant males with higher testosterone levels were found to suffer higher endoparasite loads in eastern chipmunk (*Tamias striatus*; Patterson and Shulte-Hostedde 2011). These patterns were explained by possible energy trade-offs with bold, active and/or dominant individuals investing heavily in mating (males) or breeding (females) success, resulting in reduced energy available for the immune system and/or other life history functions (Sheldon and Verhulst 1996; Pelletier et al. 2005; Millesi et al. 1998; Lane et al. 2010).

In conclusion, personality variation could be examined in the light of costs and benefits, which allow bold phenotypes to persist despite the higher parasite risk associated with bold behaviors (Ezenwa et al. 2017). Bolder, active and explorative individuals can be advantaged in territoriality or social dominance and in reproduction, but are subject to costs associated to increased encounter with predators and/or parasites, with potential negative effects of the latter on the immune system (Biro et al. 2004; Boon et al. 2008; Boyer et al. 2010; Patterson and Shulte-Hostedde 2011; meta-analysis by Smith and Blumstein 2008). We believe that our study provides some insight in how personality affects parasite transmission and, conversely, how parasites could lead to differential risk associated to individual behavior. In a next step, we will explore possible relationships between parasite infection and physiological stress and their potential influence on reproductive success and/or investment. In addition, we will investigate if these relationships are mediated by individual variation in personality of grey squirrels.

Chapter 5

The use of uterine scars to explore fecundity levels in invasive alien tree squirrels

5.1 Summary

This Chapter describes a staining technique of uterine scars that allow to determine female individual fecundity (seasonal and annual number of young born). Uterine scars count was applied on Eastern grey squirrel (*Sciurus carolinensis*) and Pallas's squirrel (*Callosciurus erythraeus*) species introduced in Italy. For grey squirrels, 25% of examined females produced a single litter and 61% two litters and females which reproduced in both seasons tended to have larger summer than spring litters (on average 2.61 and 1.94 offspring, respectively) and mean annual fecundity was 3.4 scars/female ranging from 1 to 8 births. For Pallas's squirrels, 58% of females had a spring litter, some of these also produced a summer litter (35%) and a few even a third litter in autumn (10%). Moreover, for grey squirrel there was no effect of year, eye lens weight, body size or body mass on total fecundity, while for the other invasive species heavier and older females (higher eye lens weight) had more uterine scars than younger animals with lower body mass. Furthermore, fecundity of the two invasive alien species in Italy was similar or even higher than in the native range and/or in other countries of introduction, suggesting they are well adapted to their new environment and potentially have a high capacity to spread and recover after reduction of population size. This Chapter describes a technique that allows to obtain reliable measures of females fecundity, which are necessary to understand mechanisms that lie behind ecological invasions phenomenon. Finally, this method will be used to evaluate possible relationship between female reproductive output and physiological stress, parasite load and personality (previous Chapters and Chapter 6).

5.2 Introduction

Invasive alien species (IAS) are a major threat to long-term survival of local native species (Clavero and Garcia-Berthou 2005; European Environment Agency 2012). Among mammals, many IAS have arrived in the new environment through pet trade (e.g. many tree squirrels) or by accidental or deliberate releases from fur farms (e.g. muskrat, coypu, mink) (Kolar and Lodge 2001; Bonesi and Palazon 2007; Martinoli et al. 2010; Simberloff et al. 2013; Bertolino and Lurz 2013).

Most of these species need to be controlled to avoid or reduce direct or indirect damage to native species, agriculture and forestry, buildings and infrastructure or human health (Keller et al. 2011; Simberloff et al. 2013; Genovesi et al. 2015). Control campaigns generally consist in removing a proportion of all animals present in a given population. In order for control to be effective, the number of individuals removed must be scaled to the net population growth rate to define adequate harvesting quotas (Baker 2006). Moreover, in addition to supplying practical information to directly implement control strategies, reliable estimates of population growth rates of IAS can support further investigations aimed at disclosing impacts of such species on native species and/or human health and activities.

One of the primary parameters affecting

population growth rate is the annual reproductive output of females, which is the product of the number of young weaned per litter and the number of litters produced in a year (Gurnell et al. 2004). This value is generally referred to as fecundity. In small mammals fecundity can be estimated by intensive capture-mark-recapture studies which, however, require a large investment in terms of time, manpower and funding (e.g. Wauters and Lens 1995; Wauters et al. 2008). Therefore, in species that are harvested for hunting and/or control, alternative methods have been developed. When carcasses of pregnant females are available, the number of embryos can be directly counted (Wauters et al. 1995). Otherwise, when carcasses are obtained after parturition or for species producing more than one litter per year, counting uterine scars can be an effective method (Bengtson and Siniff 1981; Lindström 1981; Rolley 1985; Bray et al. 2003). Such scars are left by the detachment of the placenta from uterine walls, hence each scar corresponds with the parturition of a single young, thus providing information on litter size at birth. Fecundity has been estimated through placental-scar counts in rodents (Nixon and McClain 1975; Martin et al. 1976), lagomorphs (Hackländer et al. 2011) and terrestrial carnivores (Allen 1983; Lindström 1994; Mowat et al. 1996; Ruette and Albaret 2011; Melero et al. 2015) and seals (Kaukala et al. 2014).

One of the issues involved in counting uterine scars resides in the difficulties to count them a long time after parturition (e.g. Martin et al. 1976; Elmeros and Hammershøj 2006; Ruette and Albaret 2011). Here we adapted a staining technique which allows to distinguish the number of uterine scars of different litters to two alien squirrels present in Italy (Eastern grey squirrel, *Sciurus carolinensis* and Pallas's squirrel, *Callosciurus erythraeus*). This method allows to estimate fecundity of female squirrels as a demographic parameter that may subsequently be used i) in studies on population dynamics of the alien species aimed at predicting necessary levels of control (i.e. number of animals to be culled) to successfully reduce population size; and ii) in spatially explicit population dynamics models to predict future range expansion and demographic trends (e.g. Lurz et al. 2001; Tattoni et al. 2006; Bertolino et al. 2008).

5.3 Materials and methods

5.3.1 Trapping

Over three years (2011-2013), from September to December, grey squirrels (*Sciurus carolinensis*) and Pallas's squirrels (*Callosciurus erythraeus*) were trapped in Lombardy as part of a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at eradicating the alien species in Italy. Traps (live-traps model 202, Tomahawk Live Trap Co., Wisconsin, USA) were set in the morning, baited with hazelnuts and checked twice to three times a day to minimize stress to captured animals during trapping. For each trapped squirrel sex and reproductive condition were recorded (Wauters and Dhondt 1989). Animals were euthanized by CO2 inhalation, following EC and AVMA guidelines (Close et al. 1996, 1997; Leary and American Veterinary Medical Association 2013). Each individual was weighed to the nearest 5 g using a Pesola spring-balance and hind foot length was measured with a thin ruler (± 0.5 mm). Each carcass was immediately placed in a sealed plastic bag and stored at -20°C for further examinations.

5.3.2 Laboratory methods

In 2014, we analyzed uterine scars of 49 female grey squirrels trapped in three different sites in Lombardy and of 40 Pallas's squirrels from the only population in Varese province, Lombardy, Northern Italy. All the analyzed animals were culled at the end of the last breeding season (mid September – early December). Many authors have underlined the importance of the timing of animal sampling for uterine (placental) scars investigation with respect to parturition date(s), since scars fade over time, thus affecting their correct recognition and determination (e.g. Martin et al. 1976; Lindstrom 1981; Allen 1983; Elmeros and Hammershøj 2006). Even in our case, preliminary trials on individuals that gave birth during summer did not evidence any scars if stained after winter diapause, indicating that after winter the process of endometrium repair is completed and uterine scars can no longer be revealed by our staining techniques. Hence, collection of animals in autumn-early winter allows to reveal all the uterine scars of the previous breeding season. During post mortem examination, the uterus was collected, placed in clean tap water and sealed in plastic bags which were stored at -20°C. From the same individuals, we also took both eyes and stored them in vials with a 10% formalin solution. After two weeks, eye lenses were removed and dried for 48 h at 80°C following the procedure described in Beale (1962). Dried eye lens weight was then used to distinguish immature subadults from adults in *C. erythraeus* (body mass < 240 g and dry eye-lens weight < 13 mg), and as a proxy for age in both species (e.g. Beale 1962). Since, in populations at low density, grey squirrels are capable of breeding already when 7-8 months-old (Gurnell et al. 2001), we did not use eye lens weight, nor body mass, to exclude animals from our analyses.

Fecundity was determined by counting the number of uterine-placental scars which are formed by the detachment of each embryo's placenta at parturition, thus allowing to estimate the total number of young born from a single female during the entire reproductive season (from February-March to July-September in these alien tree squirrels) (Koprowski 1994; Gurnell 1996; our unpubl. data). Uterine scars were identified after staining which reveals dark pigments of macrophages involved in processes of repairing the endometrium after detachment of the hemochorial placenta (Figures. 5.1, 5.2, 5.3; Wild, 1971).

After removing the connective tissue, the uterus was opened over its entire length and stained using the Turnbull reaction developed by Salewski (1964) on rats (*Rattus rattus*) and later adapted by (Bray et al. 2003) on lagomorphs. Uteri were first immersed for 10 minutes in a fresh 10% solution of ammonium sulphide (H_8N_2S), rinsed thoroughly with tap water and then soaked for 10 minutes in a solution made of equal parts of 1% chlorhydric acid (HCl) and of a 20% solution of potassium hexacyanoferrate ($K_4[Fe(CN)_6]$, $3H_2O$). The staining process was ended by flushing the uterus with cold tap water to eliminate all traces of reagents. To prevent changes in uterine scars color over time, they were counted within the next hour using a microscope equipped with a digital camera. Since the macrophages are rich in hemosiderin, they are colored from blue to beige based on the age of the scar. A scar is recognized as having a paler central crater surrounded by two darker bands. In each band there are circular depressions with darker pigmentation due to concentration of blood and macrophages, indicated as antimesometrial depressions (Figures 5.1, 5.2, 5.3).

We based the classification on the colors of crater, surrounding bands and antimesometrial depression. In particular, older scars, appear much paler than the more recent scars which show a darker staining. Since the animals have been collected in the same period and squirrels have synchronized seasonal parturitions, we assumed that the paler scars refer to the first parturition, while the darker ones to the second litter. In some Pallas's squirrels, three different shades of staining occurred, indicating that these females produced a total of three litters/year.

5.3.3 Statistical analyses

For both species, we explored the effects of phenotypic and environmental factors on individual variation in number of litters/year and on total fecundity (total number of uterine scars over the entire breeding season). Since our dependent variables were counts following a Poisson distribution, we used generalized linear models (GLM) with Poisson error structure to test effects of site of collection (study area, only for grey squirrels), sampling year, body mass, foot length and eye lens weight.

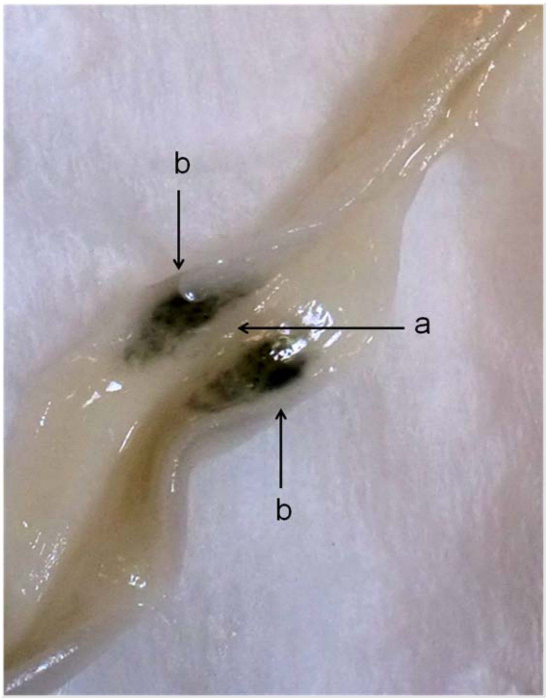


Figure 5.1 – Detail of a stained placental scar in a longitudinally dissected uterus of grey squirrel. A single scar is constituted by a pale, central crater (a) surrounded by two dark bands (b). Each of the bands shows a circular area with darker pigmentation due to blood and macrophages accumulation (antimesometrial depression).

We also explored effects of species, period (spring vs. summer litter; spring parturition from February to April; summer parturition from June to August) and the species * period interaction on litter size (number of births per litter) thus considering only females that had at least one uterine scar for a given reproductive period, using a generalized linear model with Poisson error structure. All GLM were done using the Proc Genmod procedure (SAS/STAT 9.4 software, Copyright © 2011, SAS Institute Inc., Cary, NC, USA).

5.4 Results

5.4.1 Grey squirrel

Of 49 female grey squirrels, 5 had a very small uterus, typical of immature nulliparous individuals and were not analyzed. Of the remaining 44 specimens, 6 did not have any uterine scars (0 litters, 14% of females), 11 (25%) had scars of only one litter and the remaining 27 (61%) had scars from two litters. Out of the 11 females that had a single litter/year, five reproduced only in spring and six only in summer. Overall, females produced between 0 to 8 young/year, with an average of 3.4 scars/female (Table 5.1). The highest litter size at birth (maximum number of scars for a single litter) was six (Table 5.1). There was no difference in average number of scars between spring and summer reproduction (Mann-Whitney U-test, p = 0.087). However, if we only considered the 27 females that had scars for both periods, they produced on average a larger summer than spring litter (difference 0.74 ± 0.25 young/litter, Wilcoxon matched pairs signed ranks test, p = 0.01).

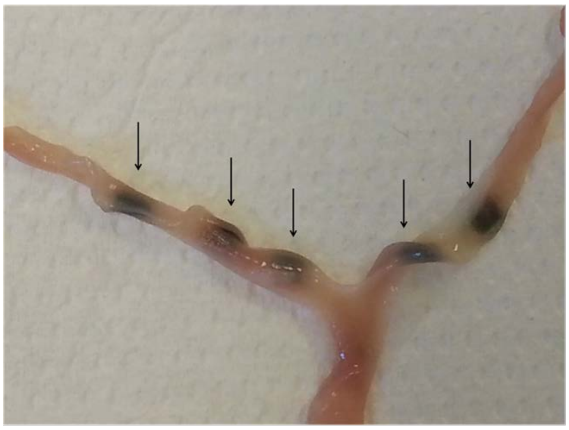


Figure 5.2 – Uterus of grey squirrel (*Sciurus carolinensis*) stained to highlight placental scars (indicated by arrows). Uniformity of staining indicates that all the five scars are referable to the same gestation/parturition.

Body mass of female grey squirrels varied between 450 and 660 g (mean ± SD = 533 ± 51 g) and right hind foot length between 56.0 and 67.0 mm (mean ± SD = 62.1 ± 2.5 mm). Neither body mass (Figure 5.4a) nor foot length significantly affected variation in total number of uterine scars, or in number of litters produced. Also we found no effects of eye lens weight, study site or year (Table 5.2a).

5.4.2 Pallas's squirrel

Of 40 female Pallas's squirrels, 9 had a small uterus, typical of immature nulliparous individuals and were not used in further analyses. Of the remaining 31 specimens, 13 did not have any uterine scar (0 litters, 42% of females), 7 (22%) had scars of only one litter, 8 (26%) of two litters, and 3 females (10%) had scars from three litters. All females that produced a summer or summer and autumn litter (thus with more recent uterine scars) had also produced an early spring litter (oldest uterine scars). Hence, out of the 18 reproductive females, all of them had a spring litter, 11 reproduced in spring and summer and three in spring, summer and autumn. Overall, females produced between 0 and 9 young/year, with an average of 3.1 scars/female (Table 5.3). The highest litter size at birth (maximum number of scars for a single litter) was 6 (Table 5.3). Mean number of uterine scars of early spring litters was higher than of summer (second) litters (Mann-Whitney U-test, p = 0.015), but this was mainly due to more females having a spring than a summer litter resulting in more specimens with 0 scars for summer litters. In fact, considering only the 11 females with both spring and summer parturition, there was no difference in average litter size (Wilcoxon test, p = 0.60).

Body mass of adult female Pallas's squirrels varied between 210 and 330 g (mean ± SD = 279 ± 28 g) and right hind foot length between 45.0 and 51.0 mm (mean ± SD = 49.1 ± 1.5 mm). Number of litters/year increased with eye lens weight, indicating that older female produced more litters (Table 5.2b).

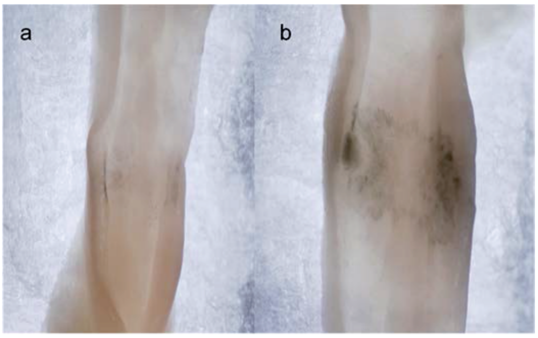


Figure 5.3 – Light-stained/paler scar referable to an older gestation (a) and dark-stained/darker scar referable to a more recent parturition (b).

There was no effect of sampling year on number of uterine scars, but the total number of uterine scars increased with a female's foot length (estimate ± SE = 0.19 ± 0.09), body mass (Figure 5.4b, estimate ± SE = 0.012 ± 0.0 04) and age (eye lens weight 0.064 ± 0.016; Table 5.2b). Body mass and eye lens weight were correlated (n = 31; r = 0.39; p = 0.029) suggesting an increase of body mass with age.

Table 5.1 – Fecundity data of female grey squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of scars (range) and mean litter size (± SD) of females which produced at least a litter (scars > 1). Data for spring and summer parturition separately, and over the entire year (total).

Litters	**Uterine scars**			**Litter size**	
	n	mean ± SD	range (min- max)	n	mean ± SD
Spring parturition	44	1.41 ± 1.33	0–6	32	1.94 ± 1.19
Summer parturition	44	1.95 ± 1.49	0–5	33	2.61 ± 1.12
Total	44	3.36 ± 1.71	0–8	38	3.89 ± 1.13

5.4.3 Grey squirrel vs Pallas's squirrel

There was a nearly significant species * period interaction ($\chi^2 = 3.75$; df = 1; p = 0.053) on litter size. Therefore, we compared litter size between the two species for spring and summer litters separately (for values see Tables 5.1 and 5.3). In spring, Pallas's squirrels produced on average larger litters than grey squirrels (Kruskal-Wallis $\chi^2 = 12.0$; df = 1; p = 0.0005), but in summer there was no difference between the two species in mean litter size (Kruskal-Wallis $\chi^2 = 0.29$; df = 1; p = 0.59). As a consequence, total litter size was higher in Pallas's squirrel than in grey squirrel (Kruskal-Wallis $\chi^2 = 8.10$; df = 1;

p = 0.004) with breeding females of the former producing on average 1.4 young more per year than grey squirrels.

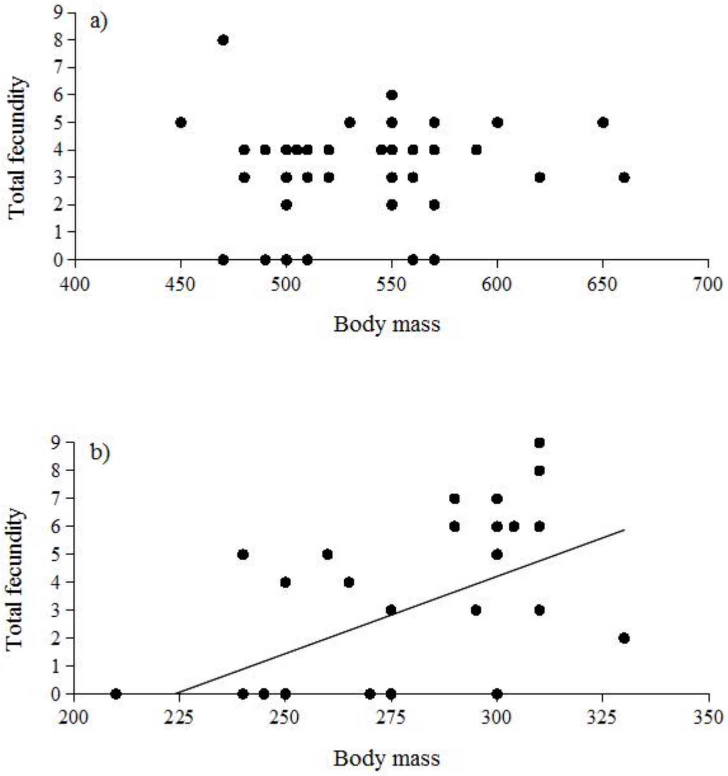


Figure 5.4 – Relationship between female body mass (g) and total fecundity (total number of uterine scars over the entire breeding season) in: a) grey squirrels; b) Pallas's squirrels.

5.5 Discussion

5.5.1 Reliability of uterine scar counts to estimate fecundity

The uterine staining technique based on variability in macrophage coloration described in this paper and applied to invasive tree squirrels allows to distinguish individual uterine scars and to discriminate scars produced earlier (spring litters) or later (summer – autumn litters) during the breeding season. The technique was successfully applied to both grey squirrels and Pallas's squirrels and allowed us to distinguish from two (grey squirrel) to even three (Pallas's squirrel) parturition events (litters) in a year and to determine number of births (litter size) for each litter separately as well as a female's total fecundity over the entire year (e.g. Nixon and McClain 1975; Martin et al. 1976; Hackländer et al. 2011).

Several studies have evaluated and discussed the reliability of uterine scar counts (also called placental scar counts, PSC); where possible this was done by comparing litter size and/or pregnancy rate estimates based on PSC with those based on counts of embryos (Lindström 1981; Allen 1983; Mowat et al. 1996; Ruette and Albaret 2011). Using rodents, hares or carnivores held in captivity, it was shown that the time elapsed since the last (or previous) parturition(s) can strongly affect the reliability of PSC as a fecundity measure since uterine scars tend to fade over time (Martin et al. 1976; Lindstrom 1981; Bray et al. 2003; Elmeros and Hammershøj 2006). All studies agree that, with or without staining, the darkest scars are those of the most recent parturition event and most authors underline that counting also “paler” scars will overestimate true litter size (e.g. Allen 1983; Mowat et al. 1996). This is partly due to paler scars indicating a site where embryo resorption occurred, or being left not by the last but by previous reproduction events (e.g. hares, foxes and lynx, Allen 1983; Mowat et al. 1996; Bray et al. 2003). Studies comparing counts of both unstained and stained uterine scars, agree that staining helps to correctly distinguish recent from older scars, both in species that produce once/year than in those potentially producing more than one litter/year (Martin et al. 1976; Bray et al. 2003; Ruette and Albaret 2011). Overall, counting of the darkest uterine scars and counts of embryos in animals of the same population culled in the same year are strongly correlated, with –in some species- a slight overestimation of litter size at birth using PSC, probably due to embryo resorption (Allen 1983; Mowat et al. 1996; Elmeros and Hammershøj 2006; Ruette and Albaret 2011). In this study, the observed variation in darkness of staining suggests that all parturitions of the year were determined and allowed us to clearly distinguish between first and second litters. Thus, since our animals were all sampled in autumns-winter, the identification of paler scars indicate that up to 9 months after parturition scars can be identified with this staining technique. However, we cannot exclude the possibility of some embryo resorption, thus we could have in some cases overestimated the actual litter size, however this could be studied experimentally only by comparing number of uterine scars and number of pups shortly after parturition (e.g. voles, foxes, mink; Martin et al. 1976; Lindström 1981; Elmeros and Hammershøj 2006).

In tree squirrels, earlier works made use of uterine scars to estimate fecundity, however details on the staining method used and on the number of litters/year are often lacking. More in detail, Tang and Alexander (1979), T'sui et al. (1982) and Tamura (1999) analyzed fecundity of *C. erythraeus* in its native range without describing the staining method and with no indication of how older scars (from earlier litters) could be distinguished from more recent ones. Also, the number of scars from single and/or multiple litters was not reported and authors only analyze data of number of embryos/litter in pregnant females (see Table 5.5). Humanson (1962) on grey squirrels in their native range, refers to the staining method used as "Prussian blue reaction" without giving further details. Similarly, Shorten (1951) on grey squirrels introduced in the UK did not describe how the uterus was stained or how uterine scars were counted and apparently no attempt was made to distinguish between older and more recent scars. Moreover, this paper only presented average number of uterine scars per female for a given litter (spring or summer/autumn litter) without range of scars or a measure of variance (SD or SE, see Table 5.4). Only Nixon and McClain (1975) distinguish between older (spring litter) and more recent scars (summer litter), using darker staining as the discriminating factor.

Table 5.2 – GLM (dependent variables: Number of litters or total annual fecundity measured by uterine scars of all litters) exploring the effects of study area (only grey squirrel), sampling year and of phenotypic parameters on variation in fecundity among (a) female grey squirrels; (b) female Pallas's squirrels.

Explanatory variables	Number of litters/year	Total annual fecundity
(a) Grey squirrel		
Body mass	$\chi^2 = 0.00$; df = 1; p = 0.95	$\chi^2 = 2.06$; df = 1; p = 0.15
Foot length	$\chi^2 = 0.01$; df = 1; p = 0.91	$\chi^2 = 0.32$; df = 1; p = 0.57
Eye lens weight	$\chi^2 = 0.43$; df = 1; p = 0.51	$\chi^2 = 0.32$; df = 1; p = 0.64
Year	$\chi^2 = 0.73$; df = 2; p = 0.70	$\chi^2 = 3.33$; df = 2; p = 0.19
Study area	$\chi^2 = 0.45$; df = 2; p = 0.80	$\chi^2 = 2.26$; df = 2; p = 0.32
(b) Pallas's squirrel		
Body mass	$\chi^2 = 2.45$; df = 1; p = 0.12	$\chi^2 = 7.51$; df = 1; p = 0.01
Foot length	$\chi^2 = 1.42$; df = 1; p = 0.23	$\chi^2 = 4.39$; df = 1; p = 0.04
Eye lens weight	$\chi^2 = 6.24$; df = 1; p = 0.01	$\chi^2 = 15.3$; df = 1; $p < 0.0001$
Year	$\chi^2 = 0.01$; df = 1; p = 0.92	$\chi^2 = 1.46$; df = 1; p = 0.23

5.5.2 Grey squirrel

In our sample of grey squirrels from N. Italy, collected over a period of 3 years, 61% of females produced two litters/year. This was higher than the average reported in a multi-year study in the native range (Ohio, 27%, Nixon and McClain 1975). These authors also documented that in their study site the percentage of females producing two litters/year varied between 0% and 36% in relation to tree seed abundance; consequently, total number of uterine scars/female varied over years and was highest in years with good seed crops (Nixon and McClain 1975). In contrast, we did not find a significant year effect on total number of uterine scars/female in this study. Medium to long-term studies have documented that grey squirrel reproductive rate varies markedly among years in both the native range and in areas in Great Britain where the species has been introduced (e.g. Koprowski 1994; Gurnell 1996; Kenward et al. 1998). Reproductive rate is density dependent (with adult female density) and increases when food abundance is high.

Density dependent variation in fecundity over time was also observed in native Baltic grey seals (*Halichoerus grypus*, Kauhala et al. 2014) and invasive American mink (*Neovison vison*) in Scotland (Melero et al. 2015). Seals showed a reduction in pregnancy rates over time correlated with an increase in population size (Kauhala et al. 2014). In the case of invasive mink, the probability of conceiving a litter and litter size increased as female density declined after culling, resulting in density dependent compensation in fecundity (Melero et al. 2015). This marked increase in fecundity in females reinvading the controlled area implies that control strategies must be sufficiently robust in order to overcome this compensation and suppress densities of invasive mammals (Melero et al. 2015). Both these studies had much larger samples sizes than ours and/or were carried out over a much longer time span. Hence, in our case, the lack of significant variation in total reproductive output between years might be due to smaller sample size and/or less annual variation in food availability in the Italian sites (deciduous woodland and park habitats) than in the woods and forests in Great Britain and North America.

In our study, females that had reproduced in both seasons tended to have a larger summer than spring litter (2.61 and 1.94 offspring respectively, see Table 5.4). Larger summer litters were also reported for grey squirrels introduced to England (Shorten 1951) and in the native range, particularly for adult (> 1-year old) females (Table 5.4). In the study in Ohio, adult females tended to produce larger litters than yearlings and, consequently, their total annual reproductive output was higher (Table 5.4 and Nixon and McClain 1975). We did not divide our sample in these two age-classes, but used eye lens weight as a continuous variable and proxy for age. However, we did not find any effect of eye lens weight on number of litters or on total fecundity. Among the grey squirrels analyzed in this study, variation in number of litters and in total number of uterine scars was not correlated with body size or body mass. Body mass and age are important factors in determining female reproductive success in other tree squirrels, for example Eurasian red squirrels (*Sciurus vulgaris*), through both indirect effects (on dominance status, home range quality) and direct effects (entering estrus, successful weaning of offspring, number of litters in lifetime) (Wauters and Dhondt, 1989, 1995; Lurz et al. 2000, 2005; Wauters et al. 2001, 2008). There can be several reasons why we did not find any effect of eye lens or body mass on number of uterine scars in this study. First, our data are few and larger datasets are necessary to investigate how individual variation in phenotypic characteristics might affect variation in reproductive rate. Second, age effects might be important only between primiparous yearlings and multiparous adults, as suggested by Nixon and McClain (1975): in this case using a continuous variable (i.e. eye lens weight) might not reveal a significant pattern. Also in red foxes (*Vulpes vulpes*), the major difference in fertility was between yearlings and adults (Ruette and Albaret 2011), while in long-lived species, for example Baltic grey seals, age-dependent pregnancy rates were evident using multi-year age-classes (Kaukala e al. 2014). Third, body mass, which is known to fluctuate seasonally in this species (Kenward and Tonkin 1986; Koprowski 1994), could be important for successful reproduction at the beginning of the breeding season, but not relevant when measured at the end of it (as in this study). Fourth, the lack of effects of body mass and age might indicate an adaptation of the IAS to the new environment: grey squirrel females invest in producing offspring independent of their age, size or body condition and their relative success (variation in number of young) is more determined by other factors not measured here.

Finally, it is interesting to note that mean litter size estimated with number of uterine scars was slightly higher for both spring and summer litters in Ohio than in this study (Table 5.4). In contrast, in our case more females reproduced twice a year and consequently mean number of uterine scars/female over the entire year was higher. Also, maximum number of scars found in a female was higher in N. Italy than in the population in the native range (Table 5.4). This high reproductive rate and breeding at an early age could be adaptations developed by spreading, introduced populations and/or density-dependent compensations to culling (e.g. Gurnell et al. 2001; Melero et al. 2015).

We found only one paper with partly comparable data on uterine scars of other introduced populations (grey squirrels in the UK, Shorten 1951). However, as above mentioned, this paper only presents average number of uterine scars per female for a given litter (spring or summer/autumn litter) without any range of scars or a measure of variance. Overall, mean number of scars for both spring and summer litters, as well as the maximum number of young/litter in summer/autumn (based on embryo counts) tended to be higher in the UK populations than in Italy (Table 5.4). It has been shown that grey squirrels in the UK spread more rapidly than in Italy (Bertolino et al. 2014), but both studies presented preliminary results of fecundity and it would be speculative trying to explain differences in fecundity between invasive populations in the two countries based on these data.

Table 5.3 – Fecundity data of female Pallas's squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of scars (range) and mean litter size (± SD) of females which produced a litter (scars > 1). Data for spring, summer and autumn parturition separately, and over the entire year (total).

Litters	**Uterine scars**			**Litter size**	
	n	mean ±SD	Range (min- max)	n	mean ±SD
Spring parturition	31	1.90 ± 1.97	0–6	18	3.28 ± 1.45
Summer parturition	31	0.90 ± 1.49	0–6	11	2.55 ± 1.44
Autumn parturition	31	0.26 ± 0.81	0–3	3	2.67 ± 0.58
Total	31	3.06 ± 3.00	0–9	18	5.28 ± 1.87

5.5.3 Pallas's squirrel

Although our dataset was small, relationships between phenotypical characteristics of females and reproductive output revealed some interesting patterns. In the introduced population in N. Italy, females invested heavily in an early spring litter. In fact, 58% of animals examined had a spring litter, and only among the females which had already produced a spring litter, some also produced a summer litter and a few even a third litter in autumn. In contrast with grey squirrels examined in this study, individual variation in total number of uterine scars in Pallas's squirrels depended on eye lens weight (age) and body mass: older females and those of higher body mass produced more offspring in a year than younger ones and/or than females of lower body mass. The preponderance of early (spring) breeding in our study site might be related to seasonal variation in food availability or quality. In March-April, during lactation of spring litters, the invasive species was observed foraging on a wide variety of tree and shrub species, and was often seen feeding on flowers of wild cherry (*Prunus avium*), a common tree species in the mixed deciduous forest where the population occurs. Cherry flowers and, in May-June, their ripe fruits are abundant and rich in carbohydrates and may constitute an energy-rich food supply for breeding females in spring and early summer (onset of summer breeding). Furthermore, although we do not yet know if and how frequent Pallas's squirrels hoard tree seeds in our study site, in similar habitats cached tree seeds are an important food source for spring breeding in native red squirrels (Wauters and Casale 1996; Zong et al. 2010, 2014).

We found few data in the literature reporting measures of fecundity in this species, either in its native or introduction range (Table 5.5). Data from the native range, based on counts of embryos in animals shot throughout the year, reveal two litters per year (concentrated in spring and summer) with only 1-2 embryos/litter (Tang and Alexander 1979; T'sui et al. 1982). These authors suggest that small litter size was related to high population density in the areas where animals were collected (commercial conifer forests, orchards and woods). In any case, all data from populations introduced to other countries show higher fecundity than in the native range (Table 5.5), suggesting that this species might be extremely adaptive to new environments and habitats, as supported by its high invasiveness (Bertolino and Lurz 2013). High fecundity in colonizing populations in Italy and Japan could also be due to low squirrel density. However, in our case, large number of animals removed for control and high estimated population density (ca. 7 individuals/ha, Mazzamuto et al. 2017) seem to contradict this. Comparing fecundity between Japan and Italy, although based on different methods, showed that in both countries females could produce up to 3 litters per year, with average and maximum litter size slightly higher in Italy than in Japan (Table 5.5) (Tamura et al. 1988; Tamura, 1999). In contrast, data from a population introduced at Antibes (France), suggest lower fecundity in this country (n = 84, mean ± SE = 1.8 ± 0.1 embryos/female, range = 1-3, Chapuis et al. unpubl. data) maybe due to poor habitat quality at the collection site.

Table 5.4 – Fecundity data of grey squirrels: Comparison between populations from the native range and introduced populations (this study).

		NATIVE RANGE			INTRODUCED POPULATIONS							
		Nixon and McClain (1975)			Shorten (1951)				This study			
Breeding season	Age	Placental scar count		Range	Age	Placental scar count		Range	Age	Placental scar count		Range
		n	mean ± SD			n	mean			n	mean ± SD	
Spring	Yearling	23	2.43 ± 0.58	1–3	All	66	2.70	1–4*	All	32	1.94 ± 1.19	1–6
	Adult	40	2.70 ± 0.89	1–4								
Summer	Yearling	35	2.51 ± 0.77	1–4	All	36	3.04	1–7*	All	33	2.61 ± 1.12	1–5
	Adult	63	3.49 ± 0.87	2–6								
Annual	Yearling	46	2.54 ± 0.75	1–4					All	38	3.89 ± 1.13	1–8
	Adult	98	3.43 ± 0.89	1–6								

n = number of squirrels examined; mean number of scars/female with SD; Range = minimum and maximum number of uterine scars per litter or over the entire year (annual); *= embryos/female, no data on placental scars range

Table 5.5 – Comparison between studies on fecundity (mean ± SD and range) in Pallas's squirrels native range (Tang and Alexander 1979; T'sui et al. 1982) and in introduced populations (Tamura et al. 1988; Tamura 1999; This study). Sample size between brackets, * no sample size given.

	NATIVE RANGE			INTRODUCED POPULATIONS		
	Tang and Alexander (1979)	T'sui et al. (1982)		Tamura et al. (1988) Tamura (1999)	This study	
Litters/year	1–2(40)	1–2(15)		1–3*	1–3(31)	
Embryos/female	1.68(40)	Spring	1.83(6)	2.4±0.7 (SD)		
		Summer	1.75(8)	range 1–4(44)		
Scars/breeding females					Spring	3.28±1.45 (1–6)(18)
					Summer	2.55±1.44 (1–6)(11)
					Autumn	2.67±0.58 (1–3)(3)

5.6 Conclusions

Our first data of uterine scar counts in female grey squirrels culled in N. Italy suggest than fecundity over an entire year was slightly larger than in populations in the native range. Hence, this IAS seems well adapted to the new environment in Italy, where habitats most commonly occupied are mixed lowland woods with presence of oaks (*Quercus* sp.) and urban, suburban or private parks where densities tend to be high (Wauters et al. 1997; Venturini et al. 2005; Bertolino et al. 2008; Martinoli et al. 2010). Furthermore, it has been reported that the grey squirrels in Italy have lost many of the macroparasite species they normally host in their native range, setting the premises for positive effects of parasite release on host fitness (Romeo et al. 2014a), which could also include a higher fecundity in animals with low helminth abundance in the Italian populations.

A similar pattern was found in the Pallas's squirrel where uterine scar counts definitively indicate a high fecundity of individual females when compared with the (scarce) data available for the native range. Also for this species, the extremely poor parasite fauna and absence of gastrointestinal helminths in most animals, suggest that parasite release might be involved in the high fecundity of the IAS in this area (Mazzamuto et al. 2016) and in other countries where it has been introduced (Dozières et al. 2010; Gozzi et al. 2014). Although there are no directly comparable fecundity data for native red squirrels, which can have large litters in some circumstances (Mari et al. 2008), estimates of reproductive rate from percentage females breeding and litter size at weaning in lowland mixed woodlands (e.g. Wauters and Lens 1995; Wauters et al. 2001) suggest that the two IAS have similar or even higher fecundity than the native species. Thus, not only grey but also Pallas's squirrels might become a risk for the long-term persistence of populations of the native red squirrel. Preliminary data on trapping success and removal of the IAS linked with occurrence/density of red squirrels (Mazzamuto et al. 2017) suggest that this is actually the case.

Our data also have implications for Spatially Explicit Population Dynamics Models (SEPMs) used to predict future range expansion of invasive squirrels. In fact, in these models, parameter input of fecundity values have a high impact on model results of future population size and distribution (Lurz et al. 2001; Guichón and Doncaster 2008). Based on fecundity values found in our study, the range of litter sizes and% females breeding used in SEPMs predicting future expansion of grey squirrels in Italy are realistic (litter size range: 2-4 in Lurz et al. 2001; 1.5 – 3.5 in Tattoni et al. 2006; 2 – 3.5 in Bertolino et al. 2008).

In conclusion, the results presented in this paper indicate that uterine scar counts are a good method to estimate reproductive output in the two introduced squirrel species and that both IAS have a high reproductive output in their new range in Italy. Therefore, and since both species negatively affect native red squirrels (Gurnell et al. 2004; Bertolino et al. 2014; Mazzamuto et al. 2017), management plans must aim to eradicate both species in Italy, or, when in the case of the grey squirrel this is considered impossible, control populations to avoid further expansion.

Chapter 6

Relationships between parasite load, physiological stress and body mass and their effects on reproduction in an invasive alien species

6.1 Summary

This Chapter focuses on relationships among parasite infection, physiological stress, reproductive success/investment and the interacting role of differences in personality traits in Eastern grey squirrel (*Sciurus carolinensis*). None of the four parasite infections predictors investigated here (*Strongyloides robustus* abundance, prevalence, and intensity, and helminths richness) was correlated with levels of physiological stress. Both methods to determine parasite loads and measure physiological stress, in terms of FGM concentrations, were described in previous Chapters and applied also here. Grey squirrels FGM concentrations did not differ between the sexes but increased with the individual's body mass, and among males, reproductively active animals with scrotal testes had higher FGM concentrations than reproductively non-active males with small abdominal testes. Levels of physiological stress were higher in autumn than in other seasons. Among females, reproductive success increased with body mass and there was no effect of FGM concentrations or parasite infection. Among males, testes mass, used and described in this Chapter as a measure of reproductive investment, was positively related to body mass but negatively with parasite infections and there was no effect of FGM concentrations. This result suggests an increased cost of reproductive investment in strongly parasitized males (high *S. robustus* abundance and intensity). Overall, there is a strong and general effect of body mass, which correlates positively with FGM concentrations, female reproductive success, male reproductive investment and parasite infections (abundance and intensity). Furthermore, a personality score measuring boldness and exploration tendency was not correlated with female reproductive success or male reproductive investment, even if bolder grey squirrels had a higher parasite load (*S. robustus* abundance and infection status) than shy animals. Individual variation in boldness/exploration scores was not correlated with variation in levels of physiological stress. This final Chapter summarizes the relationships between all factors investigated in this thesis showing that personality (boldness/exploration tendency) influences directly and positively parasite load, which in turn has a negative effect on male reproductive investment. Also, physiological stress, parasite load and reproductive success/investment were all strongly correlated with an individual's body mass. Finally, female reproductive success was only affected by body mass and not by FGM concentrations and/or parasite load. This last finding highlights the prominent role of a good body condition (body mass) that could mask the other possible more subtle effects of parasites and levels of physiological stress.

6.2 Introduction

Parasites exert subtle forces in structuring communities mediating intraspecific and interspecific interactions (Tompkins et al. 2010; Roy and Handley 2012; Telfer and Brown 2012). In general, parasite transmission, and therefore disease incidence, is determined by a combination of exposure to infected hosts, vector or infective stages, and host susceptibility (Anderson and May 1978; Telfer and Brown 2012). High levels of physiological stress, may affect susceptibility through the action of glucocorticoid hormones that can induce suppression of individual immune response (see Padgett and Glaser 2003; Martin 2009; Ezenwa et al. 2012), thereby facilitating parasitic infections (Wilson et al. 1993; Dunn et al. 2012; Poulin 2013; St. Juliana et al. 2014). Stress hormones have been shown to be involved in many body function, including expression of behavior, response to infection and diseases, and even regulation of reproduction and development (Dantzer et al. 2014). For instance, research on laboratory mammals and humans demonstrates that high and prolonged elevated glucocorticoid levels reduces survival and reproduction (Wasser et al. 1997; Creel 2001; Creel et al. 2002; Bercovitch and Ziegler 2002).

In addition, Corlatti et al. (2012) highlighted how reproductive effort, in terms of allocation of energy resources to reproduction, could lead to less energy investment in immune system reaction against pathogen increasing a host’s susceptibility (Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Hõrak et al. 2006). Indeed, several studies found a positive correlation between reproductive effort and parasitism (in Corlatti et al. 2012: e.g. zebra finches, *Taeniopygia guttata*: Deerenberg et al. 1997; collared flycatchers, *Ficedula albicollis*: Nordling et al. 1998; tree swallows, *Tachycineta bicolor*: Ardia et al. 2003; bighorn sheep, *Ovis canadensis*: Pelletier et al. 2005). In turn, the increase in parasite infection is likely to reduce resources that could be used for maintaining homeostasis (body condition, survival) and/or reproduction (Lehmann 1993; Corlatti et al. 2012).

Furthermore, recent studies showed how parasites and animal behavior are inextricably linked (Boyer et al. 2010; Ezenwa et al. 2017) and that animal personality can create between individual heterogeneities in the acquisition and spread of parasites (VanderWaal and Ezenwa 2016). Overall, during the last decades, cumulative effect of several factors has been rising as a new approach to investigate variation in individual fitness (e.g. reproduction and survival), in particular in the light of relationships with direct influences of physiological stress, behavior and parasitic infections (Sih et al. 2004; Both et al. 2005; Morand et al. 2006; Chapman et al. 2006; Smith and Blumstein 2008; Hillegass et al. 2010; Dantzer et al. 2010, 2014; Bosson et al. 2013). However, disentangling all these factors and the cause-consequence pathways is a complex matter.

Here we use one of the most successful and impacting invasive alien mammal species, the Eastern grey squirrel (*Sciurus carolinensis*) introduced to N. Italy (for study species details see Chapter 4), to investigate the following hypotheses: 1) variation in physiological stress levels leads to differential parasite infection, predicting a positive correlation between levels of physiological stress and parasite load; 2) increase in physiological stress and/or higher parasite loads are associated with lower reproductive success/investment in grey squirrels; 3) The relationship between parasite load, physiological stress and reproductive success is mediated by personality. The latter hypothesis predicts that personality will not have a direct effect on reproductive success/investment in grey squirrels, but an indirect effect through a relationship with either parasite abundance or physiological stress.

6.3 Materials and methods

6.3.1 *Study areas, trapping and handling squirrels*

We trapped Eastern grey squirrel in five study areas (Piobesi, Berroni, RS, MOR, Commande) without native red squirrels (grey only areas) in Piedmont, North Italy (681 captures of 281 individuals). Details about study areas, trapping sessions, handling, and grey squirrel density estimates are described in Chapter 4.

6.3.2 Fecal sample collection

After capture and handling, fecal samples were collected from underneath the traps (n = 193). Details about pellet collection and hormones extraction are described in Chapters 1, 2.

6.3.3 Enzyme immunoassay

193 samples were assayed on a total of 22 EIA plates. Pools of grey squirrel extracts were used as intra-assay controls at dilutions of 1:50 (~30% binding) and 1:400 (~70% binding). Average intra-assay CVs were 9.5% and 9.4% respectively for pools diluted 1:50 and 1:400. Inter-assay CVs were estimated from standards of known concentration with a high (n = 22 plates, 12.3% binding) and low (n = 22 plates, 81.6% binding) concentration that had inter-assay CVs of 16.1% and 9.3%, respectively.

6.3.4 Personality

Individual personality was determined using direct (arena test measurements) and indirect indices (trappability and trap diversity) as described in Chapter 4. However, only PC1 scores were included in models described in this Chapter, since expert-based behavior group and personality traits identified through arena test were available for only two study areas, resulting in much smaller sample size than for PC1 scores.

6.3.5 Parasitological examination

A total of 96 culled grey squirrels were examined for gastro-intestinal helminths as described in Chapter 4. On a total of 96 carcasses examined, 47 had measurements for FGM concentrations and among these 40 had also personality scores.

6.3.6 Reproductive success and investment

The analysis of uterine scars collected during post mortem examination of 49 female grey squirrel culled in October-November allowed us to determine fecundity counting the number of uterine-placental scars (Santicchia et al. 2015b and Chapter 5). This count corresponds with the total number of young born from a single female during the entire reproductive season. Uterine scars were identified after staining which reveals dark pigments of macrophages involved in processes of repairing the endometrium after detachment of the hemochorial placenta. For details and technique description see Santicchia et al. 2015b and Chapter 5.

Reproductive investment for males was estimated measuring testes mass, that is considered a good measure of their investment in reproductive activity and annual reproductive success in sciurids (Schulte-Hostedde and Millar 2004; Manjerovic et al. 2008). Testes were measured only from carcasses of grey squirrels with scrotal testes (n = 24). After removing the scrotum, the testes were weighted (0.01 g) using an electronic precision balance and size (length) was determined using precision calliper (0.1 mm).

6.4 Statistical analyses

All analyses were performed in R version 3.3.3 (R Development Core Team, 2017) using the lme4 package (version 1.1-12, Bates et al. 2015). Before model building, all the explanatory variables were examined for covariance and no collinearity issues were detected. Unless otherwise specified, all values and parameter estimates are reported as mean ± SE. Where necessary, we assessed significance of pair-wise comparisons using differences of least square means (DLSM) with Satterthwaite approximations to degrees of freedom in R package lmerTest (version 2.0-33, Kuznetsova et al. 2016). Foot length was not included in the models since it was strongly and positively correlated with body mass ($n = 222$; $r = 0.51$; $p < 0.0001$).

6.4.1 Physiological stress

For each of the models described, we conducted linear mixed-effects models (LMM) with FGM concentrations (expressed as ln transformed ng/g dry feces) as the dependent variable, squirrel identity as random intercept term to account for repeated samples on the same individuals and study area as random factor.

We first explored variation in FGM concentrations with sex, season (winter [December to March], spring-summer [April to August], or autumn [September to November]) and body mass as fixed effects. The second order interaction between season and sex was also included in the full model but it was eliminated using stepwise backward model selection (partial effect $p = 0.64$).

In order to account for the fact that the observed differences in FGM concentrations between sexes were to some extent due to differences in reproductive condition of the squirrels sampled (Dantzer et al. 2016), we investigated if FGM concentrations differed between season, body mass and reproductive condition (see Dantzer et al. 2016 and Chapter 2 for details on reproductive condition classes) in separate models for males and females. Data from the Commande study area were excluded from this model because of a strongly biased sample size towards sexually inactive squirrels (males with abdominal testes).

In addition, on a subsample of individual squirrels for which we also measured personality, we ran a model to investigate if FGM concentration was affected by variation in individual personality including sex, season, body mass, and PC1 score as fixed effects, squirrel identity as random intercept and study area as random factor.

6.4.2 Parasites infection

We explored variation in four different predictors of parasite infection of individual grey squirrels: (1) abundance of *Strongyloides robustus*; (2) intensity of infection with *S. robustus*; (3) infection status or prevalence of infection and (4) helminth richness. Each of these was used as a response variable in mixed models. For details of type of models used and error distribution see Chapter 4. In all models, we first examined the effect of FGM concentration, sex and body mass; then we ran the same models adding PC1 score as exploratory variable. Study area was included as random factor (see Chapter 4 Statistical analysis paragraph).

6.4.3 Reproductive success and investment

We analyzed testis left and right size and mass to check for correlations (Mass SX – DX: $r = 0.99$; Size SX – DX: $r = 0.98$; Mass SX – Size SX: $r = 0.97$; Mass DX – Size DX: $r = 0.96$; all $p < 0.0001$) and we decided to use only mass of the left testis as an index of reproductive investment for males.

Individual variation in reproductive success (females: total number of uterine scars/year) was examined with Generalized Linear Mixed Models (GLMM) with Poisson error distribution. We examined the effect of body mass, four predictors of parasite infection (see above for details), personality scores (PC1) and FGM concentrations. Study area was included as random factor. Next, we explored male reproductive investment (testis mass) with Linear Mixed Models (LMM) with testes mass as dependent variable and the same fixed and random effects of the previous models on females reproductive success.

6.5 Results

6.5.1 Physiological stress of grey squirrels

Grey squirrels showed a significant increase in FGM concentration with body mass (estimate 0.0016 ± 0.0007; $t_{241} = 2.31$, $p = 0.02$) (Figure 6.1). Moreover, FGM concentrations were significantly higher in autumn than in winter and in spring-summer (autumn against winter estimate 0.40 ± 0.10, $t_{220} = 4.26$, $p < 0.001$; autumn against spring-summer estimate 0.30 ± 0.14; $t_{147} = 1.95$; $p = 0.05$; spring-summer against winter $p > 0.05$; Figure 6.2).

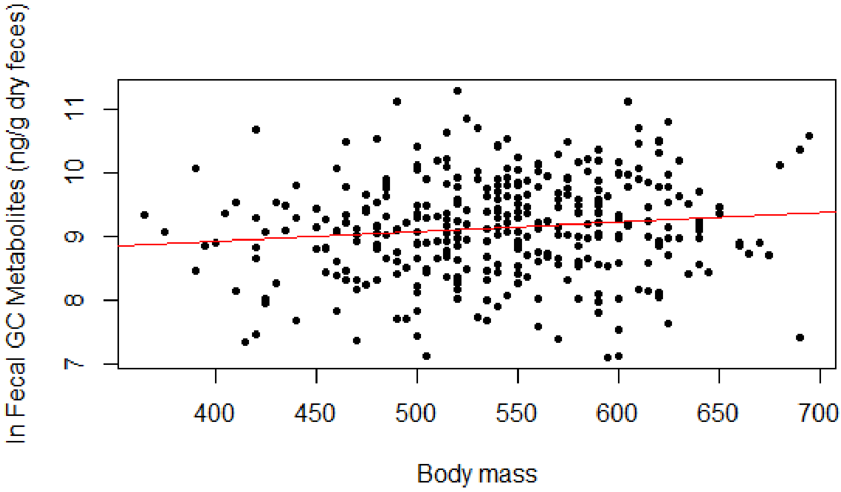


Figure 6.1 – Relationship between fecal glucocorticoid (GC) metabolite concentrations (ln ng/g dry feces) and body mass in Eastern grey squirrel (n = 193; t_{241}= 2.31, p = 0.02). Linear regression line in red.

Additional sex-specific models showed that the observed differences in FGM concentrations were affected by differences in reproductive condition in males (higher FGM for males with scrotal testes compared to males with abdominal testes, estimate 0.37 ± 0.15; $t_{129} = 2.44$; p = 0.02) (Figure 6.3). In contrast, in female grey squirrels, FGM concentrations were significantly influenced by seasonal differences (season overall effect: $F_{2,49} = 6.30$, p = 0.004; autumn against winter estimate 0.60 ± 0.16; $t_{103} = 3.55$; p < 0.001; autumn against spring-summer estimate 0.40 ± 0.20; $t_{30} = 2.24$, p = 0.03; spring-summer against winter: p = 0.42) and FGM tended to increase with body mass, although this relationship was not statistically significant ($t_{119} = 1.71$, p = 0.09). There was no effect of female reproductive condition on FGM concentrations ($F_{2,96} = 0.19$, p = 0.83).

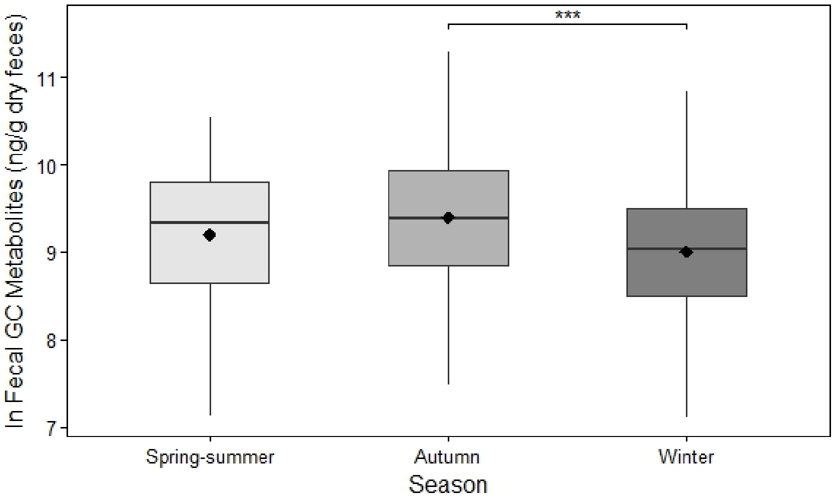


Figure 6.2 – Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eastern grey squirrel, in Spring-Summer (light-grey color; n = 62), Autumn (grey color; n = 91) and Winter (dark-grey color; n = 189). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. *** p < 0.001.

6.5.2 Relationship between physiological stress and personality

In a reduced sample of animals for which we also had a personality score (n =186), grey squirrels FGM concentrations were not affected by their boldness score (PC1 score $p = 0.91$; Figure S1). As for the entire sample, FGM concentrations varied between seasons ($F_{2,144} = 7.61$, $p = 0.0007$) and increased with a squirrel's body mass ($F_{1,254} = 4.92$, $p = 0.03$; Figure S1).

6.5.3 Relationship between parasites infection predictors, physiological stress and personality

None of the four grey squirrels infection predictors (helminth richness, *S. robustus* abundance, *S. robustus* infection status and intensity of infection) was related with the level of physiological stress of individual grey squirrels (Figure S1). In this dataset, the only significant relationship was between *S. robustus* abundance and PC1 score (estimate: 0.51 ± 0.18; $\chi^2 = 8.06$; $p = 0.005$), a result consistent with findings from Chapter 4. For detailed results of all models results see Table 6.1.

6.5.4 Reproductive success and investment

On a total of 49 females, 13 did not have any uterine scars (0 litters, 26%), 20 (41%) had scars of only one litter and the remaining 16 (33%) had scars from two litters. Overall, females produced between 0 to 7 young/year, with an average of 3.86 ± 0.32 scars/female. The highest litter size at birth (maximum number of scars for a single litter) was seven. In all models with parasite infection predictors and personality, only body mass positively affected female reproductive success (uterine scars/year; all $p < 0.05$; Table 6.2) (Figure 6.4a; Figure S1). While there was no effect of FGM concentrations or personality on female fecundity (Table 6.2; Figure S1).

Testes mass and size was measured for 24 males with active scrotal testes (mass left testis, mean ± SE: 1.46 ± 0.16 g; range: 0.33 - 2.90 g; right testis, mean ± SE: 1.47 ± 0.16 g; range: 0.34 – 2.90 g; length left testis, mean ± SE: 2.12 ± 0.09 cm; range: 1.40 – 2.92 cm; length right testis, mean ± SE: 2.13 ± 0.08 cm; range: 1.50 – 2.80 cm).

There was a general effect of body mass in all models with mass left testis as response variable (all $p < 0.05$) (Figure 6.4b; Figure S1). In addition, *Strongyloides robustus* abundance and intensity had a negative effect on testis mass (abundance estimate: - 0.02 ± 0.01, $t_{19} = -2.35$, $p = 0.03$, Figure 6.5; intensity estimate: - 0.02 ± 0.01, $t_{15} = -2.37$, $p = 0.03$; Table 6.3; Figure S1).

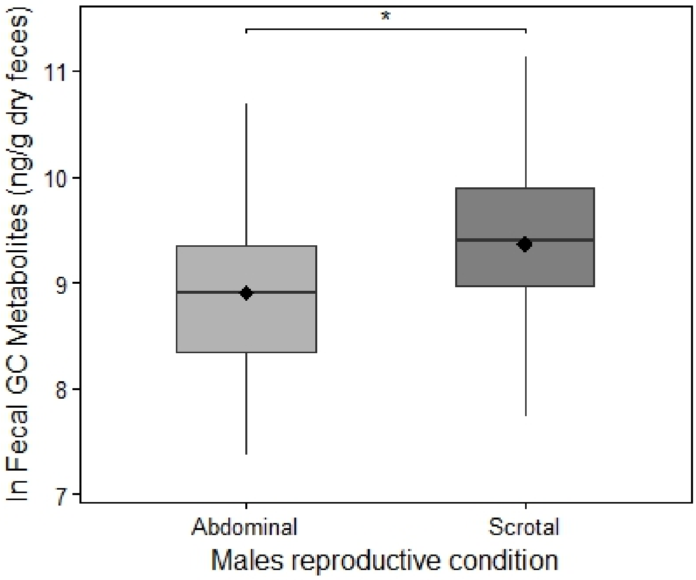


Figure 6.3 – Relationship between reproductive condition and fecal glucocorticoid (GC) metabolite concentrations in male Eastern grey squirrel. Fecal GC metabolite concentrations were measured in samples from abdominal (grey color; n = 58; "Abdominal") or scrotal (dark-grey color; n = 87; "Scrotal") testes. Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. * $p < 0.05$.

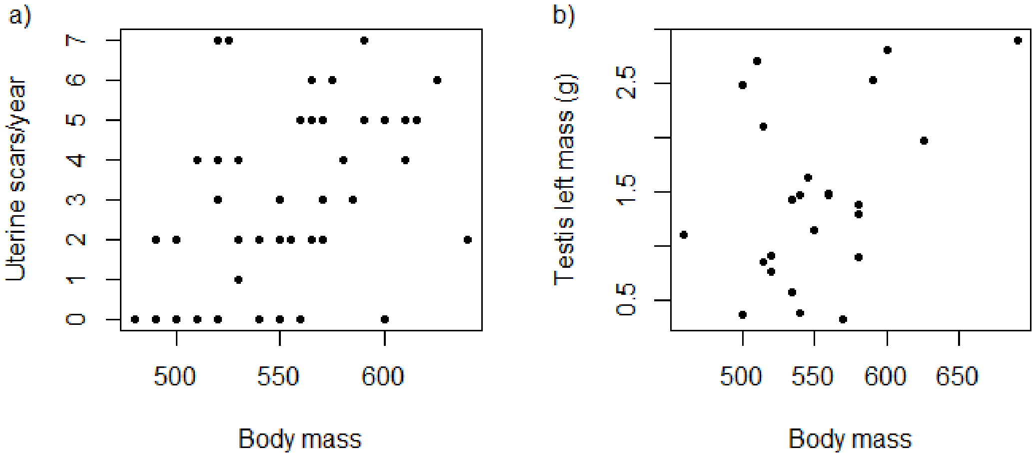


Figure 6.4 – Relationship between Eastern grey squirrel body mass and: a) females reproductive success (uterine scars/year) (n = 49; all $p < 0.05$; Table **6.2**); b) males reproductive investment (testis left mass) (n = 24; all $p < 0.05$; Table **6.3**).

6.6 Discussion

We explored, as first step, if variation in four parasite infections predictors (*S. robustus* abundance, prevalence, and intensity, and helminths richness) was affected by physiological stress, but our results showed that none of them was influenced by FGM concentration. Furthermore, we investigated variation in stress levels of Eastern grey squirrel in relation to intrinsic individual parameters, such as body mass, sex and reproductive condition. FGM concentrations did not differ between the sexes but increased with the individual's body mass, and among reproductively active animals with scrotal testes had higher FGM concentrations than reproductively non-active males with small abdominal testes. Levels of physiological stress also changed with extrinsic factors and were higher in autumn than in other seasons.

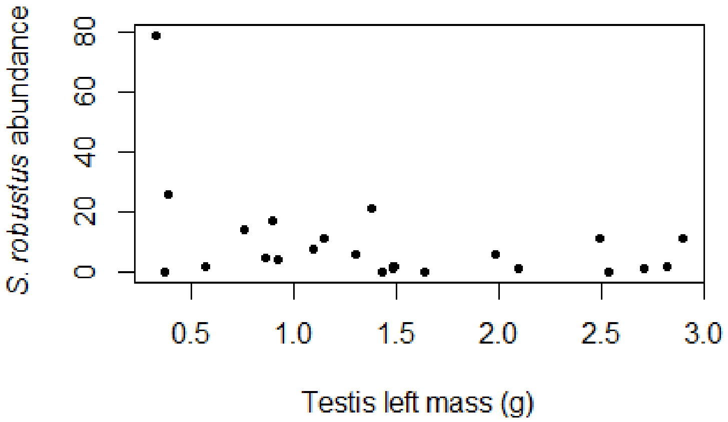


Figure 6.5 – Relationship between *S. robustus* abundance and testis left mass (g) in male Eastern grey squirrels (n = 24, t_{19}= -2.35, p = 0.03) (Table 6.3).

Secondly, we tested if the increase in physiological stress and/or higher parasite loads were associated with lower reproductive success (females)/investment (males) in grey squirrels. Our models reported that females reproductive success was affected only by body mass and there was no effect of FGM concentrations or parasite infection. Conversely, males testes mass was positively related to body mass but negatively with parasite infections and there was no effect of FGM concentrations, this result suggests that the cost of reproductive investment in males is related to a high *S. robustus* abundance and intensity.

Overall, our models showed a strong and general effect of body mass, that positively influences FGM concentrations, female reproductive success, male reproductive investment and parasite infections (abundance and intensity).

Finally, we tested the prediction that personality would not affect directly reproductive success/investment, but instead indirectly through its influence on FGM concentration and/or parasite loads. Our findings confirmed the first part of this hypothesis showing that personality score was not correlated with females reproductive success or males reproductive investment. In addition, we demonstrated that parasite load (*S. robustus* abundance and infection status) was higher in bold than in shy animals (see Chapter 4; Table 7), while there was no direct influence of personality on physiological stress.

6.6.1 Relationship between parasite infections and physiological stress

Physiological stress has been investigated in relation to its negative effect on individual immune system (Romero 2004; Dantzer et al. 2014), which might result in a less efficient immune response and, subsequently a higher susceptibility to parasitic infections (Fenton and Perkins 2010). However, in our populations of invasive alien grey squirrels we did not find any effect of levels of physiological stress on the four infection predictors that we considered (helminth richness, *S. robustus* abundance, *S. robustus* infection status and intensity of infection). This result was unexpected since there is a wide literature on relationship between parasites and physiological stress (Chapman et al. 2006; Dunn et al. 2012; Poulin 2013; St. Juliana et al.

2014; Ezenwa et al. 2017). We believe that the predicted relation did not occur due to intrinsic problems related to our study system. As already underlined in earlier chapters, the parasite fauna of alien grey squirrels is characterized by the process of parasite release, with a low macroparasite richness and a single dominant gastro-intestinal helminth (Romeo et al. 2014a). Furthermore, parasite intensity of our sample was slightly lower compared to other populations in Northern Italy (10.7 ± 2.1 against 16.9 ± 2.1 respectively, see Romeo et al. 2014a) and with respect to findings from populations in the species' natural range in the Southeastern United States (Davidson 1976), where severe hemorrhagic enteritis was recorded for infected grey squirrels with more than 150 worms, thereby activating a immune response related to high intensity of infection (e.g. *Strongyloides ratti*: Paterson and Viney 2002). This result may indicate that pathogenic effects occur with high intensity of infection, which was not reached by the majority of grey squirrels in our populations, possibly explaining the observed non-significant relationship between parasite infection and FGM concentration.

6.6.2 Variation in physiological stress levels

Grey squirrel FGM concentrations increased in relation to body mass and were higher in autumn than in winter and spring-summer. These results are consistent with those found for Golden-mantled ground squirrel (*Spermophilus saturates*) where cortisol increased at the end of the active season, in coincidence with the peak in body mass (Boswell et al. 1994). Moreover, also in Eurasian red squirrel (*Sciurus vulgaris*), higher FGM concentrations were found in winter respect to autumn and in autumn compared to spring-summer (Dantzer et al. 2016). Grey squirrels breeding seasons (matings) tend to peak during December to February and May to June in the native range; however, reproductive males with descend (scrotal) testes can be found in almost any month of the year both in the native range (Steele and Koprowski 2001) and in Italian populations. In autumn, resident grey squirrels engage intensively in caching (scatter-hoarding) nuts and large tree seeds, spending more time on the ground (Wauters et al. 2002a). This activity is likely to increase interactions among hoarders and potential cache-pilferers and might explain why FGM concentrations were most elevated in autumn in this species. Furthermore, grey squirrels are not territorial (Don 1983; Thompson 1978b), but resident adult male and female kin groups may defend core areas in autumn thereby limiting immigration (Kenward 1985; Gurnell et al. 2001), and agonistic behavior peaks in spring and autumn, the periods of greatest dispersal (Thompson 1978b). Hence, the seasonal pattern of changes in FGM concentrations seems to be related rather to the social organization, dispersal and hoarding behaviors and not to the breeding season. Overall, there is no general pattern in mammals as glucocorticoid levels are elevated during the breeding season compared to the pre- or post-breeding seasons in some species (Vera et al. 2013; Corlatti et al. 2014; Jachowski et al. 2015) but not in others (Place and Kenagy 2000; Romero et al. 2008; Delehanty and Boonstra 2011; Bauer et al. 2014; Fletcher et al. 2015). Hence, the reproductive status of an animal might influence its adrenocortical activity, thus baseline FGM levels, with subsequent effects to their assessment (Millspaugh and Washburn 2004, Goymann 2012).

We did find higher FGM concentrations in males with scrotal than abdominal testes. Scrotal testes represent an active reproductive status for males (Dantzer et al. 2016). In squirrels, reproductive behavior is characterized by mating chase, in which male squirrels compete for access to females based on male dominance hierarchy (Koprowski 1993a). In grey squirrels direct conflict between sexes is evident during mating bouts in evasive behavior, selectivity of mating sites, and removal of the copulatory plug by the female (Koprowski 1993b, c, 1996). This behavior causes high intraspecific contact, in particular among the competing males, and could lead to the observed increase in stress hormones levels.

6.6.3 Variation in reproductive success and investment

Variation among animals in levels of physiological stress has been reported to influence individual variation in fitness components (e.g. reproduction) (Cleveland et al. 2004; Both et al. 2005; Smith and Blumstein 2008; Réale et al. 2009; Patterson and Shulte-Hostedde 2011; Dantzer et al. 2013; Vetter et al. 2016). High physiological stress measured in rhesus macaques (*Macaca mulatta*) was correlated with reduced reproductive success (Cleveland et al. 2004), while high glucocorticoid levels in females North American red squirrel (*Tamiasciurus hudsonicus*) allowed them to produce faster growing offspring, indicating a positive effect of stress (Dantzer et al. 2013). In this study, Dantzer et al. (2013) experimentally elevated actual and perceived density, which induced higher maternal glucocorticoid levels. In contrast with these studies we did not find any effects of physiological stress on reproductive success/investment.

On the other hand, parasitic infections can influence reproduction in various ways. For instance, in female Cape ground squirrels (*Xerus inauris*) and Columbian ground squirrels (*Spermophilus columbianus*) reproductive success, in terms of number of offspring raised to emergence (litter size at weaning), increased in relation to experimental parasite removal (Neuhaus 2003; Hillegass et al. 2010). Also, in North American red squirrels (*Tamiasciurus hudsonicus*), reproductive success decreased in animals with higher richness of endo and ectoparasites combined (Gooderham and Shulte-Hostedde, 2011). The only significant relationship we found in our relatively small sample (n = 24 males), was a negative correlation between parasite infection (*S. robustus* abundance and intensity) and male reproductive investment (testis mass). In previous studies, abundance and intensity have been discussed as factors related to individual immunity and, thus, to susceptibility (Paterson and Viney 2002; Romeo et al. 2014b). Energy expenditure in immune system against infections may altered normal allocation of resources for reproduction (Lochmiller and Deerenberg 2000; Hõrak et al. 2006). Hence, more heavily infected male grey squirrels might have a reduced immune response, and subsequently less available energy to invest in reproduction (testes mass).

In contrast, among females we did not find an effect of individual variation in parasitic infections on annual reproductive success (number of uterine scars). We believe that the lack of an apparent cost of parasite load on reproduction could be explained by the majority of our sampled squirrels having a low parasite intensity (see Table 4.5).

The strongest relationship revealed in this study was the positive effect of an individual's body mass on its reproductive output, both in females (uterine scars/year) and males (testis mass). In tree squirrels, body mass is associated with dominance rank in both sexes, with heavier males occupying larger home ranges and mating more frequently than lower-ranked subordinate males of poorer body mass (Wauters and Dhondt 1989; Wauters et al. 1990; Koprowski 1993a). Hence, higher dominance might also be related to larger testes, thus to reproductive investment (Manjerovic et al. 2008). For females, entering in estrus is determined by reaching a good body mass that, in turn, guarantees to support reproduction effort throughout pregnancy and lactation of the young (Wauters and Dhondt 1989, 1995; Wauters et al. 2007, Hayssen 2016)

We believe the lack of significant effect of FGM concentration and parasite load on females reproductive success can be explained, at least partly, by the prominent role of body mass. As explained above, reaching a good body mass is a driving factor for female reproduction and, thus, could cover other possible more subtle effects related to other factors such as parasite infections or physiological stress. In addition, male reproductive investment might be significantly influenced by the cost related to parasite infection, that could cover other more subtle effect derived from FGM levels.

6.6.4 *Personality effect on reproductive success/investment and physiological stress*

A meta-analysis conducted by Smith and Blumstein (2008) on both wild and captive animals provided evidence of general relationship between personality and fitness. They reported that, as general trend, bolder individuals have a greater reproductive success than shyer ones, particularly in males, but survive less. Nevertheless, in wild animals they found no effect of boldness on reproductive success, which could be explained by their sex biased sample size. Conversely, they found that exploration had no effect on reproductive success. Moreover, they asserted that further studies need to be conducted to investigate possible effect of environmental condition or other forms of selection. In particular, studies conducted on eastern chipmunk (*Tamias striatus*) showed that bold males had more offspring when controlling for parasite load (Patterson and Shulte-Hostedde 2011) and docility and boldness of bighorn sheep (*Ovis canadensis*) had a negative effect on reproductive success in early life stages respect to strong positive effect found in older rams (Réale et al. 2009). In addition, Le Coeur et al. (2015) showed that variation in personality of Siberian chipmunk (*Tamias sibiricus*) was associated with variation in annual reproductive success, but that the direction of this relationship fluctuated between years, according to annual food availability. These examples highlight how relationships between personality and reproductive success can vary depending on other factors that fluctuate over time and across space.

Moreover, personality has been investigated also in relation to physiological stress (Koolhaas et al. 1999; Westergaard et al. 2003; Réale et al. 2007). For instance, in great tit (*Parus major*) more aggressive and bold birds (fast explorers) showed no stress response with respect to less aggressive (slow explorers) ones (Carere et al. 2003), and similar relationships have been investigated in several animal taxa (see Carere et al. 2010 for a review). On the contrary, in our study populations, grey squirrels personality score did not affect FGM concentrations.

In conclusion, during the last years, some studies have demonstrated that the direct relationships between variables related to stress, personality and parasite infections might be difficult to detect. For instance, host behavior and parasite connections may be indirect, thus mediated by genetics, physiology, or efficiency of the immune system (Dunn et al. 2012; Ezenwa et al. 2017). This apparent lack of clear, direct, relationships needs to be investigated deeply and opens the way to future studies that could test if more subtle (e.g. indirect) effects of FGM concentration and/or personality might affect reproductive success/investment in free-living mammals (Werner and Peacor 2003; Schmitz et al. 2004; Dunn et al. 2012; Ezenwa et al. 2017).

Table 6.1 – Minimum selected models explaining observed variation in FGM concentration and parasite infection predictors in grey squirrels. (predictor sex: male held as reference level). Significant parameters in bold.

Response variable	**Sample size**	**Predictor**	**df**	**χ^2**	**p**	**Predictor**	**df**	**χ^2**	**p**
S. robustus abundance	96 individuals	sex	1	0.79	0.37	sex	1	1.79	0.18
		body mass	1	1.85	0.17	body mass	1	2.01	0.16
		FGM conc.	1	0.005	0.95	FGM conc.	1	0.001	0.97
						PC1 score	**1**	**8.06**	**0.01**
Infection status	96 individuals	sex	1	0.27	0.61	sex	1	0.62	0.43
		body mass	1	1.004	0.32	body mass	1	1.19	0.28
		FGM conc.	1	0.04	0.84	FGM conc.	1	0.06	0.80
						PC1 score	1	1.57	0.21
Intensity	61 individuals	sex	1	1.46	0.23	sex	1	3.95	0.047
		body mass	1	1.07	0.30	body mass	1	0.24	0.63
		FGM conc.	1	0.68	0.41	FGM conc.	1	0.81	0.37
						PC1 score	**1**	**6.47**	**0.01**
Helminth richness	96 individuals	sex	1	0.38	0.54	sex	1	0.04	0.84
		body mass	1	0.05	0.83	body mass	1	0.34	0.56
		FGM conc.	1	0.12	0.73	FGM conc.	1	0.39	0.53
						PC1 score	1	0.08	0.78

Table 6.2 – Minimum selected models explaining observed variation in females grey squirrels fecundity (n = 49) (predictor sex: male held as reference level). Significant parameters in bold.

Response variable	**Predictor**	**df**	**χ^2**	**p**	**Predictor**	**df**	**χ^2**	**p**
Uterine scars/year	**body mass**	**1**	**17.15**	**< 0.0001**	**body mass**	**1**	**7.19**	**0.01**
	S. robustus abundance	1	0.75	0.39	*S. robustus* abundance	1	0.78	0.38
					PC1 score	1	0.90	0.34
Uterine scars/year	**body mass**	**1**	**16.47**	**< 0.0001**	**body mass**	**1**	**6.30**	**0.01**
	Infection status	1	1.11	0.29	Infection status	1	0.59	0.44
					PC1 score	1	0.42	0.52
Uterine scars/year	**body mass**	**1**	**9.34**	**0.002**	**body mass**	**1**	**8.53**	**0.003**
	Intensity	1	1.02	0.31	Intensity	1	1.14	0.29
					PC1 score	1	0.39	0.53
Uterine scars/year	**body mass**	**1**	**16.78**	**< 0.0001**	**body mass**	**1**	**6.05**	**0.01**
	Helminth richness	1	0.66	0.42	Helminth richness	1	0.47	0.49
					PC1 score	1	0.61	0.43
Uterine scars/year	body mass	1	0.25	0.62	**body mass**	**1**	**6.32**	**0.01**
	FGM conc.	1	0.09	0.77	PC1 score	1	0.69	0.41

Table 6.3 – Minimum selected models explaining observed variation in males grey squirrels reproductive investment (n = 24) (predictor sex: male held as reference level). Significant parameters in bold.

Response variable	**Predictor**	**df**	**χ²**	**p**	**Predictor**	**df**	**χ²**	**p**
Testis mass	**body mass**	**1**	**10.63**	**0.001**	**body mass**	**1**	**8.79**	**0.003**
	***S. robustus* abundance**	**1**	**5.51**	**0.02**	***S. robustus* abundance**	**1**	**4.44**	**0.04**
					PC1 score	1	0.25	0.62
Testis mass	**body mass**	**1**	**5.29**	**0.02**	**body mass**	**1**	**4.51**	**0.03**
	Infection status	1	1.27	0.26	Infection status	1	1.47	0.23
					PC1 score	1	1.20	0.27
Testis mass	**body mass**	**1**	**6.45**	**0.01**	**body mass**	**1**	**6.31**	**0.01**
	Intensity	**1**	**5.63**	**0.02**	**Intensity**	**1**	**4.56**	**0.03**
					PC1 score	1	0.68	0.41
Testis mass	**body mass**	**1**	**6.42**	**0.01**	**body mass**	**1**	**5.64**	**0.02**
	Helminth richness	1	0.12	0.73	Helminth richness	1	0.20	0.65
					PC1 score	1	0.95	0.33
Testis mass	**body mass**	**1**	**19.23**	**< 0.0001**	**body mass**	**1**	**5.65**	**0.02**
	FGM conc.	1	0.09	0.76	PC1 score	1	0.82	0.36

Conclusions

The aim of the present study is to investigate the interactions between Invasive Alien Species (IAS) and native species, and improve the knowledge on biological invasions impact in the light of potential influence of parasitic infections, physiological stress and personality on individual fitness (reproductive success/investment). Several studies highlighted how invasive propensity and, consequently, successful invasion are favored in (host) species that preferentially allocate resources to growth and reproductive effort, such as number of offspring per breeding season, over costly immune defence mechanisms (Kolar and Lodge 2001; Sakai et al. 2001; Lee and Klasing 2004; Telfer and Brown 2012). Only recently, variation in individual fitness (e.g. reproduction and survival) has been investigated in the light of relationships with direct or cumulative effects/influences of physiological stress, behavior and parasitic infections (Sih et al. 2004; Both et al. 2005; Morand et al. 2006; Hillegass et al. 2010; Dantzer et al. 2010, 2014; Bosson et al. 2013). Parasites have a key role in invasion processes but still little is known about their effect on invasive and native host populations (Daszak et al. 2000; Prenter et al. 2004). Furthermore, their interaction with other factors, such as physiological stress and personality, could influence individual fitness, thereby facilitating or limiting invasion success (Dunn et al. 2012).

In particular, this study investigated the interspecific competition between the North American invasive Eastern grey squirrel (*Sciurus carolinensis*) and the native Eurasian red squirrel (*Sciurus vulgaris*).

In my project I went through preliminary steps that allowed to identify techniques and methods used in successive analyses; firstly, I investigated each single factor separately, then I explored their cumulative effects.The first necessary step, that consequently allowed to evaluate physiological stress in native species in relation to invasive species, is validation of an enzyme immunoassay to measure fecal glucocorticoid metabolites in Eurasian red squirrel. The first chapter show how this assay can detect changes in FGM concentrations in response to changes in reproductive condition or seasonal/habitat factors and can be a useful tool to detect physiological stress in wildlife. Successively, I investigated native species physiological stress in relation to invasive species presence and how it changes during experimental removal of Eastern grey squirrel. The second chapter asserts that the sole presence of Eastern grey squirrel is sufficient to increase stress hormones levels in Eurasian red squirrel and suggest that also physiological stress should be considered as one of the mechanisms involved in invasive-native species interactions. This finding confirm the first hypothesis.

The second step defined the indirect and direct methods to measure personality in squirrels, this step allowed to investigate, in further analyses, the potential mediatory role of personality on relationships between factors involved. The third chapter shows that indirect indices derived from capture-mark-recapture data are a reliable method to measure personality and, the fourth chapter, highlights that they are consistent with direct measurements obtained with arena tests. Furthermore, analyzing the relationship between personality and parasites I demonstrated that bold Eastern grey squirrel are more likely to be infected, confirming the third hypothesis.

The third step presented a staining technique to count placental uterine scars in female grey squirrels. The fifth chapter indicates that uterine scars counts are a good method to estimate reproductive success in Eastern grey squirrels and consider how, in the new range, IAS have a higher reproductive output than in their native range, adding knowledge on Eastern grey squirrel population growth, hence its capacity to spread. Next, the sixth chapter, highlights the relationships between physiological stress and parasitic infections, showing that none of the four infection predictors are influenced by physiological stress, probably due to sample size characteristics. This finding does not support the second hypothesis. Moreover, I tested the potential influence of physiological stress and/or parasite loads on Eastern grey squirrel reproductive success (females) and investment (males), showing how male testes mass is negatively correlated to parasitic infection, but not to stress hormones levels. This result supports partly the fourth hypothesis. However, for female grey squirrels relationships are different. Variation in, female reproductive success is influenced significantly only by individual variation in body mass. Contrary to predictions in the fourth hypothesis, physiological stress and parasite infections are not related to reproductive output. In addition, these findings show a prominent role of body mass on Eastern grey squirrel reproductive output. Finally, I analyzed the mediatory role of personality, whose predicted absence of direct relationship with reproductive success/investment is confirmed in the last chapter. However, the tested indirect influence of personality, through effects on physiological stress, is not supported by the results. Thus, the fifth hypothesis, is demonstrated only regarding the direct effect of personality on parasite load.

Summarizing the overall relationships of parasites, physiological stress, personality on Eastern grey squirrel reproductive output, I can confirm that personality influences directly and positively parasite load, that in turn has a negative (costly) effect on male reproductive investment. Certainly, the strong and significant relationship of body mass, thus individual condition, with physiological stress and parasite load, and its marked effect on females reproductive success and males reproductive investment, confirms findings from earlier studies on different species of native tree squirrels.

In conclusion, as abovementioned, successful invasions are favored in (host) species that preferentially allocate resources to growth and reproductive effort. I can confirm that, also regarding Eastern grey squirrel, reproductive success is mainly and strongly influenced by female body mass, the same is true for male reproductive investment, which in turn is influenced indirectly by grey squirrel personality and directly by parasite load. This thesis has demonstrated for the first time that Eastern grey squirrel impact on Eurasian red squirrel populations occurs also, through increase in physiological stress in the native species. Moreover, this study shows that individual condition is a prominent factor that drives invasion success (reproductive output), but the overall effect can be mediated by parasites, which are confirmed to have a strong role in biological invasion. Finally, the role of variation in personality among individuals of the alien species affects the prevalence of macroparasite infections, but its indirect effects in trait-mediated competition remains, so far, less clear.

References

Able DJ (1996). The contagion indicator hypothesis for parasite-mediated sexual selection. Proceedings of the National Academy of Sciences. 93: 2229-33.

Allen SH (1983). Comparison of red fox litter sizes determined from counts of embryos and placental scars. Journal of Wildlife Management. 47: 860-863.

Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, Cunningham AA, Dobson AP, Ezenwa V, Jones KE, Pederson AB, Poss M, Pulliam JRC (2003). Social organization and parasite risk in mammals: integrating theory and empirical studies. Annual Review of Ecology, Evolution and Systematics. 34: 517-547.

Anderson RM, May RM (1978). Regulation and stability of host-parasite population interactions. 1. regulatory processes. Journal of Animal Ecology. 47: 219-247.

Anson JR, Dickman C, Boonstra R, Jessop TS (2013). Stress triangle: Do introduced predators exert indirect costs on native predators and prey ? PLoS ONE. 8: e60916.

Archer J (1973). Tests for emotionality in rats and mice: A review. Animal Behaviour. 21: 205-235.

Ardia DR, Schat KA, Winkler DW (2003). Reproductive effort reduces longterm immune function in breeding tree swallows (*Tachycineta bicolor*). Proceedings of the Royal Society of London B: Biological Sciences. 270: 1679-1683.

Aschauer A, Hoffmann IE, Millesi E (2006). Endocrine profiles and reproductive output in European ground squirrels after unilateral ovariectomy. Animal Reproductive Science. 92: 392-400.

Baenninger R (1966). Waning of an aggressive motivation in *Betta splendens*. Psychological Science. 4: 241-242.

Baker S (2006). Eradication of coypus (*Myocastor coypus*) from Britain: the elements required for a successful campaign, in: Koike F, Clout MN, Kawamichi M, De Poorter Iwastsuki, K. (Eds.), Assesment and Control of Biological Invasion Risk. Shoukadoh Book Sellers, Kyoto, Japan and IUCN, Gland, Switzerland, 2006., pp: 142–147.

Banks PB, Dickman CR (2007). Alien predation and the effects of multiple levels of prey naiveté. Trends in Ecology & Evolution. 22: 229-230.

Barber I, Dingemanse NJ (2010). Parasitism and the evolutionary ecology of animal personality. Philosophical Transactions of the Royal Society B: Biological Sciences. 365: 4077-4088.

Barber I, Mora AB, Payne EM, Weinersmith KL, Sih A (2016). Parasitism, personality and cognition in fish. Behavioural Processes. 141: 205-219.

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 Parasitologica. 42: 102-114.

Bates D, Mächler M, Bolker B, Walker S (2015). Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67.

Bauer CM, Hayes LD, Ebensperger LA, Romero LM (2014). Seasonal variation in the degu (*Octodon degus*) endocrine stress response. General and Comparative Endocrinology. 197: 26-32.

Beale DM (1962). Growth of the eye lens in relation to age in fox squirrels. Journal of Wildlife Management. 26: 208-211.

Bell AM (2007). Future directions in behavioural syndromes research. Proceedings of the Royal Society of London B: Biological Sciences. 274: 755-761.

Bell AM, Stamps JA (2004). Development of behavioural differences between individuals and populations of sticklebacks, *Gasterosteus aculeatus*. Animal Behaviour. 68: 1339-1348.

Bell AM, Hankison SJ, Laskowski KL (2009). The repeatability of behaviour: a meta analysis. Animal Behaviour. 77: 771-783.

Bengtson JL, Siniff DB (1981). Reproductive aspects of female crabeater seals (*Lobodon carcinophagus*) along the Antarctic Penisula. Canadian Journal of Zoology. 59: 92-102.

Bercovitch FB, Ziegler TE (2002). Current topics in primate socioendocrinology. Annual Review of Anthropology. 31: 45-67.

Berger S, Wikelski M, Romero LM, Kalko EKV, Rödl T (2007). Behavioral and physiological adjustments to new predators in an endemic island species, the Galápagos marine iguana. Hormones and Behavior. 52: 653-663.

Bergeron P, Montiglio PO, Réale D, Humphries MM, Gimenez O, Garant D (2013). Disruptive viability selection on adult exploratory behaviour in eastern chipmunks. Journal of Evolutionary Biology. 26: 766-774.

Bergmüller R, Taborsky M (2010). Animal personality due to social niche specialisation. Trends in Ecology & Evolution. 25: 504-511.

Bertolino S (2009). Animal trade and non-indigenous species introduction: the world-wide spread of squirrels. Diversity and Distributions. 15: 701–708.

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

Bertolino S, Lurz PWW, Sanderson R, Rushton SP (2008). Predicting the spread of the American grey squirrel (*Sciurus carolinensis*) in Europe: A call for a co-ordinated European approach. Biological Conservation. 141: 2564-2575.

Bertolino S, Montezemolo NC, Preatoni DG, Wauters LA, Martinoli A (2014). A grey future for Europe: *Sciurus carolinensis* is replacing native red squirrels in Italy. Biological Invasions. 16: 53-62.

Biro PA, Stamps JA (2008). Are animal personality traits linked to life-history productivity? Trends in Ecology & Evolution. 23: 361-368.

Biro PA,Abrahams MV, Post RJ, Parkinson EA (2004). Predators select against high growth rates and risk taking behaviour in domestic trout populations. Proceedings of the Royal Society of London B: Biological Sciences. 271: 2233-2237.

Bisi F, von Hardenberg J, Bertolino S, Wauters LA, Imperio S, Preatoni DG, Mazzamuto MV, Provenzale A, Martinoli A (2016). Current and future conifer seed production in the Alps: testing weather factors as cues behind masting. European Journal of Forest Research. 135: 743-754.

Blumstein DT, Holland B-D, Daniel JC (2006). Predator discrimination and 'personality' in captive Vancouver Island marmots (*Marmota vancouverensis*). Animal Conservation. 9: 274-282.

Bohn SJ, Webber QMR, Florko KRN, Paslawski KR, Peterson AM, Piche JE, Menzies AK, Willis CKR (2017). Personality predicts ectoparasite abundance in an asocial sciurid. Ethology. 0: 1-11.

Bonesi L, Palazon S (2007). The American mink in Europe: Status, impacts, and control. Biological Conservation. 134: 470-483.

Bonier F, Martin PR, Moore IT, Wingfield JC (2009). Do baseline glucocorticoids predict fitness?. Trends in Ecology & Evolution. 24: 634-642.

Boon AK, Réale D, Boutin S (2007). The interaction between personality, offspring fitness and food abundance in North American red squirrels. Ecology Letters. 10: 1094-1104.

Boon AK, Réale D, Boutin S (2008). Personality, habitat use, and their consequences for survival in North American red squirrels *Tamiasciurus hudsonicus*. Oikos. 117: 1321-1328.

Boonstra R, Hik D, Singleton GR, Tinnikov A (1998). The impact of predator-induced stress on the snowshoe hare cycle. Ecological Monographs. 68: 371–394.

Boonstra R, Hubbs AH, Lacey EA, McColl CJ (2001). Seasonal changes in glucocorticoid and testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon. Canadian Journal of Zoology. 79: 49-58.

Boronow KE, Langkilde T (2010). Sublethal effects of invasive fire ant venom on a native lizard. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology. 313: 17-23.

Bosson CO, Palme R, Boonstra R (2009). Assessment of the stress response in Columbian ground squirrels: laboratory and field validation of an enzyme immunoassay for fecal cortisol metabolites. Physiological and Biochemical Zoology. 82: 291-301.

Bosson CO, Palme R, Boonstra R (2013). Assessing the impact of live-capture, confinement, and translocation on stress and fate in eastern gray squirrels. Journal of Mammalogy. 94: 1401-1411.

Boswell T, Woods SC, Kenagy GJ (1994). Seasonal changes in body mass, insulin, and glucocorticoids of free living golden-mantled ground squirrels. General and comparative endocrinology. 96: 339-46.

Both C, Dingemanse NJ, Drent PJ, Tinbergen JM (2005). Pairs of extreme avian personalities have highest reproductive success. Journal of Animal Ecology. 74: 667-674.

Boutin S, Larsen KW (1993). Does food availability affect growth and survival of males and females differently in a promiscuous small mammal, *Tamiasciurus hudsonicus*? Journal of Animal Ecology. 62: 364-370.

Boutin S, Wauters LA, McAdam AG, Humphries MM, Tosi G, Dhondt AA (2006). Anticipatory reproduction and population growth in seed predators. Science. 314: 1928- 1930.

Boyer N, Réale D, Marmet J, Pisanu B, Chapuis JL (2010). Personality, space use and tick load in an introduced population of Siberian chipmunks *Tamias sibiricus*. Journal of Animal Ecology. 79: 538-547.

Bray Y, Marboutin É, Péroux R, Ferron J (2003). Reliability of stained placental-scar counts in European hares. Wildlife Society Bulletin. 31: 237-246.

Brearley G, Rhodes J, Bradley A, Baxter G, Seabrook L, Lunney D, Liu Y, McAlpine C (2013). Wildlife disease prevalence in human-modified landscapes. Biological Reviews. 88: 427-442.

Bremner-Harrison SB, Prodohl PA, Elwood RW (2004). Behavioural trait assessment as a release criterion: boldness predicts early death in a reintroduction programme of captive-bred swift fox (*Vulpes velox*). Animal Conservation. 7: 313-320.

Breuner CW, Patterson SH, Hahn TP (2008). In search of relationships between the acute adrenocortical response and fitness. General and Comparative Endocrinology. 157: 288-295.

Broennimann O, Treier UA, Müller-Schärer H, Thuiller W, Peterson AT, Guisan A (2007). Evidence of climatic niche shift during biological invasion. Ecology Letters. 10: 701-709.

Bronstein PM (1972). Open-field behavior of the rat as a function of age. Journal of Comparative and Physiological Psychology. 80: 335-341.

Brownie AC (1992). The metabolism of adrenal cortical steroids Pp. 209-224 in The Adrenal Gland (V.H.T. James, ed.). Raven Press Ltd, New York, NY.

Buchanan KI, Goldsmith AR (2004). Noninvasive endocrine data for behavioural studies: the importance of validation. Animal Behaviour. 67: 183-185.

Burnham KP, Anderson DR (2002). Model selection and multi-model inference: a practical information-theoretic approach, Springer, New York.

Burns JG (2008). The validity of three tests of temperament in guppies (*Poecilia reticulata*). Journal of Comparative Psychology. 122: 344-356.

Busch DS, Hayward LS (2009). Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation. 142: 2844-2853.

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997). Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology. 83: 575–583.

Cabezas S, Blas J, Marchant TA, Moreno S (2007). Physiological stress levels predict survival probabilities in wild rabbits. Hormones and Behavior. 51: 313–320.

Cadi A, Joly P (2003). Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). Canadian Journal of Zoology. 81: 1392-1398.

Campbell DT, Fiske DW (1959). Convergent and discriminant validation by the multitrait-multimethod matrix. Psychological Bulletin. 56: 81-105.

Carere C, Groothuis TGG, Möstl E, Daan S, Koolhaas JM (2003). Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. Hormones and Behavior. 43: 540-548.

Carere C, Caramaschi D, Fawcett TW (2010). Covariation between personalities and individual differences in coping with stress: Converging evidence and hypotheses. Current Zoology. 56: 728-740.

Carter AJ, Goldizen AW, Tromp SA (2010). Agamas exhibit behavioral syndromes: bolder males bask and feed more but may suffer higher predation. Behavioral Ecology. 21: 655-661.

Carter AJ, Feeney WE, Marshall HH, Cowlishaw G, Heinsohn R (2013). Animal personality: what are behavioural ecologists measuring ? Biological Reviews. 88: 465-475.

Caso JR, Leza JC, Menchen L (2008). The effects of physical and psychological stress on the gastrointestinal tract: lessons from animal models. Current Molecular Medicine. 8: 299-312.

Cavigelli SA, McClintock MK (2003). Fear of novelty in infant rats predicts adult corticosterone dynamics and an early death. Proceedings of the National Academy of Sciences. 100: 16131-16136.

Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, McClintock MK (2005). Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. Journal of Endocrinology. 184: 153-163.

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

Chapman CA, Wasserman MD, Gillespie TR, Speirs ML, Lawes MJ, Saj TL, Zieglers TE (2006). Do food availability, parasitism, and stress have synergistic effects on red colobus populations living in forest fragments ? American Journal of Physical Anthropology. 131: 525-534.

Chapple DG, Simmonds SM, Wong BBM (2012). Can behavioral and personality traits influence the success of unintentional species introductions? Trends in Ecology & Evolution. 27: 57-64.

Chase DA, Flynn EE, Todgham AE (2016). Survival, growth and stress response of juvenile tidewater goby, *Eucyclogobius newberryi*, to interspecific competition for food. Conservation Physiology. 4: cow013.

Clavero M, Garciaberthou E (2005). Invasive species are a leading cause of animal extinctions. Trends in Ecology & Evolution. 20: 110-110.

Cleveland A, Westergaars GC, Trenkle MK, Higley JD (2004). Physiological predictors of reproductive outcome and mother–infant behaviors in captive Rhesus macaque females (*Macaca mulatta*). Neuropsychopharmacology. 29: 901-910.

Clinchy M, Sheriff MJ, Zanette LY (2013). Predator-induced stress and the ecology of fear. Functional Ecology. 27: 56-65.

Close B, Banister K, Baumans V, Bernoth EM, Bromage N, Bunyan J, Erhardt W, Flecknell P, Gregory N, Hackbarth H, Morton D, Warwick C (1996). Recommendations for euthanasia of experimental animals: Part 1. Laboratory Animals. 30: 293-316.

Close B, Banister K, Baumans V, Bernoth EM, Bromage N, Bunyan J, Erhardt W, Flecknell P, Gregory N, Hackbarth H, Morton D, Warwick C (1997). Recommendations for euthanasia of experimental animals: Part 2. Laboratory Animals. 31: 1-32.

Cockrem JF (2013). Individual variation in glucocorticoid stress responses in animals. General and Comparative Endocrinology. 181: 45-58.

Cockrem JF, Barrett DP, Candy EJ, Potter MA (2009). Corticosterone responses in birds: individual variation and repeatability in Adelie penguins (*Pygoscelis adeliae*). General and Comparative Endocrinology. 163: 158-168.

Cooke SJ, Sack L, Franklin CE, Farrell AP, Beardall J, Wikelski M, Chown SL (2013). What is conservation physiology? Perspectives on an increasingly integrated and essential science. Conservation Physiology. 1: cot001.

Corlatti L, Béthaz S, von Hardenberg A, Bassano B, Palme R, Lovari S (2012). Hormones, parasites and male mating tactics in Alpine chamois: identifying the mechanisms of life history trade-offs. Animal Behaviour. 84: 1061-1070.

Corlatti L, Palme R, Lovari S (2014). Physiological response to etho-ecological stressors in male Alpine chamois: timescale matters! Naturwissenschaften. 101: 577-586.

Cote I, Poulin R (1995). Parasitism and group-size in social mammals: a meta-analysis. Behavioral Ecology. 6: 159-65.

Cote J, Clobert J, Brodin T, et al. (2010). Personality-dependent dispersal: characterization, ontogeny and consequences for spatially structured populations. Philosophical Transactions of the Royal Society B: Biological Sciences. 365: 4065-4076.

Creel S (2001). Social dominance and stress hormones. Trends in Ecology & Evolution. 16: 491-497.

Creel S, Fox JE, Hardy J, Sands B, Garrot B, Peterson RO (2002). Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology. 16: 809-814.

Creel S, Dantzer B, Goymann W, Rubenstein DR (2013). The ecology of stress: effects of the social environment. Functional Ecology. 27: 66-80.

Crespi EJ, Williams TD, Jessop TS, Delehanty B (2013). Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals ? Functional Ecology. 27: 93-106.

Dall SRX, Houston AI, McNamara JM (2004). The behavioural ecology of personality: consistent individual differences from an adaptive perspective. Ecology Letters. 7: 734-739.

Dammhahn M, Almeling L (2012). Is risk taking during foraging a personality trait? A field test for cross-context consistency in boldness. Animal Behaviour. 84: 1131-1139.

Daniewski W, Jezierski T (2003). Effectiveness of divergent selection for open-field activity in rabbits and correlated response for body weight and fertility. Behavior Genetics. 33: 337-345.

Dantzer B, McAdam AG, Palme R, Fletcher QE, Boutin S, Humphries MM, Boonstra R (2010). Fecal cortisol metabolite levels in free-ranging North American red squirrels: assay validation and the effects of reproductive condition. General and Comparative Endocrinology. 167: 279-286.

Dantzer B, McAdam AG, Palme R, Boutin S, Boonstra R (2011). How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. General and Comparative Endocrinology. 174: 124-131.

Dantzer B, Newman AEM, Boonstra R, Palme R, Boutin S, Humphries MM, McAdam AG (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. Science. 340: 1215-1217.

Dantzer B, Fletcher QE, Boonstra R, Sheriff MJ (2014). Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species ? Conservation Physiology. 2: cou023.

Dantzer B, Santicchia F, van Kesteren F, Palme R, Martinoli A, Wauters LA (2016). Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season. Journal of Mammalogy. 97: 1385-1398.

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. Proceedings of the Helminthological Society of Washington. 43: 211-217.

Deerenberg C, Arpanius V, Daan S, Bos N (1997). Reproductive effort decreases antibody responsiveness. Proceedings of the Royal Society of London B: Biological Sciences. 264: 1021-1029.

Dehnhard M, Clauss M, Lechner-Doll M, Meyer HHD, Palme R (2001). Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. General and Comparative Endocrinology. 123: 111- 120.

Delehanty B, Boonstra R (2009). Impact of live trapping on stress profiles of Richardson's ground squirrel (*Spermophilus richardsonii*). General and Comparative Endocrinology. 160: 176-82.

Delehanty B, Boonstra R (2011). Coping with intense reproductive aggression in male arctic ground squirrels: the stress axis and its signature tell divergent stories. Physiological and Biochemical Zoology. 84: 417-428.

Di Febbraro M, Martinoli A, Russo D, Preatoni D, Bertolino S (2016). Modelling the effects of climate change on the risk of invasion by alien squirrels. Hystrix, the Italian Journal of Mammalogy. 27. doi:10.4404/hystrix-27.1-11776

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. Ecological Research. 23: 529-542.

Di Pierro E, Ghisla A, Wauters LA, Molinari A, Martinoli A, Gurnell J, Tosi G (2011). The effects of seed availability on habitat use by a specialist seed predator. European Journal of Wildlife Research. 57: 585-595.

Dickens MJ, Romero LM (2013). A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology. 191: 177-189.

Dickens MJ, Earle KA, Romero LM (2009). Initial transference of wild birds to captivity alters stress physiology. General and Comparative Endocrinology. 160: 76-83.

Dickens MJ, Delehanty B, Romero LM (2010). Stress: an inevitable component of animal translocation. Biological Conservation. 143: 1329-1341.

Dingemanse NJ, de Goede P (2004). The relation between dominance and exploratory behavior is context-dependent in wild great tits. Behavioral Ecology. 15: 1023-1030.

Dingemanse NJ, Réale D (2005). Natural selection and animal personality. Behaviour. 142: 1165-1190.

Dingemanse NJ, Bouwman KM, van de Pol M, van Overveld T, Patrick SC, Matthysen E, Quinn JL (2012). Variation in personality and behavioural plasticity across four populations of the great tit *Parus major*. Journal of Animal Ecology. 81: 116-126.

Dochtermann NA, Jenkins SH (2007). Behavioural syndromes in Merriam's kangaroo rats (*Dipodomys merriami*): a test of competing hypotheses. Proceedings of the Royal Society of London B: Biological Sciences. 274: 2343-2349.

Don BAC (1983). Home range characteristics and correlates in tree squirrels. Mammal Review. 13: 123-132.

Dozières A, Pisanu B, Gerriet O, Lapeyre C, Stuyck J, Chapuis J-L (2010). Macroparasites of Pallas's squirrels (*Callosciurus erythraeus*) introduced into Europe. Veterinary Parasitology. 172: 172-176.

Dunn JC, Cole EF, Quinn JL (2011). Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. Behavioral Ecology and Sociobiology. 65: 1459-1471.

Dunn AM (2009). Parasites and biological invasions. Advances in Parasitology. 68: 161-184.

Dunn AM, Perkins SE (2012). Editorial: Invasions and infections. Functional Ecology. 26: 1234-1237.

Dunn AM, Torchin ME, Hatcher MJ, et al. (2012). Indirect effects of parasites in biological invasions. Functional Ecology. 26: 1262-1274.

Edwards PD, Boonstra R (2016). Coping with pregnancy after 9 months in the dark: Post-hibernation buffering of high maternal stress in arctic ground squirrels. General and Comparative Endocrinology. 232: 1-6.

Ehrenfeld JG (2010). Ecosystem consequences of biological invasions. Annual Review of Ecology, Evolution and Systematics. 41: 59-80.

Elmeros M, Hammershøj M (2006). Experimental evaluation of the reliability of placental scar counts in American mink (*Mustela vison*). European Journal of Wildlife Research. 52: 132-135.

Ergon T, Gardner B (2014). Separating mortality and emigration: modelling space use, dispersal and survival with robust-design spatial capture-recapture data. Methods in Ecology and Evolution. 5: 1327-1336.

European Environment Agency (2012). The impacts of invasive alien species in Europe. EEA Technical report No. 16.

Ezenwa VO (2004). Host social behavior and parasitic infection: a multifactorial approach. Behavioral Ecology. 15: 446-54.

Ezenwa VO, Ekernas LS, Creel S (2012). Unravelling complex associations between testosterone and parasite infection in the wild. Functional Ecology. 26: 123-133.

Ezenwa VO, Archie EA, Craft ME, Hawley DM, Martin LB, Moore J, White L (2017). Host behaviour–parasite feedback: an essential link between animal behaviour and disease ecology. Proceedings of the Royal Society of London B: Biological Sciences. 283: 2015-3078.

Fanson KV, Best EC, Bunce A, Fanson BG, Hogan LA, Keeley T, Narayan EJ, Palme R, Parrotti ML, Sharp TM, Skogvold K, Tuthill L, Webster KN, Bashaw M (2017). One size does not fit all: Monitoring faecal glucocorticoid metabolites in marsupials. General and Comparative Endocrinology. 244: 146-156.

Fenton A, Perkins SE (2010). Applying predator-prey theory to modelling immune- mediated, within-host interspecific parasite interactions. Parasitology. 137: 1027-1038.

Festa-Bianchet M, Jorgenson JT, Bérubé CH, Portier C, Wishart WD (1997). Body mass and survival of bighorn sheep. Canadian Journal of Zoology. 75: 1372-1379.

Fletcher QE, Boonstra R (2006). Impact of live trapping on the stress response of the meadow vole (*Microtus pennsylvanicus*). Journal of Zoology. 270: 473-478.

Fletcher QE, Speakman JR, Boutin S, McAdam AG, Woods SB, Humphries MM (2012). Seasonal stage differences overwhelm environmental and individual factors as determinants of energy expenditure in free-ranging red squirrels. Functional Ecology. 26: 677- 687.

Fletcher QE, Dantzer B, Boonstra R (2015). The impact of reproduction on the stress axis of free-living male northern red backed voles (*Myodes rutilus*). General and Comparative Endocrinology. 224: 136-147.

Fraser DF, Gilliam JF, Daley MJ, Le AN, Skalski GT (2001). Explaining leptokurtic movement distributions: intrapopulation variation in boldness and exploration. American Naturalist. 158: 124-135.

Gallup GG Jr, Capper SA (1970). Preference for mirror-image stimulation in finches (*Passer domesticus domesticus*) and parakeets (*Melopsittacus undulatus*). Animal Behaviour. 18: 621-624.

Genovesi P, Carboneras C, Vilà M, Walton P (2015). EU adopts innovative legislation on invasive species: a step towards a global response to biological invasions? Biological Invasions. 17: 1307-1311.

Glen AS, Dickman CR (2008). Niche overlap between marsupial and eutherian carnivores: does competition threaten the endangered spotted-tailed quoll ? Journal of Applied Ecology. 45: 700–707.

Gooderham K, Shulte-Hostedde A (2011). Macroparasitism influences reproductive success in red squirrels (*Tamiasciurus hudsonicus*). Behavioral Ecology. 22: 1195-1200.

González-Bernal E, Brown GP, Shine R (2014). Invasive Cane toads: social facilitation depends upon an individual's personality. PLoS ONE. 9: e102880.

Gosling SD (2001). From mice to men: what can we learn about personality from animal research? Psychological Bulletin. 127: 45-86.

Goymann W (2005). Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Annals of the New York Academy of Sciences. 1046: 35-53.

Goymann W (2012). On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods in Ecology and Evolution. 3: 757-765.

Goymann W, Möstl E, Van't Hof T, East ML, Hofer H (1999). Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. General and Comparative Endocrinology. 114: 340-348.

Gozzi CA, Guichón LM, Benitez VV, Troyelli A, Navone GT (2014). Gastro-intestinal helminths in the red-bellied squirrel introduced in Argentina: accidental acquisitions and lack of specific parasites. Hystrix, the Italian Journal of Mammalogy. 25: 101-106.

Graham SP, Freidenfelds NA, McCormick GL, Langkilde T (2012). The impacts of invaders: Basal and acute stress glucocorticoid profiles and immune function in native lizards threatened by invasive ants. General and Comparative Endocrinology. 176: 400-408.

Graham SP, Freidenfelds NA, Thawley CJ, Robbins TR, Langkilde T (2017). Are invasive species stressful? The glucocorticoid profile of native lizards exposed to invasive fire ants depends on the context. Physiological and Biochemical Zoology. 90: 328-337.

Groothuis TGG, Carere C (2005). Avian personalities: characterization and epigenesist. Neuroscience & Biobehavioral Reviews. 29: 137-150.

Guenther A, Trillmich F (2015). Within-litter differences in personality and physiology relate to size differences among siblings in cavies. Physiology & Behavior. 145: 22-28.

Guichón ML, Doncaster PC (2008). Invasion dynamics of an introduced squirrel in Argentina. Ecography. 31: 211-220.

Gurnell J (1996). The effects of food availability and winter weather on the dynamics of a grey squirrel population in Southern England. Journal of Applied Ecology. 33: 325-338.

Gurnell J, Pepper H (1993). A critical look at conserving the British red squirrel (*Sciurus vulgaris*). Mammal Review. 23: 127-137.

Gurnell J, Wauters LA, Preatoni D, Tosi G (2001). Spacing behaviour, kinship and dynamics of the grey squirrel in a newly colonised deciduous woodland in north Italy. Canadian Journal of Zoology. 79: 1533-1543.

Gurnell J, Wauters LA, Lurz PW, Tosi G (2004). Alien species and interspecific competition: effects of introduced eastern grey squirrels on red squirrel population dynamics. Journal of Animal Ecology. 73: 26-35.

Gurnell J, Lurz PWW, Wauters LA (2015). Years of interactions and conflict in Europe: competition between Eurasian red squirrels and North American grey squirrel. In: Red squirrels: Ecology, Conservation & Management in Europe. Shuttleworth CM, Lurz PWW, Hayward MW, England, pp 19–37.

Gyuris E, Hankó JF, Feró O, Barta Z (2016). Personality and ectoparasitic mites (*Hemipteroseius adleri*) in firebugs (*Pyrrhocoris apterus*). Behavioural Processes. 122: 67-74.

Hackländer K, Zeitlhofer C, Ceulemans T, Suchentrunk F (2011). Continentality affects body condition and size but not yearly reproductive output in female European hares (*Lepus europaeus*). Mamm. Biol. - Z. Für Säugetierkd. 76: 662-664.

Haigh A, Butler F, O'Riordan R, Palme R (2017). Managed parks as a refuge for the threatened red squirrel (*Sciurus vulgaris*) in light of human disturbance. Biological Conservation. 211: 29-36.

Hall CS (1934). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. Journal of Comparative Psychology. 18: 385-403.

Hall ML, van Asten T, Katsis AC, Dingemanse NJ, Magrath MJL, Mulder RA (2015). Animal personality and pace-of-life syndromes: do fast-exploring fairy-wrens die young ? Frontiers in Ecology and Evolution. 3: 28.

Hämäläinen, A., Heistermann M, Fenosoa ZSE, Kraus C (2014). Evaluating capture stress in wild gray mouse lemurs via repeated fecal sampling: method validation and the influence of prior experience and handling protocols on stress responses. General and Comparative Endocrinology. 195: 68-79.

Hammond TT, Palme R, Lacey EA (2015). Contrasting stress responses of two co- occurring chipmunk species (*Tamias alpinus* and *T. speciosus*). General and Comparative Endocrinology. 211: 114-122.

Hammond-Tooke CA, Poulin R, Nakagawa S (2012). Parasitism and behavioural syndromes in the fish *Gobiomorphus cotidianus*. Behaviour. 149: 601-622.

Handa RJ, Burgess LH, Kerr JE, O'Keefe JA (1999). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Hormones and Behavior. 28: 464-476.

Hänninen L, Pastell M (2009). CowLog: Open-source software for coding behaviors from digital video. Behavior Research Methods. 41: 472-476.

Harper JM, Austad SN (2001). Effect of capture and season on fecal glucocorticoid levels in deer mice (*Peromyscus maniculatus*) and red-backed voles (*Clethrionomys gapperi*). General and Comparative Endocrinology. 123: 337-344.

Harris BN, Saltzman W, De Jong TR, Milnes MR (2012). Hypothalamic–pituitary–adrenal (HPA) axis function in the California mouse (*Peromyscus californicus*): Changes in baseline activity, reactivity, and fecal excretion of glucocorticoids across the diurnal cycle. General and Comparative Endocrinology. 179: 436-450.

Hatcher MJ, Dick JTA, Dunna AM (2012). Disease emergence and invasions. Functional Ecology. 26: 1275-1287.

Hawley DM, Etienne RS, Ezenwa VO, Jolles AE (2011). Does animal behavior underlie covariation between hosts' exposure to infectious agents and susceptibility to infection? Implications for disease dynamics. Integrative and Comparative Biology. 51: 528-539.

Hayssen V (2016). Reproduction in grey squirrels: from anatomy to conservation in The Grey Squirrel: Ecology and Management of an Invasive Species in Europe. Eds. Shuttleworth CM, Lurz PWW, Gurnell J (2016). European Squirrel Initiative, Stoneleigh Park, Warwickshire, UK. pp 115-130.

Heiblum R, Aizenstein O, Gvaryahu G, Voet H, Robinzon B, Snapir N (1998). Tonic immobility and open field responses in domestic fowl chicks during the first week of life. Applied Animal Behaviour Science. 60: 347-357.

Heistermann M, Palme R, Ganswindt A (2006). Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. American Journal of Primatology. 68: 257-273.

Herman JP, Adams D, Prewitt C (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Endocrinology. 61: 180–190.

Hillegass MA, Waterman JM, Roth JD (2010). Parasite removal increases reproductive success in a social African ground squirrel. Behavioral Ecology. 21: 696-700.

Holm S (1979). A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics. 6: 65-70.

Hõrak P, Tummeleht L, Talvik H (2006). Predator threat, copulation effort and immunity in male rats (*Rattus norvegicus*). Journal of Zoology. 268: 9-16.

Horváth G, Martín J, López P, Garamszegi LZ, Bertók P, Herczeg G (2016). Blood parasite infection intensity covaries with risk-taking personality in male Carpetan rock lizards (*Iberolacerta cyreni*). Ethology. 122: 355-363.

Hothorn T, Bretz F, Westfall P (2008). Simultaneous inference in general parametric models. Biometrical Journal. 50: 346-363.

Humanson GL (1962). Animal tissue techniques. W. H. Freeman and Co., San Francisco. 468 pp.

Huntingford FA (1976). Relationship between anti-predator behavior and aggression among conspecifics in the three-spined stickleback, *Gasterosteus aculeatus*. Animal Behaviour. 24: 245-260.

Jachowski DS, Washburn BE, Millspaugh JJ (2015). Revisiting the importance of accounting for seasonal and diel rhythms in fecal stress hormone studies. Wildlife Society Bulletin. 39: 738-745.

Jessop TS, Anson JR, Narayan E, Lockwood T (2015). An introduced competitor elevates corticosterone responses of a native lizard (*Varanus varius*). Physiological and Biochemical Zoology. 88: 237-245.

Kauhala K, Ahola MP, Kunnasranta M (2014). Decline in the pregnancy rate of baltic grey seal females during the 2000s. Annales Zoologici Fennici. 51: 313-324.

Keller RP, Geist J, Jeschke JM, Kühn I (2011). Invasive species in Europe: ecology, status, and policy. Environmental Sciences Europe. 23: 23.

Kelley AD, Humphries MM, McAdam AG, Boutin S (2015). Changes in wild red squirrel personality across ontogeny: activity and aggression regress towards the mean. Behaviour. 152: 1291-1306.

Kenagy GJ, Place NJ (2000). Seasonal changes in plasma glucocorticosteroids of free- living female yellow-pine chipmunks: effects of reproduction and capture and handling. General and Comparative Endocrinology. 117: 189-199.

Kenward RE (1985). Ranging behaviour and population dynamics in grey squirrels. In Behavioural ecology: ecological consequences of adaptive behaviour: 319-330. Sibly RM, Smith RH (Eds). Oxford: Blackwell Scientific Publications.

Kenward RE, Tonkin JM (1986). Red and grey squirrels: some behavioural and biometric differences. Journal of Zoology. 209: 279-281.

Kenward RE, Hodder KH, Rose RJ, Walls CA, Parish T, Holm JL, Morris PA, Walls SS, Doyle FI (1998). Comparative demography of red squirrels (*Sciurus vulgaris*) and grey squirrels (*Sciurus carolinensis*) in deciduous and conifer woodland. Journal of Zoology. 244: 7-21.

Kolar CS, Lodge DM (2001). Progress in invasion biology: predicting invaders. Trends in Ecology & Evolution. 16: 199-204.

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt CG, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ (1999). Coping styles in animals: current status in behavior and stress-physiology. Neuroscience & Biobehavioral Reviews. 23: 925-935.

Koprowski JL (1993a). Alternative reproductive tactics in male eastern gray squirrels: “making the best of a bad job”. Behavioral Ecology. 4: 165-171.

Koprowski JL (1993b). Do estrous female gray squirrels, *Sciurus carolinensis*, advertise their receptivity ? Canadian Field Naturalist. 106: 392-394.

Koprowski JL (1993c). The role of kinship in field interactions of juvenile grey squirrels. Canadian Journal of Zoology. 71: 224-226.

Koprowski JL (1994). *Sciurus carolinensis.* Mammalian Species. 480: 1-9.

Koprowski JL (1996). Natal philopatry, communal nesting, and kinship in fox squirrels and eastern gray squirrels. Journal of Mammalogy. 77: 1006-1016.

Korsten P, Mueller JC, Hermannstadter C, Bouwman KM, Dingemanse NJ, Drent PJ, Liedvogel M, Matthysen E, Van Oers K, Van Overveld T, Patrick SC, Quinn JL, Sheldon BC, Tinbergen JM, Kempenaers B (2010). Association between DRD4 gene polymorphism and personality variation in great tits: a test across four wild populations. Molecular Ecology. 19: 832-843.

Kortet R, Hedrick AV, Vainikka A (2010). Parasitism, predation and the evolution of animal personalities. Ecology Letters. 13: 1449-1458.

Krause SK, Van Vuren DH, Laursen C, Kelt DA (2015). Behavioral effects of an immunocontraceptive vaccine on eastern fox squirrels. Journal of Wildlife Management. 79: 1255-1263.

Krebs CJ (1989). Ecological Methodologies. Harper and Row, New York, NY. 654 p.

Kuznetsova A, Brockhoff PB, Christensen RHB (2016). lmerTest: Tests in linear mixed effects models. R package version 2.0-33. https://CRAN.R-project.org/package=lmerTest.

Lane JE, Boutin S, Speakman JR, Humphries MM (2010). Energetic costs of male reproduction in a scramble competition mating system. Journal of Animal Ecology. 79: 27-34.

Lantová P, Šíchová K, Sedláček F, Lanta V (2011). Determining behavioural syndromes in voles – The effects of social environment. Ethology. 117: 124-132.

Le Coeur C, Thibault M, Pisanu B, Thibault S, Chapuis J-L, Baudry E (2015). Temporally fluctuating selection on a personality trait in a wild rodent population. Behavioral Ecology. 26: 1285-1291.

Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, et al. (2013). AVMA guidelines for the euthanasia of animals: 2013 edition.

Lee KA, Klasing KC (2004). A role for immunology in invasion biology. Trends in Ecology & Evolution. 19: 10.

Lehmann T (1993). Ectoparasites: direct impact on host fitness. Parasitology Today. 9: 8-13.

Lepschy M, Touma C, Hruby R, Palme R (2007). Non-invasive measurement of adrenocortical activity in male and female rats. Laboratory Animals. 41: 372-387.

Lepschy M, Touma C, Palme R (2010). Fecal glucocorticoid metabolites: how to express yourself–comparison of absolute amounts versus concentrations in samples from a study in laboratory rats. Laboratory Animals. 44: 192-198.

Lindström ER (1981). Reliability of placental scar counts in the Red fox (*Vulpes vulpes* L.) with special reference to fading of the scars. Mammal Review. 11: 137-149.

Lindström ER (1994). Placental scar in the red fox (*Vulpes vulpes* L.) revisited. Mamm. Biol. - Z. Für Säugetierkd. 59: 169-173.

Lochmiller RL, Deerenberg C (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity ? Oikos. 88: 87-98.

Loehle C (1997). The pathogen transmission avoidance theory of sexual selection. Ecological Modelling. 103: 231-250.

Lurz PWW, Garson PJ, Wauters LA (1997). Effects of temporal and spatial variation in habitat quality on red squirrel dispersal behavior. Animal Behaviour. 54: 427-435.

Lurz PWW, Garson PJ, Wauters LA (2000). Effects of temporal and spatial variations in food supply on the space and habitat use of red squirrels (*Sciurus vulgaris* L.). Journal of Zoology. 251: 167-178.

Lurz PWW, Rushton SP, Wauters LA, Bertolino S, Currado I, Mazzoglio P, Shirley MDF (2001). Predicting grey squirrel expansion in North Italy: a spatially explicit modelling approach. Landscape Ecology. 16: 407-420.

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

Malange J, Izar P, Japyassú H (2016). Personality and behavioural syndrome in *Necromys iasiurus* (Rodentia: Cricetidae): notes on dispersal and invasion processes. Acta Ethologica. 19: 189-195.

Manjerovic MB, Kinahan AA, Waterman JM, Bennett NC, Bateman PW (2008). Structure and allometry of genitalia in males and females of a social African ground squirrel with high polygynandry. Journal of Zoology. 275: 375-380.

Manogue KR, Leshner AI, Candland DK (1975). Dominance status and adrenocortical reactivity to stress in squirrel monkeys (*Saimiri sciureus*). Primates. 16: 457-463.

Mari V, Martini S, Romeo C, Molinari A, Martinoli A, Tosi G, Wauters LA (2008). Record litter size in the Eurasian Red Squirrel (*Sciurus vulgaris*). Hystrix, the Italian Journal of Mammalogy. 19: 61-65.

Martin JGA, Réale D (2008). Temperament, risk assessment and habituation to novelty in eastern chipmunks, *Tamias striatus*. Animal Behaviour. 75: 309-318.

Martin LB (2009). Stress and immunity in wild vertebrates: Timing is everything. General and Comparative Endocrinology. 163: 70-76.

Martin KH, Stehn RA, Richmond ME (1976). Reliability of placental scar counts in the prairie vole. Journal of Wildlife Management. 40: 264-271.

Martinoli A, Bertolio S, Preatoni DG, Balduzzi A, Marsan A, Genovesi P, Tosi G, Wauters LA (2010). Headcount 2010: the multiplication of the grey squirrel populations introduced to Italy. Hystrix, the Italian Journal of Mammalogy. 21: 127-136.

Mateo JM (2007). Ecological and hormonal correlates of antipredator behavior in adult Belding's ground squirrels (*Spermophilus beldingi*). Behavioral Ecology and Sociobiology. 62: 37-49.

Mateo JM, Cavigelli SA (2005). A validation of extraction methods for noninvasive sampling of glucocorticoids in free living ground squirrels. Physiological and Biochemical Zoology. 78: 1069-1084.

Mazzamuto MV, Pisanu B, Romeo C, Ferrari N, Preatoni D, Wauters LA, Chapuis J-L, Martinoli A (2016). Poor parasite community of an invasive alien species: macroparasites of Pallas's squirrel in Italy. Annales Zoologici Fennici. 53: 103-112.

Mazzamuto MV, Bisi F, Wauters LA, Preatoni DG, Martinoli A (2017). Interspecific competition between alien Pallas's squirrels and Eurasian red squirrels reduces density of the native species. Biological Invasions. 19: 723-735.

McInnes CJ, Coulter L, Dagleish MP, Deane D, Gilray J, Percival A, et al. (2013). The emergence of squirrelpox in Ireland: Squirrelpox in Ireland. Animal Conservation. 16: 51-59.

Melero Y, Robinson E, Lambin X (2015). Density- and age-dependent reproduction partially compensates culling efforts of invasive non-native American mink. Biological Invasions. 17: 2645-2657.

Millesi E, Huber S, Dittami J, Hoffman I, Daan S (1998). Parameters of mating effort and success in male European ground squirrels, *Spermophilus citellus*. Ethology. 104: 298-313.

Millspaugh JJ, Washburn BE (2004). Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology. 138: 189-199.

Minderman J, Reid JM, Hughes M, Denny MJH, Hogg S, Evans PGH, Whittinghama MJ (2010). Novel environment exploration and home range size in starlings *Sturnus vulgaris*. Behavioral Ecology. 21: 1321-1329.

Misslin R, Cigrang M (1986). Does neophobia necessarily imply fear or anxiety. Behavioural Processes. 12: 45–50.

Moller AP, Dufva R, Allander K (1993). Parasites and the evolution of host social-behavior. Advances in the Study of Behavior. 22: 65-102.

Monestier C, Morellet N, Gaillard J-M, Cargnelutti B, Vanpé C, Hewison AJM (2015). Is a proactive mum a good mum? A mother's coping style influences early fawn survival in roe deer. Behavioral Ecology. 26: 1395-1403.

Montiglio PO, Pelletier F, Palme R, Garant D, Réale D, Boonstra R (2012a). Noninvasive monitoring of fecal cortisol metabolites in the eastern chipmunk (*Tamias striatus*): validation and comparison of two enzyme immunoassays. Physiological and Biochemical Zoology. 85: 183-193.

Montiglio PO, Garant D, Pelletier F, Réale D (2012b). Personality differences are related to long-term stress reactivity in a population of wild eastern chipmunks, *Tamias striatus*. Animal Behaviour. 84: 1071-1079.

Mooney HA, Cleland EE (2001). The evolutionary impact of invasive species. Proceedings of the National Academy of Sciences. 98: 5446-5451.

Moore J (2002). Parasites and the Behavior of Animals. Oxford University Press, Oxford..

Morand S, Krasnov B R, Poulin R (2006). Micromammals and macroparasites: from evolutionary ecology to management. Tokyo: Springer.

Möstl E, Palme R (2002). Hormones as indicators of stress. Domestic Animal Endocrinology. 23: 67-74.

Möstl E, Rettenbacher S, Palme R (2005). Measurement of corticosterone metabolites in birds' droppings: an analytical approach. Annals of the New York Academy of Sciences. 1046: 17-34.

Mowat G, Boutin S, Slough BG (1996). Using placental scar counts to estimate litter size and pregnancy rate in lynx. Journal of Wildlife Management. 60: 430-440.

Munerato MS, Marques JA, Caulkett NA, Tomás W, Zanetti ES, Trovati RG, Pereira GT, Palme R (2015). Hormonal and behavioural stress responses to capture and radio- collar fitting in free-ranging pampas deer (*Ozotoceros bezoarticus*). Animal Welfare. 24: 437-446.

Mutzel A, Dingemanse NJ, Araya-Ajoy YG, Kempenaers B (2013). Parental provisioning behaviour plays a key role in linking personality with reproductive success. Proceedings of the Royal Society of London B: Biological Sciences. 280: 20131019.

Nagy MZ, Holm M (1970). Open-field behavior of C_3H mice: Effect of early handling, field illumination, and age at testing. Psychonomic Science. 19: 273-275.

Nakagawa S, Schielzeth H (2010). Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biological Reviews. 85: 935-956.

Narayan EJ, Cockrem JF, Hero JM (2013). Sight of a predator induces a corticosterone stress response and generates fear in an amphibian. PLoS One. 8: e73564.

Narayan EJ, Jessop TS, Hero JM (2015). Invasive cane toad triggers chronic physiological stress and decreased reproductive success in an island endemic. Functional Ecology. 29: 1435-1444.

Natoli E, Say L, Cafazzo S, Bonanni R, Schmid M, Pontier D (2005). Bold attitude makes male urban feral domestic cats more vulnerable to Feline Immunodeficiency Virus. Neuroscience & Biobehavioral Reviews. 29: 151-157.

Naya DE, Ebensperger LA, Sabat P, Bozinovic F (2008). Digestive and metabolic flexibility allows female degus to cope with lactation costs. Physiological and Biochemical Zoology. 81: 186-194.

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.

Neuhaus P (2003). Parasite removal and its impact on litter size and body condition in Columbian ground squirrels (*Spermophilus columbianus*). Proceedings of the Royal Society of London B: Biological Sciences. 270 Suppl 2: 13-5.

Nicolaus M, Tinbergen JM, Ubels R, Both C, Dingemanse NJ (2016). Density fluctuations represent a key process maintaining personality variation in a wild passerine bird. Ecology Letters. 19: 478-486.

Nixon CM, McClain MW (1975). Breeding seasons and fecundity of female gray squirrels in Ohio. Journal of Wildlife Management. 39: 426-438.

Nordling D, Andersson M, Zohari S, Gustafsson L (1998). Reproductive effort reduces specific immune response and parasite resistance. Proceedings of the Royal Society of London B: Biological Sciences. 265: 1291-1298.

Oldham J, Morlock H (1970). The effects of open-field size on activity in the Mongolian gerbil. Psychonomic Science. 20: 290.

O'Teangana D,Reilly S, Montgomery WI, Rotchford J (2000). The distribution and status of the red squirrel (*Sciurus vulgaris*) and Grey squirrel (*Sciurus carolinensis*) in Ireland. Mammal Review. 30: 45-56.

Ouyang JQ, Hau M, Bonier F (2011). Within seasons and among years: when are corticosterone levels repeatable ? Hormones and Behavior. 60: 559-564.

Padgett DA, Glaser R (2003). How stress influences the immune response. Trends in Immunology. 24: 444-448.

Pakkala JJ, Norris DR, Newman AE (2013). An experimental test of the capture- restraint protocol for estimating the acute stress response. Physiological and Biochemical Zoology. 86: 279-284.

Palme R (2005). Measuring fecal steroids - Guidelines for practical application. Annals of the New York Academy of Sciences. 1046: 75-80.

Palme R (2016). Publication group “Non-invasive FCM” available at http://www.researcherid.com/rid/A-3499-2008, checked on February 9 2016.

Palme R, Möstl E (1997). Measurement of cortisol metabolites in feces of sheep as a parameter of cortisol concentration in blood. International Journal of Mammalian Biology. 62: 192-197.

Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E (2005). Stress hormones in mammals and birds: Comparative aspects regarding metabolism, excretion and noninvasive measurement in fecal samples. Annals of the New York Academy of Sciences. 1040: 162-171.

Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013). Steroid extraction: get the best out of fecal samples. Wiener Tierärztliche Monatsschrift. 100: 238-246.

Palmer GH, Koprowski JL, Pernas T (2007). Tree squirrels as invasive species: conservation and managements implications. Managing Vertebrate Invasive Species: Proceedings of an International Symposium (Witmer GW, Pitt WC, Fagerstone KA, Eds). USDA/APHIS/WS, National Wildlife Research Center, Fort Collins, CO. 2007.

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

Patterson LD, Schulte-Hostedde AI (2011). Behavioural correlates of parasitism and reproductive success in male eastern chipmunks, *Tamias striatus*. Animal Behaviour. 81: 1129-1137.

Pelletier F, Page KA, Ostiguy T, Festa-Bianchet M (2005). Fecal counts of lungworm larvae and reproductive effort in bighorn sheep, *Ovis Canadensis*. Oikos. 110: 473-480.

Pinheiro J, Bates D (2000). Linear Mixed-Effects Models: Basic Concepts and Examples. In: Mixed-Effects Models in S and S-PLUS. Springer New York, pp 3–56.

Place NJ, Kenagy GJ (2000). Seasonal changes in plasma testosterone and glucocorticosteroids in free-living male yellow-pine chipmunks and the response to capture and handling. Journal of Comparative Physiology B. 170: 245-251.

Poulin R (2013). Parasite manipulation of host personality and behavioural syndromes. The Journal of Experimental Biology. 216: 18-26.

Prenter J, MacNeil C, Dick JTA, Dunn AM (2004). Roles of parasites in animal invasions. Trends in Ecology & Evolution. 19: 7.

Pribbenow S, Jewgenow K, Vargas A, Serra R, Naidenko S, Dehnhard M (2014). Validation of an enzyme immunoassay for the measurement of fecal glucocorticoid metabolites in Eurasian (*Lynx lynx*) and Iberian lynx (*Lynx pardinus*). General and Comparative Endocrinology. 206: 166-177.

R Development Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raouf SA, Smith LC, Brown MB, Wingfield JC, Brown CR (2006). Glucocorticoid hormone levels increase with group size and parasite load in cliff swallows. Animal Behaviour. 71: 39-48.

Réale D, Festa-Bianchet M (2003). Predator-induced selection on temperament in bighorn ewes. Animal Behaviour. 65: 463-470.

Réale D, Gallant BY, Leblanc M, Festa-Bianchet M (2000). Consistency of temperament in bighorn ewes and correlates with behaviour and life history. Animal Behaviour. 60: 589-597.

Reeder DM, Kosteczko NS, Kunz TH, Widmaier EP (2004). Changes in baseline and stress-induced glucocorticoid levels during the active period in free-ranging male and female little brown myotis, *Myotis lucifugus* (Chiroptera: Vespertilionidae). General and Comparative Endocrinology. 136: 260-269.

Réale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007). Integrating animal temperament within ecology and evolution. Biological Reviews. 82: 291-318.

Réale D, Martin J, Coltman DW, Poissant J, Festa-Bianchet M (2009). Male personality, life-history strategies and reproductive success in a promiscuous mammal. Journal of Evolutionary Biology. 22: 1599-1607.

Réale D, Dingemanse NJ, Kazem AJN, Wright J (2010). Evolutionary and ecological approaches to the study of personality. Philosophical Transactions of the Royal Society B: Biological Sciences. 365: 3937-3946.

Revelle W (2017). psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA, https://CRAN.R-project.org/package=psych Version = 1.7.5.

Rödel HG, Meyer S (2011). Early development influences ontogeny of personality types in young laboratory rats. Developmental Psychobiology. 53: 601-613.

Rödel HG, Zapka M, Talke S, Kornatz T, Bruchner B, Hedler C (2015). Survival costs of fast exploration during juvenile life in a small mammal. Behavioral Ecology and Sociobiology. 69: 205-217.

Rodrigues D, Wauters LA, Romeo C, Mari V, Preatoni D, Mathias MdaL, Tosi G, Martinoli A (2010). Living on the edge: can Eurasian red squirrels (*Sciurus vulgaris*) persist in extreme high-elevation habitats?. Arctic, Antarctic, and Alpine Research. 42: 106-112.

Rolley RE (1985). Dynamics of a harvested bobcat population in Oklahoma. Journal of Wildlife Management. 49: 283-292.

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.

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

Romeo C, Wauters LA, Ferrari N, Lanfranchi P, Martinoli A, Pisanu B, Preatoni DG, Saino N (2014a). Macroparasite fauna of alien grey squirrels (*Sciurus carolinensis*): Composition, variability and implications for native species. PloS ONE. 9: e88002.

Romeo C, Wauters LA, Cauchie S, Martinoli A, Matthysen E, Saino N, Ferrari N (2014b). Faecal egg counts from field experiment reveal density dependence in helminth fecundity: *Strongyloides robustus* infecting grey squirrels (*Sciurus carolinensis*). Parasitology Research. 113: 3403-3408.

Romeo C, Ferrari N, Lanfranchi P, Saino N, Santicchia F, Martinoli A, Wauters LA (2015). Biodiversity threats from outside to inside: effects of alien grey squirrel (*Sciurus carolinensis*) on helminth community of native red squirrel (*Sciurus vulgaris*). Parasitology Research. 114: 2621-2628.

Romeo C, McInnes CJ, Dale TD, Shuttleworth CM, Bertolino S, Wauters LA, Ferrari N. Absence of squirrelpox virus in Italian grey squirrels: one less threat to red squirrel conservation ? Animal Conservation. Submitted.

Romero LM (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology. 128: 1-24.

Romero LM (2004). Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution. 19: 249-255.

Romero LM, Wingfield JC (1999). Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Comparative Biochemistry and Physiology B. 122: 13-20.

Romero LM, Reed JM (2005). Collecting baseline corticosterone samples in the field: is under 3 min good enough ? Comparative Biochemistry and Physiology A. 140: 73-79.

Romero LM, Wingfield JC (2015). Tempests, Poxes, Predators, and People: Stress in Wild Animals and How they Cope. Oxford University Press, Oxford, UK.

Romero LM, Meister CJ, Cyr NE, Kenagy GJ, Wingfield JC (2008). Seasonal glucocorticoid responses to capture in wild free-living mammals. American Journal of Physiology. 294: 614-622.

Roy HE, Handley LJ (2012). Networking: a community approach to invaders and their parasites. Functional Ecology. 26: 1238-1248.

Rózsa L, Reiczigel J, Majoros G (2000). Quantifying parasites in samples of hosts. Journal of Parasitology. 86: 228-232.

Ruette S, Albaret M (2011). Reproduction of red fox *Vulpes vulpes* in western France: does staining improve estimation of litter size from placental scar counts ? European Journal of Wildlife Research. 57: 555-564.

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

Sakai AK, Allendorf FW, Holt JS, et al. (2001). The population biology of invasive species. Annual Review of Ecology and Systematics. 32: 305-332.

Salewski E (1964). Staining method for a macroscopic test for implantation points in the uterus of the rat. Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmakol. 247-367.

Salmaso F, Molinari A, Di Pierro E, Ghisla A, Martinoli A, Preatoni D, Serino G, Tosi G, Bertolino S, Wauters LA (2009). Estimating and comparing food availability for tree-seed predators in typical pulsed-resource systems: Alpine conifer forests. Plant Biosystems. 143: 258-267.

Santicchia F, Romeo C, Martinoli A, Lanfranchi P, Wauters LA, Ferrari N (2015a). Effects of habitat quality on parasite abundance: do forest fragmentation and food availability affect helminth infection in the Eurasian red squirrel?. Journal of Zoology. 296: 38-44.

Santicchia F, Romeo C, Grilli G, Vezzoso S, Wauters LA, Mazzamuto MV, Martinoli A, Ferrari N (2015b). The use of uterine scars to explore fecundity levels in invasive alien tree squirrels. Hystrix, the Italian Journal of Mammalogy. 26: 95-101.

Santicchia F, Gagnaison C, Martinoli A, Matthysen E, Bertolino S, Wauters LA. Habitat-dependent effects of personality on survival and reproduction in red squirrels. Behavioral Ecology and Sociobiology. Submitted.

Sapolsky RM, Romero LM, Munck AU (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews. 21: 55-89.

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. Journal of Parasitology. 93: 1476-1486.

Schmitz OJ, Vlastimil K, Ofer O (2004). Trophic cascades: the primacy of trait-mediated indirect interactions. Ecology Letters. 7: 153-163.

Schulte-Hostedde AI, Millar JS (2004). Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success. Behavioral Ecology and Sociobiology. 55: 272.

Schwarz GE (1978). Estimating the dimension of a model. Annals of Statistics. 6: 461-464.

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

Sheldon B, Verhulst S (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology & Evolution. 11: 317-321.

Sheriff MJ, Krebs CJ, Boonstra R (2009). The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. Journal of Animal Ecology. 78: 1249-1258.

Sheriff MJ, Krebs CJ, Boonstra R (2010). Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story ? General and Comparative Endocrinology. 166: 614-619.

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011). Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia. 166: 869-887.

Sheriff MJ, Wheeler H, Donker SA, Krebs CJ, Palme R, Hik DS, Boonstra R (2012). Mountain-top and valley-bottom experiences: the stress axis as an integrator of environmental variability in arctic ground squirrel populations. Journal of Zoology. 287: 65-75.

Shonfield J, Humphries MM, Boutin S, McAdam AG, Taylor RW (2012). Territorial defence behaviour in red squirrels is influenced by local density. Behaviour. 149: 369-390.

Shorten M (1951). Some aspects of the biology of the gray squirrel (*Sciurus carolinensis*) in Great Britain. Proceedings of the Zoological Society of London. 121: 427-459.

Shutt K, Setchell JM, Heistermann M (2012). Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): Validation of a fecal glucocorticoid assay and methods for practical application in the field. General and Comparative Endocrinology. 179: 167-177.

Shuttleworth CM, Lurz PWW, Gurnell J (2016). The Grey Squirrel: Ecology and Management of an Invasive Species in Europe. European Squirrel Initiative, Stoneleigh Park, Warwickshire, UK.

Sih A, Kats LB, Maurer EF (2003). Behavioural correlations across situations and the evolution of antipredator behaviour in a sunfish-salamander system. Animal Behaviour. 65: 29-44.

Sih A, Bell AM, Johnson JC, Ziemba RE (2004). Behavioral syndromes: an integrative overview. The Quarterly Review of Biology. 79: 241-277.

Sih A, Ferrari MCO, Harris DJ (2011). Evolution and behavioural responses to human-induced rapid environmental change. Evolutionary Applications. 4: 367-387.

Sih A, Cote J, Evans M, Fogarty S, Pruitt J (2012). Ecological implications of behavioural syndromes. Ecology Letters. 15: 278-289.

Sikes RS, Gannon WL (2011). Guidelines of the American Society of Mammalogists for the use of wild mammals in research. Journal of Mammalogy. 92: 235-253.

Simberloff D, Martin J-L, Genovesi P, Maris V, Wardle DA, Aronson J, et al. (2013). Impacts of biological invasions: what's what and the way forward. Trends in Ecology & Evolution. 28: 58-66.

Smith BR, Blumstein DT (2008). Fitness consequences of personality: a meta-analysis. Behavioral Ecology. 19: 448-455.

St Juliana JR, Khokhlova IS, Wielebnowski N, Kotle BP, Krasnov BR (2014). Ectoparasitism and stress hormones: strategy of host exploitation, common host–parasite history and energetics matter. Journal of Animal Ecology. 83: 1113-1123.

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

Stoffel MA, Nakagawa S, Schielzeth H (2017). rptR: Repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods in Ecology and Evolution.

Strauss A, White A, Boots M (2012). Invading with biological weapons: the importance of disease-mediated invasions. Functional Ecology. 26: 1249-1261.

Strayer DL (2012). Eight questions about invasions and ecosystem functioning. Ecology Letters. 15: 1199-1210.

Stritch C, Naulty F, Zintl A. et al. (2015). Squirrelpox virus reservoir expansion on the east coast of Ireland. European Journal of Wildlife Research. 61: 483-486.

Svendsen GE, Armitage KB (1973). Mirror-image stimulation applied to field behavioral studies. Ecology. 54: 623-627.

T'sui WH, Lin FY, Huang CC (1982). The reproductive biology of the red-bellied tree squirrel, *Callosciurus erythraeus*, at Ping-Lin, Taipei Hsien. Proceedings of the National Science Council, Republic of China, Part B Basic Science. 6: 443-451.

Tamura N (1999). Seasonal change in reproductive states of the Formosan squirrel on Izu-Oshima Island, Japan. Mammal Study. 24: 121-124.

Tamura N, Hayashi F, Miyashita K (1988). Dominance hierarchy and mating behavior of the Formosan squirrel, *Callosciurus erythraeus thaiwanensis*. Journal of Mammalogy. 320-331.

Tang D, Alexander PS (1979). The reproductive cycle of the red-bellied tree squirrel (Callosciurus erythraeus) in Taiwan. Biological Bulletin Tunghai University. 51: 1-7.

Tattoni C, Preatoni DG, Lurz PWW, Rushton SP, Tosi G, Bertolino S, Martinoli A, Wauters LA (2006). Modelling the expansion of a grey squirrel population: implications for squirrel control. Biological Invasions. 8: 1605-1619.

Taylor W (1971). The excretion of steroid hormone metabolites in bile and feces. Vitamins and Hormones. 29: 201-285.

Telfer S, Brown K (2012). The effects of invasion on parasite dynamics and communities. Functional Ecology. 26: 1288-1299.

Thompson DC (1978). The social system of the grey squirrel. Behaviour. 64: 305-328.

Tompkins DM, Sainsbury AW, Nettleton P, Buxton D, Gurnell J (2002). Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. Proceedings of the Royal Society of London B: Biological Sciences. 269: 529-533.

Tompkins DM, Dunn AM, Smith MJ, Telfer S (2011). Wildlife diseases: from individuals to ecosystems. Journal of Animal Ecology. 80: 19-38.

Touma C, Palme R (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Annals of the New York Academy of Sciences. 1046: 54-74.

Touma C, Sachser N, Möstl E, Palme R (2003). Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. General and Comparative Endocrinology. 130: 267-278.

Touma C, Palme R, Sachser N (2004). Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Hormones and Behavior. 45: 10-22.

Trizio I, Crestanello B, Galbusera P, Wauters LA, Tosi G, Matthysen E, Hauffe HC (2005). Geographical distance and physical barriers shape the genetic structure of Eurasian red squirrels in the Italian Alps. Molecular Ecology. 14: 469-481.

van Overveld T, Matthysen E (2010). Personality predicts spatial responses to food manipulations in free-ranging great tits (*Parus major*). Biology Letters. 6: 187-190.

van Overveld T, Adriaensen F, Matthysen E (2015). No evidence for correlational selection on exploratory behaviour and natal dispersal in the great tit. Evolutionary Ecology. 29: 137-156.

VanderWaal KL, Ezenwa VO (2016). Heterogeneity in pathogen transmission: mechanisms and methodology. Functional Ecology. 30: 1606-1622.

Venturini M, Franzetti B, Genovesi P, Marsan A, Spanò S (2005). Distribuzione e consistenza della popolazione di scoiattolo grigio *Sciurus carolinensis* Gmelin, 1788 nel levante genovese. Hystrix, the Italian Journal of Mammalogy. 16: 53-58.

Vera F, Zenuto RR, Antenucci CD (2013). Seasonal variations in plasma cortisol, testosterone, progesterone and leukocyte profiles in a wild population of tuco-tucos. Journal of Zoology. 289: 111-118.

Vetter SG, Brandstätter C, Macheiner M, Suchentrunk F, Gerritsmann H, Bieber C (2016). Shy is sometimes better: personality and juvenile body mass affect adult reproductive success in wild boars, *Sus scrofa*. Animal Behaviour. 115: 193-205.

Walsh RN, Cummins RA (1976). The open-field test: A critical review. Psychological Bulletin. 83: 482-504.

Wasser SK, Bevis K, King G, Hanson E (1997). Noninvasive physiological measures of disturbance in Northern Spotted Owl. Conservation Biology. 11: 1019-1022.

Wauters LA, Dhondt AA (1987). Activity budget and foraging behaviour of the red squirrel (*Sciurus vulgaris* Linnaeus, 1758) in a coniferous habitat. Mamm. Biol. - Z. Für Säugetierkd. 52: 341-353.

Wauters LA, Dhondt AA (1989). Body weight, longevity and reproductive success in red squirrels (*Sciurus vulgaris*). Journal of Animal Ecology. 58: 637-651.

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

Wauters LA, Dhondt AA (1992). Spacing behaviour of red squirrels, *Sciurus vulgaris*: variation between habitats and the sexes. Animal Behaviour. 43: 297-311.

Wauters LA, Dhondt AA (1993). Immigration pattern and success in red squirrels. Behavioral Ecology and Sociobiology. 33: 159-167.

Wauters LA, Dhondt AA (1995). Lifetime reproductive success and its correlates in female Eurasian red squirrels. Oikos. 72: 402-410.

Wauters LA, Lens L (1995). Effects of food availability and density on red squirrel (*Sciurus vulgaris*) reproduction. Ecology. 76: 2460-2469.

Wauters LA, Casale P (1996). Long-term scatterhoarding in the red squirrel (*Sciurus vulgaris*). Journal of Zoology. 238: 195-207.

Wauters LA, Gurnell J (1999). The mechanism of replacement of red squirrels by grey squirrels: a test of the interference competition hypothesis. Ethology. 105: 1053-1071.

Wauters LA, Dhondt AA, De Vos R (1990). Factors affecting male mating success in red squirrels (*Sciurus vulgaris*). Ethology Ecology & Evolution. 2: 195-204.

Wauters LA, Hutchinson Y, Parkin DT, Dhondt AA (1994). The effects of habitat fragmentation on demography and on the loss of genetic variation in the red squirrel. Proceedings of the Royal Society of London B: Biological Sciences. 225: 107-111.

Wauters LA, De Crombrugghe SA, Nour N, Matthysen E (1995). Do female roe deer in good condition produce more sons than daughters. Behavioral Ecology and Sociobiology. 37: 189-193.

Wauters, LA, Somers L, Dhondt A (1997). Settlement behaviour and population dynamics of reintroduced red squirrels *Sciurus vulgaris* in a park in Antwerp, Belgium. Biological Conservation. 82: 101-107.

Wauters LA, Gurnell J, Preatoni D, Tosi G (2001). Effects of spatial variation in food availability on spacing behaviour and demography of Eurasian red squirrels. Ecography. 24: 525-538.

Wauters LA, Tosi G, Gurnell J (2002a). Interspecific competition in tree squirrels: do introduced grey squirrels (*Sciurus carolinensis*) deplete tree seeds hoarded by red squirrels (*S. vulgaris*) ? Behavioral Ecology and Sociobiology. 51: 360-367.

Wauters LA, Gurnell J, Martinoli A, Tosi G (2002b). Interspecific competition between native Eurasian red squirrels and alien grey squirrels: does resource partitioning occur ? Behavioral Ecology and Sociobiology. 52: 332-341.

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

Wauters LA, Bertolino S, Adamo M, Van Dongen S, Tosi G (2005). Food shortage disrupts social organization: the case of red squirrels in conifer forests. Evolutionary Ecology. 19: 375-404.

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.

Wauters LA, Verbeylen G, Preatoni D, Martinoli A, Matthysen E (2010). Dispersal and habitat cuing of Eurasian red squirrels in fragmented habitats. Population Ecology. 52: 527-536.

Wauters LA, Preatoni D, Martinoli A, Verbeylen G, Matthysen E (2011). No sex bias in natal dispersal of Eurasian red squirrels. Mammalian Biology. 76: 369-372.

Wauters LA, Amori G, Aloise G, Gippoliti S, Agnelli P, Galimberti A, Casiraghi M, Preatoni D, Martinoli A (2017). New endemic mammal species for Europe: *Sciurus meridionalis* (Rodentia, Sciuridae). Hystrix, the Italian Journal of Mammalogy. 28. doi:10.4404/hystrix-28.1-12015.

Weiser MJ, Handa RJ (2009). Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic–pituitary–adrenal axis via estrogen receptor alpha within the hypothalamus. Neuroscience. 159: 883-895.

Weiss A, Adams MJ (2013). Differential behavioral ecology. In: Animal personalities: behavior, physiology and evolution (C. Carere and D. Maestripieri). Chicago: University of Chicago Press. p. 96-123.

Werner EE, Peacor SD (2003). A review of trait-mediated indirect interactions in ecological communities. Ecology. 84: 1083-1100.

Westergaard GC, Suomi SJ, Chavanne TJ, Houser L, Hurley A, Cleveland A, Snoy PJ, Higley JD (2003). Physiological correlates of aggression and impulsivity in free-ranging female primates. Neuropsychopharmacology. 28: 1045-1055.

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*. The American Midland Naturalist. 131: 43.

White A, Lurz P, Bryce J, Tonkin M, Ramoo K, Bamforth L, et al. (2016). Modelling disease spread in real landscapes: Squirrelpox spread in Southern Scotland as a case study. Hystrix, the Italian Journal of Mammalogy. 27. doi:10.4404/hystrix-27.1-11657

Wikelski M, Cooke SJ (2006). Conservation physiology. Trends in Ecology & Evolution. 21: 38-46.

Wild AE (1971). Transmission of proteins from mother to conceptus in the grey squirrel (*Sciurus carolinensis*). Immunology. 20: 789-797.

Wilson DS, Coleman K, Clark AB, Biederman L (1993). Shy-bold continuum in pumpkinseed sunfish (*Lepomis gibbosus*): an ecological study of a psychological trait. Journal of Comparative Psychology. 107: 250-260.

Wilson RC, Vacek T, Lanier DL, Dewsbury DA (1976). Open-field behavior in muroid rodents. Behavioral Biology. 17: 495-506.

Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD (1998). Ecological bases of hormone-behavior interactions: the “emergency life history stage”. Integrative and Comparative Biology. 38: 191–206.

Wolak ME, Fairbairn DJ, Paulsen YR (2011). Guidelines for estimating repeatability. Methods in Ecology and Evolution. 3: 129-137.

Wolf M, van Doorn GS, Leimar O, Weissing FJ (2007). Life-history trade-offs favour the evolution of animal personalities. Nature. 447: 581-584.

Woodruff JA, Lacey EA, Bentley GE, Kriegsfeld LJ (2013). Effects of social environment on baseline glucocorticoid levels in a communally breeding rodent, the colonial tuco-tuco (*Ctenomys sociabilis*). Hormones and Behavior. 64: 566–572.

Zhao Q-S, Hu Y-B, Liu P-F, Chen L-J, Sun Y-H (2016). Nest site choice: a potential pathway linking personality and reproductive success. Animal Behaviour. 118: 97-103.

Zong C, Wauters LA, Van Dongen S, Mari V, Romeo C, Martinoli A, Preatoni DG, Tosi G (2010). Annual variation in predation and dispersal of Arolla pine (*Pinus cembra* L.) seeds by Eurasian red squirrels and other seed-eaters. Forest Ecology and Management. 260: 587-594.

Zong C, Mei S, Santicchia F, Wauters LA, Preatoni DG, Martinoli A (2014). Habitat effects on hoarding plasticity in the Eurasian red squirrel (*Sciurus vulgaris*). Hystrix, the Italian Journal of Mammalogy. 25: 14-17.

Zuk M, Stoehr AM (2002). Immune defense and host life history. American Naturalist. 160: 9-22.

Zuur AF, Ieno EN, Elphick CS (2010). A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution. 1: 3–14.

Supplementary material

S.1 Chapter 2 “Stress in biological invasions: alien grey squirrels increase physiological stress in native Eurasian red squirrels”

S.1.1 Study site information

Detailed data on forest structure and composition of the alpine sites in the Alps are given elsewhere (Rodrigues et al., 2010; Wauters et al., 2008). The lowland sites (150-250 m elevation) are mature mixed deciduous woodlands dominated by oaks (*Quercus robur*, *Q. petraea*, *Q. rubra*) and hornbeam (*Carpinus betulus*), with some birch (*Betula* sp.), bird cherry (*Prunus avium*), elm (*Ulmus minor*), sweet chestnut (*Castanea sativa*), black locust (*Robinia pseudoacacia*) and some conifers (*Pinus sylvestris*, *Picea abies*, *Taxus baccata* and some ornamental species).

Table S1 – Location of study sites in Lombardy and Piedmont, North Italy and periods in which squirrels were captured for collection of fecal samples (sample size: total fecal samples collected and per sex) to measure fecal glucocorticoid metabolite (FGM) concentrations. In all sites, the density of red squirrels (highest density of different trapping periods) was estimated by MNA, and minimum density of grey squirrels (in red-grey sites) was estimated from removals (see methods for details). For each site mean FGM concentration (ng/g dry feces) and ln-transformed FGM concentration are presented.

Site-type	**Coordinates**	**Dates captures**	**Sample size (M, F)**	**Red squirrel density (ha^{-1})**	**Grey squirrel density (ha^{-1})**	**Mean FGM (ng/g)**	**ln mean FGM (ng/g dry feces)**
Red-only							
Vanzago (pre March 2015)	45°31′N, 8°58′E	Dec 2014-March 15	23 (12, 11)	0.28	n/a	34565	10.451
Castelbarco (pre March 2014)	45°35′N, 9°31′E	Dec 2013	6 (3, 3)	0.33	n/a	25291	10.138
Valfurva	46°27′N, 10°31′E	May 2014-Oct 2015	72 (38, 34)	0.60	n/a	22548	10.023
Bormio	46°27′N, 10°30′E	Sep 2014-Oct 2015	10 (7, 3)	0.24	n/a	29795	10.302
Cancano	46°33′N, 10°15′E	Sep 2014-Sep 2015	14 (8, 6)	0.13	n/a	17367	9.762
Red-grey							
Vanzago	45°31′N, 8°58′E	Jan 2016	13 (6, 7)	0.28	0.24	78119	11.266
Castelbarco	45°35′N, 9°31′E	March 2014-Dec 2015	52 (35, 17)	0.30	0.67	70669	11.166
Le Foppe	45°61′N, 9°49′E	March 2014-Jul 2015	19 (11, 8)	1.11	0.32	71170	11.173
Lambro 30	45°62'N, 9°28'E	Aug 2014-Feb 2016	6 (3, 3)	0.26	0.32	51670	10.853
Lambro 02-05	45°67′N, 9°26′E	Feb 2014-Nov 2015	17 (9, 8)	0.38	2.28	57067	10.952
Passatempo	45°00′N, 7°78′E	Dec 2013-Nov 2015	16 (6, 9)	0.38	0.98	134745	11.811
Staffarda	44°71′N, 7°41′E	Nov 2013-Feb 2015	13 (8, 4)	0.26	0.11	96146	11.474

S.1.2 Models testing for habitat effect

Since red-only sites occurred in two habitats (mountain conifer or lowland deciduous forest) and red-grey sites in only one habitat (lowland deciduous forest), the habitat could produce confounding effect and was not independent from study site (only red squirrels vs. red and grey squirrels present). Therefore, we tested the effect of habitat-type (red-only conifer, red-only deciduous, red-grey deciduous) on FGM concentrations in a separate model, including sex, reproductive condition nested in sex, season, daytime and body mass as fixed effects. ID nested in study site and study site nested in habitat type were included as random intercepts.

Considering habitat-type instead of invasion status, patterns in the difference in FGM concentrations did not change. Red squirrels had the lowest FGM concentrations in conifer forests (n = 96, mean ± SD = 22550 ± 19125 ng/g dry feces) and slightly higher FGM concentrations in deciduous red-only sites (n = 29, 32650 ± 23468 ng/g dry feces). Differences between these two habitats were not significant (estimate 0.58 ± 0.28; $t_6 = 2.06$; $p = 0.084$). In contrast, red squirrels had significantly higher FGM concentrations in the red-grey deciduous sites (n = 135, 78130 ± 61074 ng/g dry feces) than in the coniferous and deciduous red-only sites (coniferous red-only against deciduous red-grey 1.21 ± 0.19; $t_4 = 6.33$; $p = 0.003$; deciduous red-only against deciduous red-grey 0.63 ± 0.24; $t_6 = 2.64$; $p = 0.041$). Thus, changing study site by habitat-type did not change the overall results. Also in this model, sex ($t_{201} = 0.33$; $p = 0.74$) daytime ($t_{223} = 0.71$; $p = 0.48$) and body mass ($t_{196} = 0.38$; $p = 0.70$) had no significant effects, and neither had reproductive condition nested in sex (all $p > 0.05$). The season effect was significant (autumn vs spring-summer 0.34 ± 0.14; $t_{218} = 2.54$; $p = 0.012$; autumn vs winter 0.42 ± 0.17; $t_{154} = 2.49$; $p = 0.014$).

S.1.3 Model testing for colonization by grey squirrels

Table S2 – Linear mixed-effects model with FGM concentrations (transformed using the natural logarithm, ln of ng/g dry feces) as the dependent variable and squirrel identity (ID) nested in study site as a random intercept term to account for repeated samples on the same individuals. Study site nested in colonization status was added as random intercept to account for potential differences in FGM concentrations between sites, and reproductive condition nested in sex was added as a fixed effect to account for potential changes in FGM concentrations with reproductive activity (see methods). We investigated the effects of colonization (yes or no), time-period (before vs. after, the dates of colonization), and their interaction, and included sex, daytime and body mass in the full model.

Full model	**Estimate ± SE**	**t**	**df**	**p**
Time-period		3.69	169	0.0003
Colonization status		3.24	160	0.0015
Sex*	0.04 ± 0.24	0.18	166	0.86
Body mass	-0.0002 ± 0.0021	0.09	169	0.93
Daytime†	-0.04 ± 0.13	0.34	162	0.73
Colonization status*Time-period		1.60	171	0.11
Fem lactating**	0.30 ± 0.24	1.20	155	0.23
Fem not breeding**	-0.34 ± 0.23	1.44	173	0.15
Males breeding**	-0.01 ± 0.15	0.09	167	0.93
Final model	**Estimate ± SE**	**t**	**df**	**p**
Time-period		3.93	173	0.00012
Colonization status		2.82	167	0.005
Sex*	0.16 ± 0.13	1.30	114	0.20
Colonized sites‡	0.74 ± 0.18	3.93	173	0.0004
Non-colonized sites‡	0.21 ± 0.15	1.27	155	0.21

*females held as reference level; †afternoon held as reference level; ‡interaction between time-period and colonization status: time-period 1 held as reference level; ** Pregnant females or non breeding males held as reference level.

S.1.4 Model testing for the effect of grey squirrel removal

Table S3 – Linear mixed-effects model with FGM concentrations (ln transformed) as dependent variable and squirrel identity (ID) nested in study site as a random intercept term to account for repeated samples on the same individuals. Reproductive condition (RC) nested in sex was added as a fixed effect to account for potential changes in FGM concentrations with reproductive activity. We investigated the effects of grey squirrel removal considering treatment period (initial sampling, no grey squirrels removed yet = period 1; intermediate sampling, with a low proportion of grey squirrels removed = period 2; final sampling with a higher proportion of grey squirrels removed = period 3; see methods and Table 2.1), study site, sex, and daytime as factors, and body mass as an explanatory variable, and two factor interactions between the period by study site, and period by sex. Full model: Type III ANOVA with Satterthwaite approximation for degrees of freedom. For final model after stepwise backward model selection eliminating non-significant interactions and fixed effects, we present the LMM with parameter estimates.

Full model	**Estimate ± SE**	**F**	**df**	**p**
Period		3.24	2, 111	0.043
Sex		2.02	1, 111	0.16
Study site	0.04 ± 0.24	0.99	3, 111	0.40
Body mass	-0.0002 ± 0.0021	0.75	1, 111	0.39
Daytime	-0.04 ± 0.13	0.72	1, 111	0.40
Period * sex		0.21	2, 111	0.81
Period * study site	0.30 ± 0.24	0.91	6, 111	0.49
RC nested in sex	-0.01 ± 0.15	1.79	3, 111	0.15
Final model	**Estimate ± SE**	**t**	**df**	**p**
Period 2*	-0.58 ± 0.23	2.53	121	0.013
Period 3*	-0.50 ± 0.23	2.18	121	0.031
Sex†	-1.15 ± 0.76	1.52	121	0.13
Site Vanzago‡	0.21 ± 0.22	0.93	121	0.36
Site Lambro‡	-0.25 ± 0.30	0.83	121	0.41
Site Passatempo‡	0.36 ± 0.25	1.40	121	0.16
Fem lactating**	0.60 ± 0.36	1.67	121	0.097
Fem non breeding**	0.19 ± 0.32	0.59	121	0.56
Males breeding**	1.46 ± 0.72	2.03	121	0.045

*Period 1 held as reference level; †females held as reference level; ‡Study site Castelbarco held as reference level; **Pregnant females or non breeding males held as reference level.

S.2 Chapter 4 "The price of being bold: personality affects endoparasite infection in introduced Eastern grey squirrels"

Table S1 – Ethogram for open field and mirror-image stimulation behavioral tests.

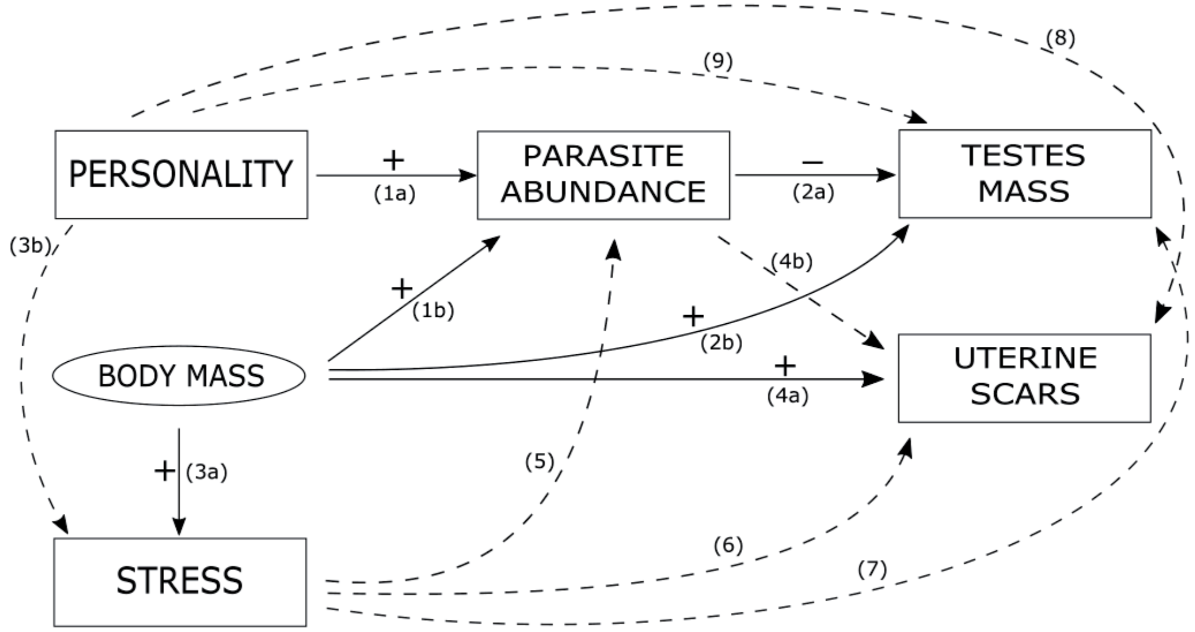
Open Field Test (OFT)		**Mirror Image Stimulation Test (MIS)**	
Behavior	Behavior description	Behavior	Behavior description
Scratch	Scratch or chew floors or walls	Scratch	Scratch or chew floors or walls
Hang	Hang on walls	Hang	Hang on walls
Groom	Grooming activity	Groom	Grooming activity
Locomotion	Jump, walk	Locomotion	Jump, walk
Rise	Rise up on hind legs	Rise	Rise up on hind legs
Scan	Head moving	Scan	Head moving
Sniff	Sniff the corner of arena	Sniff	Sniff the corner of arena
Head dip	Put head in holes in the floor	Head dip	Put head in holes in the floor
Immobile	No movement	Back	Immobile in back half of arena furthest from mirror
		Front	Immobile in front half of arena closest to mirror
		Slow	Slow approach towards mirror, with hind legs stretched out behind
		No-aggressive	Non aggressive contact with the mirror
		Attack	Strike the mirror with front legs or head
		Watch	Immobile, watching directly to mirror

Table S2 – Expert-based behavior groups defined for the behaviors identified in the OFT and MIS experiments. Each expert-based group corresponds with a personality trait.

Expert-based group	**Behaviors**
OFT	
ACTIVITY	Locomotion, Rise, Scan
EXPLORATION	Sniff, Head dip, Scratch
SHYNESS	Immobile, Hang
MIS	
SOCIABILITY	Front, Slow, No-aggressive
AVOIDANCE	Back, Hang
ALERT	Watch
ACTIVITY (OTHER)	Rise, Locomotion, Sniff, Head dip, Scratch, Scan

S.3 Chapter 6 "Relationships between parasite load, physiological stress and body mass and their effects on reproduction in an invasive alien species"

Figure S1. Relationship between personality (PC1 score), FGM concentration, body mass, parasite infection (*Strongyloides robustus* abundance), females reproductive success (uterine scars/year) and males reproductive investment (testis left mass). Solid lines represent significant relationships, dotted lines represent non-significant relationships. Numbers and letters between brackets are related to models tested.



Relationships: (1 a,b): Chapter 4, paragraph "*Relationship between body mass, personality and parasite infection*", Table 4.7; **(2 a,b; 7; 9):** Chapter 6, paragraph "*Reproductive success and investment*", Table 6.3; **(3 a,b):** Chapter 6, paragraph "*Relationship between physiological stress and personality*"; **(4 a,b; 6; 8):** Chapter 6, paragraph "*Reproductive success and investment*", Table 6.2; **(5):** Chapter 6, paragraph "*Relationship between parasites infection predictors, physiological stress and personality*", Table 6.1.

Peer-reviewed papers

Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season (2016). Journal of Mammalogy. 97(5): 1385-1398. doi:10.1093/jmammal/gyw095

Dantzer B, **Santicchia F**, van Kesteren F, Palme R, Martinoli A, Wauters LA.

Stress in biological invasions: alien grey squirrels increase physiological stress in native Eurasian red squirrels. Journal of Animal Ecology. Submitted.

Santicchia F, Dantzer B, van Kesteren F, Palme R, Martinoli A, Ferrari N, Wauters LA.

Habitat-dependent effects of personality on survival and reproduction in red squirrels. Behavioral Ecology and Sociobiology. Submitted.

Santicchia F, Gagnaison C, Martinoli A, Matthysen E, Bertolino S, Wauters LA.

The price of being bold: personality affects endoparasite infection in introduced Eastern grey squirrels. Oikos. Submitted.

Santicchia F, Romeo C, Ferrari N, Matthysen E, Vanlauwe L, Martinoli A, Wauters LA.

The use of uterine scars to explore fecundity levels in invasive alien tree squirrels. 2015. Hystrix, the Italian Journal of Mammalogy. 26(2):95-101. doi:10.4404/hystrix-26.2-11428

Santicchia F, Romeo C, Grilli G, Vezzoso S, Wauters LA, Mazzamuto MV, Martinoli A, Ferrari N.

Acknowlegements

My first thanks goes to Lucas Wauters, for his irreplaceable mentoring, guidance and for his “Belgian” way to think;

I am grateful to Adriano Martinoli for giving me the chance to follow my project;

Thanks to Damiano Preatoni for his precious assistance;

Many thanks to Nicola Ferrari and Claudia Romeo for their expertise during our “parasitological brainstorming”, for teaching and introducing me to parasitology and for providing space to carry out lab analyses;

I am grateful all the people of the Environment Analysis and Management Unit (University of Insubria) for their support and to make me feel that “we work in a wonderful team!”;

Thanks to Erik Matthysen and all the people of the Evolutionary Ecology Research Group for their kind welcome, their support and the warm and friendly working environment during my 3 months at the University of Antwerp;

Many thanks to Gerardo Fracasso, Cecilia Baldoni, Tommaso Biglino and Simone Messina for helping me to survive “up in the North”;

Thanks to Ben Dantzer and Freya van Kesteren and all the people of the Ben Dantzer lab at the University of Michigan, for their precious help and for teaching me the secrets of stress hormones analysis;

Thanks to Kristel Sanchez and Susanna Campbell to hosting me in Ann Arbor;

Many thanks to John Koprowski, Melissa Merrick and Marina Morandini and all the people of the Conservation Research Laboratory at University of Arizona for their warm welcome and for hosting me and bringing me to Mount Graham (“The Legend”);

This work was supported by the EU and realized under the LIFE09 NAT/IT/000095 EC-SQUARE Project, ASPER (Alpine Squirrel Population Ecology Research) project, lab analyses were funded by ESI (European Squirrel Initiative), and part of fieldwork was funded by National Geographic;

Thanks to Regione Lombardia, Regione Piemonte, Regione Valle d’Aosta and thanks to Provinces of: Milano, Varese, Sondrio, Cuneo and Torino. Thanks to Oasi WWF “Bosco di Vanzago” and Andrea Longo, Parco Valle Lambro, Parco Adda Nord, Villa Castelbarco, Parco Nazionale dello Stelvio, Parco Nazionale del Gran Paradiso, Parco Regionale delle Orobie Valtellinesi. Thanks to all other parks and provinces that allowed field collection;

Many thanks to Filippo Gautier and his family for being a precious assistant and giving me a warm hospitality;

Thanks to Riccardo Sella, Tenuta Berroni, Le Commande and all others private estate owners for their support to this research;

I am grateful to all the students involved in the data collection and lab analysis;
Thanks to all the researchers and colleagues that I have met and with whom I collaborated during this 3 years project;

I thank Mum and Dad to always be there for me and Marco, who believes in me.

Chapter 1 is a pre-copyedited, author-produced version of an article accepted for publication in Journal of Mammalogy following peer review. The version of record [Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season (2016). Journal of Mammalogy. 97(5): 1385-1398.] is available online at: URL: https://academic.oup.com/jmammal/article/97/5/1385/2219258 and DOI: 10.1093/jmammal/gyw095