Effects of perceived predation risk on extra-pair mating in blue tits (*Cyanistes caeruleus*)

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Front page: Blue tit chicks (*Cyanistes caeruleus*) in nest box. (Photo: Sigrunn Eliassen)

Til pappa

Som fra jeg var et lite frø har inspirert og fascinert

med sin kunnskap om, og kjærlighet for, naturen.

En ekte hobbybiolog

Til minne
kjære
Mommo

Contents

Abstract

Trivers' theory of sexual conflict in parental investment has laid the groundwork for immense quantities of research, especially within the avian taxa. Although male benefits of extra-pair copulation (EPC) are argued to be well-understood, female benefits are harder to explain. Nevertheless, EPC is thought to be female driven and so current research is focusing on possible explanations for female benefits. In 2014, Eliassen & Jørgensen suggested that female-driven EPC is based on the spreading of male interest among neighbouring families. EPC creates incentives for male cooperation in a neighbourhood, contributing to benefits for females and her offspring. We tested this, and more specifically looked at possible sex differences between parents regarding antipredator behaviour and extra-pair paternity (EPP) rates. The study was conducted on a blue tit population in southern France, Montpellier. We exposed parental birds to a taxidermy predator or novel object before egg laying. Antipredator behaviour of exposed nests and naïve neighbours was later observed during the chick rearing stage. DNA samples were collected from parents and offspring to determine EPP rates. We found that both parents reduced their antipredator behaviour in nests that contained extra-pair young, both in the form of alarming less and taking fewer risks by keeping a farther distance. We did not find any significant sex differences between parents. Our results showed no difference in EPP rates between predator-exposed and control nests (object-exposed). We did not find any evidence of a difference in antipredator behaviour during chick rearing stage between the different exposed nests, although due to a modest dataset this experiment should be repeated in consecutive years before making any firm conclusions.

Keywords

1 Introduction

The difference in relative investment of males and females in reproduction is core to understanding life history theory and sexual selection. Trivers (1972) proposed two strikingly accurate predictions concerning the conflict between sexes and reproductive strategies. The optimal breeding strategy for a male is to increase his opportunities for additional matings outside his social nest, and he is therefore expected to display life history and behavioural traits that will help him obtain this. For females, Trivers argued that the strategy should be to choose a mate that will increase the "quality" (phenotypic and/or genetic) of her offspring. Consequently, there is a conflict between the sexes and their optimal reproductive strategies, which can be seen as a "quantity versus quality conflict".

Reproductive investments in birds have been extensively studied, including the fitness consequences of clutch size, body size, breeding, mating strategies and mate choice, and the way they are traded off against one another (Montgomerie, 1988). Despite this, little is known about the interactions between nest defence and other aspects of reproductive investments by parents (review by Lima, 2009). Although clutch size is strongly correlated with reproductive success, all factors affecting fledgling health are insignificant if faced with predation and complete brood failure. Nest predation is therefore thought to be the most important factor (Ricklefs, 1969; Martin, 1995; Thompson, 2007).

Nest defence improves offspring survival, but also increases the chance that a parent is injured or dies (Shields, 1984; Mutzel et al., 2012). While the joint feeding and protection efforts of both parents benefit the young, each parent suffers a reduction in future reproductive success as a consequence of their individual efforts in their current brood (Houston et al., 2005). Parents are therefore expected to base their risk-taking decisions on a compromise between current and future broods and consider both the threat to their offspring as well as the threat to themselves (Mahr et al., 2014). In addition, there may be a trade-off between nest defence and other aspects of parental care. When predation risk is high, parents are allocating time and energy to protect their brood, which then

cannot be spent looking for food or feeding chicks (Brunton, 1990). Parental defence behaviour should therefore depend on the risk posed by a predator, the ability of parents to reduce such a risk, and at what cost.

Social monogamy in the form of bi-parental care is essential to many bird species to successfully raise their brood to fledglings as well as defending their nest from predators (Clutton-Brock, 1991). This does not mean, however, that males and females are selected in similar ways to invest in parental care. Trivers' (1972) theory on investment by parents in sexual reproduction tells us that the optimal effort for each parent could be equal, yet may differ between the two sexes. In the past decades, a lot of research has focused on the possible explanations behind the observed difference in parental care investment (Brunton, 1990). For birds breeding in pairs, the widespread occurrence of extra-pair copulation (from now on referred to as EPC) could further affect differences in parental efforts. The apparent monogamous mating systems found in many bird species were for a long time assumed to also indicate genetic monogamy between the paired mates (Lack, 1968; Ford, 1983). However as molecular methods became increasingly accessible, paternity tests made it evident that extra-pair mating was a widespread occurrence (Mock, 1985; reviewed by Griffith et al., 2002). This is especially striking in birds where social monogamy is found in over 85 % of species, but where only 25 % are also genetically monogamous (Bennet & Owens, 2002). It is now common to classify mating systems at both a social and a genetic level (Culina et al., 2015).

Following Trivers' logics, males in socially monogamous species can enhance their reproductive success with the low-cost strategy of EPC. EPC is a beneficial strategy as it can increase the number of offspring through extra-pair paternity (from now on referred to as EPP), and can be obtained through mating with neighbouring females without having to provide care at her nest. Furthermore, EPC means that some nests can have a large proportion of extra-pair offspring (sometimes exceeding 50%, Westneat et al., 1990). For the social males of EPP nests, there is no apparent fitness benefit of providing care to unrelated offspring. On the contrary, males are usually expected to reduce their

parental investment as an adaptive response to their female partner's EPC (Sheldon & Mangel, 2014). Therefore, while male benefits make EPC seem logical as it results in higher quantity of offspring, females are predicted to pay the cost in terms of reduced paternal care and protection. However, in many species females are observed to freely engage in EPC's, even pursue them. This suggests that EPC might not only benefit males, but also females (Kempenaers et al., 1992; 1997), and has traditionally been viewed as an evolutionary puzzle. Among bird species engaging in EPC, sperm competition was shown to be a widespread phenomenon (Review by Cheng & Bruggers, 1984), but the most important factor is now thought to be the role of female choice (e.g. Wagner, 1992; Gray, 1997; Kempenaers et al., 1997).

Hypotheses such as the genetic compatibility hypothesis (Tregenza & Wedell, 2000), higher fitness of offspring (Kempenaers et al., 1992; 1997) and the sexy son hypothesis (Weatherhead & Robertson, 1979) have long been thought to be the main driver for female EPC. Some researchers also argue that EPC suggests a role for fertility insurance, although not as the only explanation (Krokene et al., 1998). Even so, the overwhelming accumulation of research is showing little to no support for these current hypotheses and is now forcing researchers to look into other explanations for female EPC (in blue tits; Strohbach et al., 1998; Charmantier & Perret, 2004; in tree swallows; Barber et al., 2005; reviewed by Akçay & Roughgarden, 2007). Explanations for EPC focusing on direct (ecological) benefits such as assistance in antipredator defence from extra-pair males in the neighbourhood, or access to neighbouring territories for food resources, (Gray, 1997) are now receiving more attention.

Birds exhibit a wide variety of breeding strategies and cooperative behaviours. Although social monogamy with shared parental care between a pair is common (Clutton-Brock, 1991), birds show a wide range of parental behaviour. This range from helpers at the nest (*i.e.* Florida Scrub Jay, *Aphelocoma coerulescens*: Woolfenden, 1975), large groups of individuals incubating and defending one single nest at the time (*i.e.* acorn woodpecker, *Melanerpes formicivorous*: MacRoberts & MacRoberts, 1976), or large colonies of

birds with sub structured smaller clans overlapping in territory, exhibiting helping behaviour (*i.e.* white-fronted bee eater, *Merops bullockoides*: Hegner et al.,1982), to mention a few. With such a great variety in breeding strategies, one can assume an equal amount of variety in antipredator behaviour. Here, we focus on socially monogamous pairs which exhibit cooperative defence behaviour within pairs, to see if there is also a cooperation in antipredator defence between neighbouring pairs of birds (*i.e.* between nests).

How does EPC relate to investment in nest defence? On the one hand, even if a female engages in EPC all offspring in the nests will be hers, meaning she will risk complete brood failure and loss of all offspring that season if faced with a predator attack, regardless of EPC rate. One the other hand, males will benefit less from defending their social nest if it contains extra-pair young (from now on referred to as EPY), as compared to a nest with no EPY (Lubjuhn et al., 1993). In such a situation, it will not be beneficial for the male to defend unrelated young if it could result in injury or death (see review in Westneat & Stewart, 2003; Arnqvist & Kirkpatrick, 2005). Furthermore, the average social male will most likely have offspring in several of his neighbours' nests (Review by Akçay & Roughgarden, 2007), resulting in his offspring being spread over a larger area, protected by other parents. This means that even if a male were to experience complete brood failure in his current nest (*i.e.* the social nest), chances are some of his offspring will still survive in neighbouring nests. We can therefore hypothesise that the social male's interest in defending his nest, and thus taking a risk for his own fitness, will depend on both the rate of EPC he engages in as well as his female's EPC behaviour.

With red-winged blackbirds as her study species, Gray (1997) published the first study investigating if male neighbours offer any protection to their assumed neighbouring offspring. The hypothesis was that antipredator response would differ between EPC males and non-EPC males, even in the same neighbourhood. When exposed to a well-known model predator, males showed a considerable more aggressive behaviour towards the predator threat of a neighbouring territory if they had copulated with the breeding female in that

given territory. Gray also found a significantly higher fledging success for females engaging in EPC than those who did not, with lower depredation rates in EPC clutches as well as lower starvation rates in broods with EPY. Although this study was performed on a polygynous species, she suggests that socially monogamous species living in high-density areas would benefit from neighbour assistance when faced with predation risk. Her findings laid the groundwork for hypothesizing that socially monogamous females might acquire direct benefits from engaging in EPC. This could be argued to be the result of by-product mutualism (Connor, 1986; 1995), where the cooperative parent will benefit from helping his extra-pair offspring on a cuckolded neighbour's territory. The female recipient thus benefits from his help as a by-product of the helper's own selfish parental behaviour.

In 2014, Eliassen and Jørgensen published a paper suggesting a new way of thinking regarding cooperative behaviour in socially monogamous species. The *nicer neighbourhood hypothesis* suggests that males will relax territory defence against neighbouring males when engaging in EPC with neighbouring females. With higher levels of EPP, males' incentives for participating in collective vigilance and antipredator behaviour may also increase (Eliassen & Jørgensen, 2014). Cooperative group defence in the form of mobbing, warning calls or nest defence might help reduce the overall nest predation in an area (Skutch, 1976). In theory one can expect that monogamous pairs which do not experience a direct threat to their nest will not risk detection of own offspring, and possible death or injury by defending neighbouring nests (Collias & Collias, 1978; Winkler, 1994). This trade-off between individual cost and collective benefits changes if males have EPY in other nests. Males with EPP spread across several neighbouring nests will benefit from investing in safety and productivity of the entire neighbourhood rather than just his social nest (Eliassen & Jørgensen, 2014). More eyes on the lookout should therefore result in earlier predator detection and so reduce the overall brood failure rate. For females, the possible benefits of cooperation in predator defence might outweigh the potential risk of lost care from the social male. In such a case, EPC would be the most beneficial

strategy also from a female's perspective. It is therefore suggested that it is in the self-interest of both parents to have cooperative behaviour among neighbouring individuals as long as the benefits of such cooperation outweigh the costs.

Blue tits (*C. caeruleus*) live in socially monogamous pairs throughout the chick rearing stage, cooperating in both feeding and defending of the nest, but also frequently engage in EPC. For blue tits, variation in nest defence investment has been found to vary in relation to their own, not offspring's, risk (Mahr et al., 2014), as well as showing adaptive flexibility in parental behaviour when facing a threat (Mutzel et al., 2012). Blue tits are an appropriate species for testing the nicer neighbourhood hypothesis, as they are a common nest box breeder often breeding in close proximity to each other.

A field experiment was performed over two consecutive years (2014-2016) to test whether exposure to a predator early in the mating season would affect extra-pair copulation rates, as well as affecting antipredator responses from parents when exposed to a new threat later in the chick rearing season. This thesis focuses on data from one year (2016), and more specifically explores: a) if manipulated, early predator exposure affects EPP rates and subsequent antipredator behaviour during chick rearing; b) if antipredator behaviour is related to EPP; c) if there is a difference in antipredator behaviour (*i.e.* alarm intensity and risk taking) between males and females during chick rearing stage; and d) if antipredator behaviour is affected by the social context.

2 Material and methods

This MSc project was a cooperation between the University of Bergen, the University of Montpellier and the Centre National de la Recherche Scientifique (CNRS). Fieldwork and sampling were performed in Montpellier, France, whilst lab work, data analyses and write up were undertaken in Bergen, Norway.

Study site and nest boxes

This study was conducted in May 2016 in the La Rouvière woodland (43° 40'N, 03° 40'E), 16 km north-west from the city of Montpellier in southern France (**Figure 1**). The study area covers 300 hectares of typical Mediterranean forested habitat with interspersed patches of deciduous downy oak (*Quercus pubescens*) as well as evergreen holm oak (*Quercus ilex*) (Blondel et al., 2006). There are 227 nest boxes distributed throughout the La Rouvière woodland, all specifically designed for blue tits (*C. caeruleus*) or great tits (*Parus major*) with nest box holes of 25mm and 28mm, respectively. All nest boxes are placed on metal poles approximately 2 m high, with a meshed metal cage surrounding the nest box to avoid predation (**Figure 2**).

Figure 1: *Map of France, Montpellier and the area in La Rouvière (red mark).* ***Figure 2****. Nest box erected on metal pole with metal cage surrounding the nest box.*

Nest boxes in La Rouvière have been monitored annually since 1991, by researchers from the CEFE (Centre d'Ecologie Fonctionnelle et Evolutive), in Montpellier. The average distance between neighbouring nest boxes is 45 meters (Charmantier et al., 2015). In 2016, the average distance between occupied nest boxes was 86 meters, as some were empty. Nest boxes were checked weekly throughout the season to determine laying date, clutch size and fledging date; adults were ringed throughout the season while chicks were ringed at day 15 post-hatching. All nest boxes were emptied of old nest material at the end of the season to prepare for next year's birds.

Study species

The Eurasian Blue tit (*C. caeruleus*) is a small non-migratory passerine bird in the Paridae family with a distinct blue and yellow plumage (**Figure 3a**). The sexes are similar in morphology although males have a brighter blue coloured crown under UV light (Hunt et al., 1998). It is a widespread and common species throughout subarctic and temperate Europe and western Asia and is categorized as "least concern" on the IUCN red list (BirdLife international, 2016).

Blue tits are socially monogamous, but commonly participate in EPC at the start of the breeding season, before raising their chicks together with a social partner. The breeding season ranges from April to late June; some birds have repeat clutches after a failure, but second clutches are very rare (Blondel et al. 2006). Nests are built by the female, using moss, plant fibres, dried grasses, hair and feathers, creating a cup either in a hole in a tree or artificial cavities like nest boxes. The female lays 7-14 eggs (one egg per day) before incubating for approximately two weeks. When chicks hatch, both parents engage in feeding throughout the rest of the chick rearing stage, a 19-24 day long nesting period, followed by a couple of weeks of post-fledging feeding outside the nest.
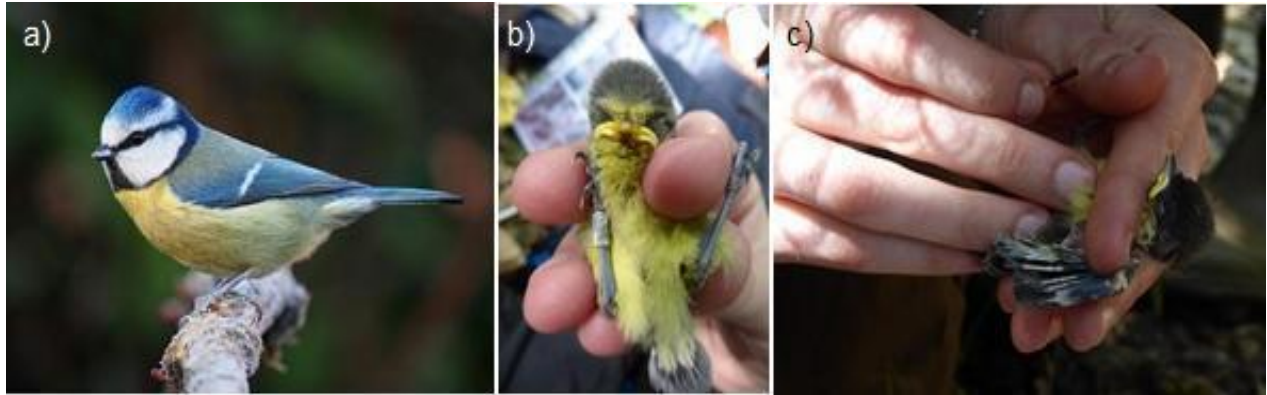
Figure 3: a) Adult blue tit with its characteristic blue and yellow plumage. b) Blue tit chick equipped with metal identification ring. c) Blood sample collection from a 15-day-old chick.

Ringing and identification of sexes

All adults were caught using nest box traps prior to observations. They were equipped with marked metal rings provided by the C.R.B.P.O. (Centre de Recherches sur la Biologie des Populations d'Oiseaux, Natural History Museum, Paris) (Charmantier et al., 2015), and registered for future identification. Nestlings between six and 15 days were also equipped with metal identification rings on their right leg (**Figure 3b**). In addition, adults were colour-ringed, making it easier to identify their sex at a distance. Identification of sex was thus possible with the help of binoculars, as females had an orange ring on their left leg, while males had a blue ring on their right leg.

Blood samples

Blood samples were collected from all chicks and adults included in our study. Blood samples (5-40 µL) were collected from the brachial vein in the wing of both the adult male and female in a nest, and from either the metatarsal or the brachial vein of nestlings depending on their age (**Figure 3c**). All blood samples were immediately placed in Queens Conservation buffer in the field and brought back to Norway for analysis. Catching of adults and blood sampling were never performed on the same day as behavioural observations.

DNA extraction and genotyping

Extra-pair paternity was analysed from 21 nests, sampling 42 individual adults and 208 chicks. DNA extractions were done with help from Louise Lindblom (UiB), using DNeasy Blood and Tissue Kits (Qiagen) and following methods adapted from the standard blood protocol. Genotyping of the chicks for paternity assignment was mostly performed by A. Mennerat, using 10 microsatellite markers based on protocols previously developed by collaborators in Montpellier (See Porlier et al. 2012), and further optimised. Loci were separately amplified by polymerase chain reaction (PCR) using fluorescently-labelled primers (10 reactions per individual sample), before being pooled in two sets of colour- and length-compatible, diluted PCR products stored at −20 °C. The samples were finally run in an ABI sequencer by Geir Dahle (Institute of Marine Research, Bergen). A. Mennerat performed the analysis of genotype data for paternity assignment by comparing each chick's genotype to that of the males and females. All chicks matched the genotype of the breeding female (*i.e.* there was no case of brood parasitism). To exclude paternity, at least two mismatches between microsatellites of father and chick had to be detected (following Charmantier & Perret 2004). For all EPP assigned to a genotyped male, the identified EP male matched the genotype of a chick completely.

Anti-predator behaviours

The test was carried out in a two-stage experiment: a manipulation of perceived predation risk during mating (*i.e.* after nest completion but before egg laying, late March/ early April) and behavioural observations during the chick rearing stage (May).

Manipulation of perceived predation risk

When manipulating the perceived predation risk, we used taxidermy specimens of two nest predators that are common at the study site: the red squirrel (*Sciurus vulgaris*) and black rat (*Rattus rattus*). The model predator was placed directly

under the nest box (n=8, hereafter referred to as "predator-exposed" nests) for 60-90 minutes on two consecutive days (one predator species per day in random order). Previous observations have confirmed adult blue tits perceiving taxidermy specimens as a threat as they have previously been observed responding by approaching them and emitting alarm calls (A. Mennerat, pers. com).

To control for the effects of disturbance to the nests, a control group of nests were matched to the first group in terms of expected laying date, and exposed to the same procedure. The only difference was that the observer placed a neutral object (a white plastic box) under the nest box instead of a taxidermy predator (n= 5, hereafter referred to as "object-exposed" nests). There was also a third group of nests that acts as a control for all exposed nests, and these were left undisturbed (n= 9, hereafter referred to as "unexposed" nests) (**Appendix, Figure A4**). Although the unexposed nests were not presented with any object at the mating stage, they were still monitored, and had adults and chicks ringed and blood-sampled during chick rearing season as described above. Reproduction was monitored in all nests following standard protocols, as part of a long-term study carried out by colleagues in Montpellier (see e.g. Blondel et al. 2006).

Behavioural observations during chick rearing

For the second phase of the experiment, antipredator defence behaviour was observed during the chick-rearing stage. To get quantitative information on the extent to which adults take part in defence at neighbouring nest boxes, nests from each of the two experimental groups were paired with the nearest nest occupied by blue tits. Each pair of nests thus included one experimental nest (either a predator-exposed or an object-exposed nest) and one neighbouring nest (unexposed). All pairs of nests were observed on two consecutive days. For all observations, two observers took the role as predators (hereby referred to as the "threat"). The first observation was performed walking away from one of the nests (nest A) towards the neighbouring nests (nest B); whereas on the next day the observers would walk from nest B towards nest A (**Figure 4**). By doing so, both

nests would experience a threat moving away from their nest (*i.e.* from A to B), and a threat walking towards their nest (B to A and vice versa). This allowed us to estimate the intensity of the behavioural defence in response to increasing versus decreasing danger.
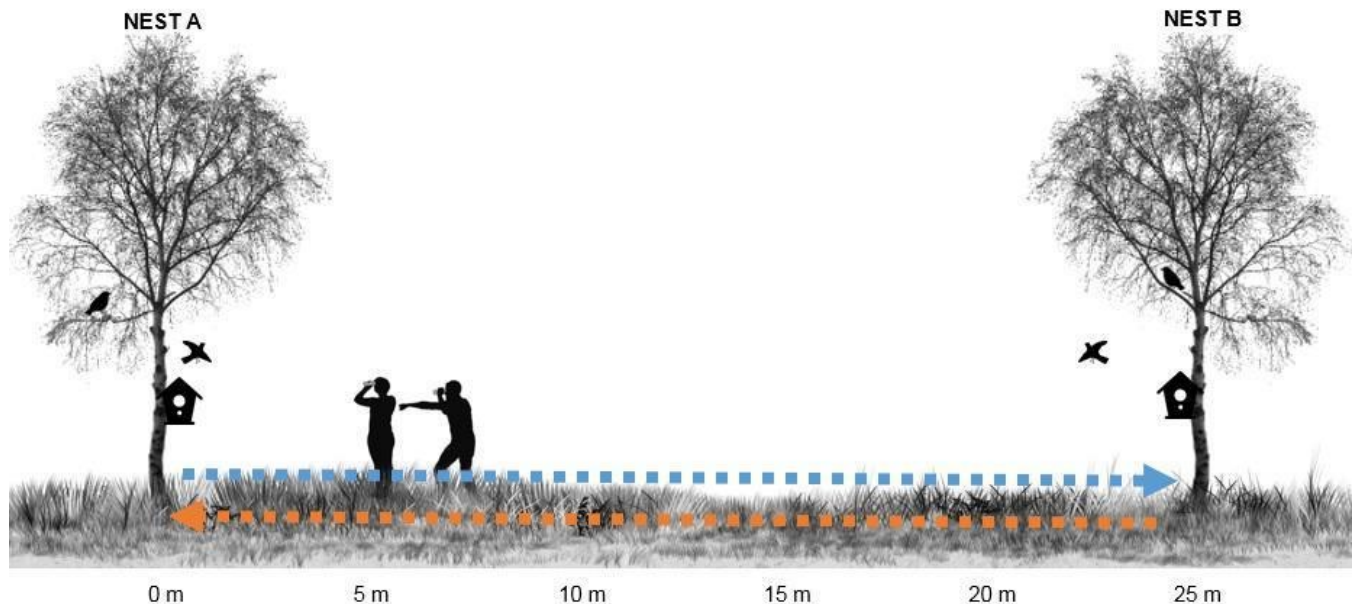
Figure 4: *Illustration of how observations were carried out in the field by starting at one nest (nest A) and moving towards a neighbouring nest (nest B), then from nest B to nest A the following day. Observers moved 5 meters at the time and recorded all behaviour from nearby adult birds for 3 min at each stop (0, 5, 10 m…).*

Each observation session started by playing back a distress call from a starling (using a Samsung Galaxy S6 phone) lasting a total of 10 seconds. During each series of observations, antipredator behaviour was measured as frequency of alarm calls and distance birds kept to threat.[1] All observations were recorded after the starling playback, for a total of 180 seconds (*i.e.* 3 minutes) at every 5-meter interval, while moving from one nest to the neighbouring nest. The distance between observation points was increased to 10 or 15 meters when no alarm calls were recorded for more than two sessions in a row (which typically happened when neighbouring nests were far apart and the observers were at mid-distance). 5-meter distances were resumed as soon as alarm-calling responses were recorded again.

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[1] *For each pair of nests, one nest would have previously been predator-exposed or object-exposed while the other nest would be an unexposed neighbouring nest (see Appendix, **Figure A4**).*

Following the playback, the birds' response was observed for three minutes, and the following information was recorded using a digital voice recorder: sex (visible from colour rings), duration of alarm calls, distance to the observers (*i.e.* the threat), as well as commenting on the overall behaviour of the pair. Distance to the observer was grouped into categories because it was challenging to give exact estimates of distance in the field as birds were moving around during the observations. We therefore used distance groups of 1 to 4 (1 = 0-2 meters, 2 = 2-5 meters, 3 = 5-10 meters, 4 = >10 meters). Distance was recorded at the start of each new session for all observed birds, and subsequently every time a bird moved to a new distance group. This made it possible to calculate the total time spent alarming in the different distance categories. All observations started beneath a nest as soon as both parents were observed flying out of the nest box, as to make sure both parents would have an equal chance of detecting the predator. All observations were distinguished between males and females whenever there was a positive confirmation of ring colour, and classified as "unidentified" if no ring was detected. Birds were also classified as unidentified whenever we had a vocal response from a blue tit that we could not see. This data was combined with paternity data provided by A Mennerat (unpublished data). Data on the number of chicks were provided by CEFE (Montpellier).

Statistical analysis

All statistical tests were performed using the statistical program "R", version 3.3.2 (R Core Team, 2015).

EPY and experimental treatment

To test if EP status (presence/absence of EPY in the nest) was affected by the manipulation of perceived predation risk during the mating season in March/April, a Fisher's exact test was performed to compare predator-exposed and object-exposed nests. To rule out the possibility that disturbance alone

could affect EP status, another Fisher's exact test was performed comparing experimentally exposed nests (*i.e.* both predator- and object-exposed nests) to their neighbouring unexposed nests for EPY. We compared the number of EPY and the proportion of EPY in predator-exposed *vs* object-exposed broods with generalized linear models fitted with a Poisson and a binomial distribution, respectively.

Behavioural analyses

For both dependent variables, *alarm time* and *threat distance*, we performed forward model selection based on the Akaike information criterion (AIC), combined with p-values obtained when comparing models (Sakamoto et al., 1986). We added explanatory variables one by one until AIC stopped decreasing. Variables with a minor effect on AIC (decreasing it by < 2 units) were kept in the final model if they had a statistically significant effect at $p<0.05$ (Rice, 1989).

As behavioural data consisted of observations made walking from one nest to another, blue tits in pairs of neighbouring nests could not be considered to behave independently from each other. Similarly, adult pairs of breeding birds at a given nest were not assumed to behave independently. Finally, there are repeated measures of individuals throughout the observations (pseudo-replication) as we observe the same nest from several distances within one observation (A to B). For these reasons, generalised linear mixed-effect models (GLMER in the lme4 package) were used with nest pair, nest ID and individual ID (ring number) as random effect factors, nested with the hierarchy of pair/nest/ ID for both alarm time and threat distance (Bates et al., 2015).

We tested which factors affected *alarm time* and *threat distance* throughout all observations. All figures are made with plots from the package "ggplots" in R-3.3.2 (Wickham, 2009).

Alarm time

A GLMER fitted with a Poisson distribution (in lme4 package; Bates et al., 2015) was used for alarm time as a dependent variable and reflects the average

duration spent alarming by individuals during each 3-minute session (thereafter referred to as "alarm time"). Explanatory variables were sex, response order (first or second), number of individuals heard, nest distance, walking direction (towards), EPP (presence/absence), time of day, experimental group (predator/object exposed), weather and brood size.

Threat distance

A GLMER fitted with a binomial distribution and an logit link function, using binomial models with time spent at close distance as "success" and time spent at further distance as "failure" (close=1, far=0) (in lme4 package; Bates et al. ,2015). To analyse threat distance we used the following reasoning. Alarming at distance 1 or 2 (*i.e.* less than 5 meters from the threat) means the bird is close enough to risk being injured or killed in a situation of real threat, whilst staying at distance 3 or 4 (*i.e.* 5 meters and more) means little risk while alarming. Distance 1 and 2 were therefore pooled into a "close" category, while distance 3 and 4 were grouped into a "far" category. Explanatory variables were response order (first or second), number of individuals heard, nest distance, walking direction, number of EPY, time of day, age, experimental group (predator/object exposed), weather and brood size.

The initial aim of each model was to test whether manipulation of perceived predation risk in early season affected subsequent alarm-calling behaviour at both nest level and within a neighbourhood. For each of the two response variables (alarm time and threat distance), we included nest pair status (predator-exposed & unexposed vs object-exposed & unexposed) and individual nest status (predator-exposed, object-exposed or unexposed) as factors. To test whether EPP affected defence behaviour we also included EP status (presence or number of EPY in the brood), as well as sex and its interaction with EP status. The distance from the nest at which the observation took place, as well as the direction in which the observers walked (decreasing vs increasing danger) were also accounted for. Age was included as a measure of possible previous breeding experience and brood size (number of chicks) were added as covariates, as these

are known to affect investment in defence behaviour in small passerine birds (Culina et al., 2015). Weather during the observations, as well as time of the day, were accounted for as the detectability and activity of birds might vary accordingly (Carr & Lima, 2010). Finally, to explore the collective component of alarm-calling behaviour we included both the number of individual blue tits heard alarming during the each 3-minute session and the response order (*i.e.* whether the focal individual was the first one to respond after hearing the distress call or not).

In our results and for the sake of clarity the model with alarm time as response variable is referred to as "GLMER1", whilst the model with threat distance is referred to as "GLMER2".

3 Results:

A total of 274 observation sessions of 180 seconds each were performed on 11 pairs of nests on 7-13 May 2016. Due to nest failure, there are 12 pairs of nests in total but only 23 nests observed (one nest contained no living young and no adults were responding). Because of the early season brood failure in one of our nests (an unexposed nest), one predator-exposed nest was paired with an object-exposed nest, and there is therefore some data missing for two unexposed neighbours.

a) *Does manipulated early predator exposure affect EPP rates and subsequent antipredator behaviour?*

Comparisons between predator-exposed and object-exposed nests show no significant differences in clutch size, brood size or mean chick weight (**Table 1**). However, in the predator-exposed group, egg laying started later (3 days on average) than in the object-exposed group, and they ultimately produced a lower number of fledglings.

Table 1: *Summary statistics of egg-laying date, clutch size (number of eggs), brood size (number of chicks), mean chick weight and chicks fledged (measured by counting dead chicks in nest after chick fledging), for predator-exposed, object-exposed and unexposed nests. For t-test: predator- and object-exposed nests were used. Unexposed nests were chosen later on in the season and were not matched for breeding time.*

		Predator-exposed (n=7)	Object-exposed (n=5)	P-value (t-test) Predator vs Object	Unexposed (n=9)
Egg-laying date (1=March 1st)	Mean ± SD	31.9 ± 2.0	28.6 ± 0.9	0.01	35.2 ± 4.4
	Range	28 - 34	27 - 29		32 - 44
Clutch size	Mean ± SD	11.4 ± 0.5	11.2 ± 1.3	0.73	10.4 ± 2.1
	Range	11-12	10-13		8-14
Brood size	Mean ± SD	10.4 ± 2.1	10.8 ± 0.8	0.68	9.8 ± 2.2
	Range	6-12	10-12		7-14
Mean chick weight (g)	Mean ± SD	9.9 ± 0.9	10.5 ± 0.6	0.20	10.1 ± 1.1
	Range	9.0-11.3	9.74-11.1		8.9-11.9
Chicks fledged	Mean ± SD	7.6 ± 2.8	10.2 ± 1.1	0.05	4.3 ± 3.6
	Range	5-12	9-12		0-8

Extra-pair status (presence/absence of EPY) in the nest differed neither between predator-exposed and object-exposed nests (Fisher's exact test= 0.576, p= 0.35), nor between exposed and unexposed nests (Fisher's exact test= 0.396, p= 0.21), **Figure 5a & b**, respectively).
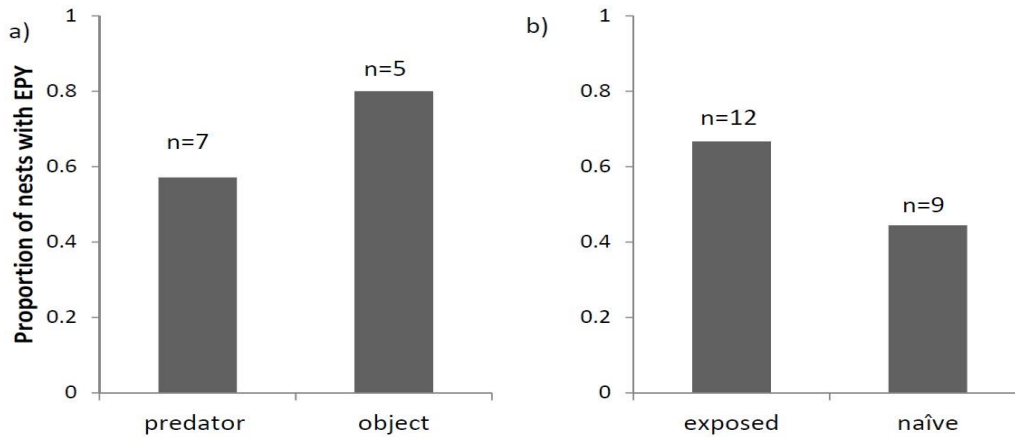
Figure 5*: a) proportion of nests with EPY in the predator-exposed and object-exposed groups (Fisher's exact test= 0.576, p = 0.35). b) proportion of nests with EPY found in all exposed and all unexposed nests (Fisher's exact test= 0.396, p = 0.21).*

Neither the number of EPY in nests nor the proportion of nestlings that were sired by EP males differed between predator-exposed or object-exposed nests (number of EPY: GLM (Poisson), df = 1, z = -1.16, p = 0.25; proportion of EPY: GLM (Binomial), df = 1, z = -0.65, p = 0.52, **Table 2**).

Table 2*. Summary statistics for number of EPY and proportion of EPY in predator-exposed, object-exposed and unexposed nests.*

		Predator-exposed (n=7)	**Object-exposed** (n=5)	**Unexposed** (n=9)
Number EPY	Mean ± SD	0.9 ± 1.5	1.6 ± 1.1	0.8 ± 0.8
	Range	0-4	0-3	0-2
Proportion EPY	Mean ± SD	0.1 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
	Range	0-0.7	0-0.3	0-0.3

We also compared alarm time (GLMER1) and threat distance (GLMER2) between the two groups using GLMER models, and did not find them to be significantly

affected by the manipulation of predation risk (GLMER1, GLMER2: all P= > 0.05, **Table 3a & 3b**).

a) *Is antipredator behaviour related to EPP?*

Nests with EPY showed parents alarming for a shorter duration than nests without EPY (GLMER1, EPY presence/absence: p= 0.001, **Figure 6a**). The more EPY present in a nest, the shorter time was spent alarming close to the threat (GLMER2, number of EPY: p= <0.001, **Figure 6b**). There was no significant interaction with sex (GLMER1, sex: p= 0.41; GLMER2, sex: P = 0.34, but see trend in **Figure 6b**). Alarm time did not vary according to experimental status, brood size or weather conditions (GLMER1, **Table 3a**), nor did threat distance vary according to experimental status, brood size, weather conditions or time of day (GLMER2, **Table 3b**).[2]
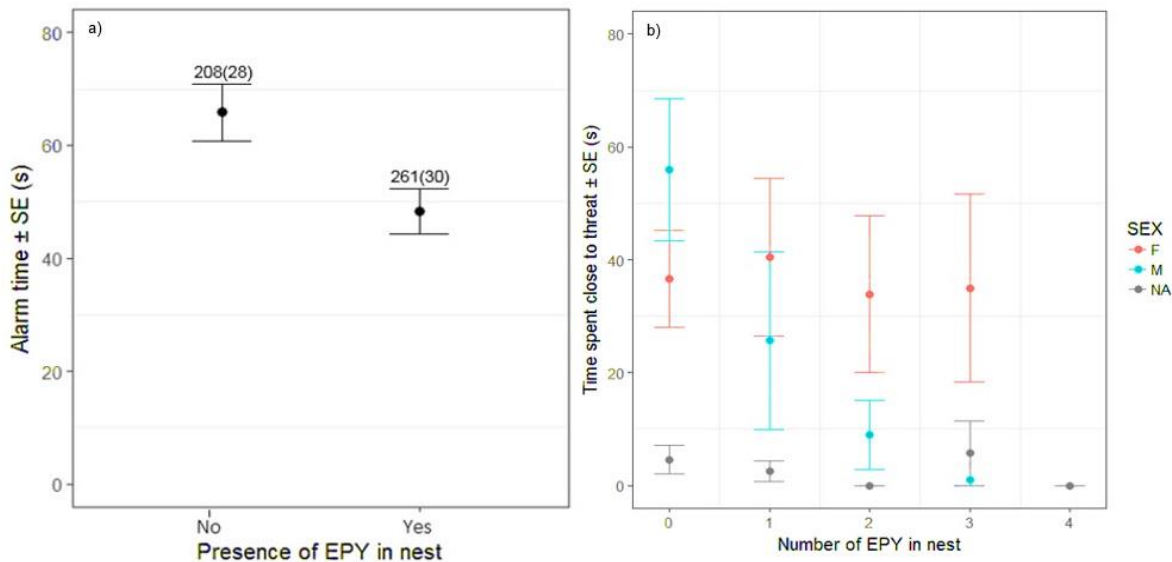
Figure 6 *a) Average alarm time for all responding birds without (NO) or with (YES) the presence of extra-pair young (EPY) in nest. Sample size is listed as total number of observations first, with total number of individuals in parentheses. b) Average alarm time spent at close distance (<5m) to the threat for males (M) and females (F) in nest with different numbers of extra-pair young. The more EPY in nest the less birds alarm. No significant sex differences were found. NA= represent birds observed but for which sex could not be assigned.*

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[2] The unexposed nests were chosen later on in the season and were not matched for breeding time like predator- and object-exposed nests were and are therefore not used for comparison.

Table 3. *Results from generalized linear mixed-effects models for a) alarm time GLMER (poisson): (GLMER1, alarm time) and b) threat distance GLMER (binomial): (GLMER2, threat distance). Estimate = regression parameter estimate; SE = standard error of estimate. Only final models following AIC-based model selection are represented in this table.*

a) **Alarm time (GLMER1)**

Explanatory variable	P -value	Estimate	SE
Sex	0.061	-1.094	0.584
Responds first	**<0.001**	-1.619	3.276
Sex x Responds first	**< 0.001**	-0.318	0.066
Number of ind. heard	**<0.001**	0.263	0.023
Nest distance	**<0.001**	-0.006	0.001
Direction towards	**<0.001**	1.143	0.211
EPP presence/absence	**0.001**	-2.022	0.622
Time of day	**0.019**	0.450	0.193
Experimental group	0.680	-0.319	0.774
Weather rain	0.704	0.663	1.747
Weather sun	0.976	-0.051	1.747
Weather wind	0.773	-0.503	1.747
Brood size	0.509	0.139	0.211

b) **Threat distance (GLMER2)**

Explanatory variable	p-value	Estimate	SE
Responds first	**<0.001**	3.346	0.211
Number of ind. heard	**<0.001**	-1.087	0.129
Nest distance	**<0.001**	0.562	0.012
Direction	**0.003**	2.264	0.645
Number of EPY	**<0.001**	4.329	1.156
Time of day	0.074	-1.251	0.700
Age	**0.047**	1.448	0.734
Experimental group	0.560	1.418	2.437
Weather rain	0.653	-27.181	60.559
Weather sun	0.725	-21.242	60.559
Weather wind	0.718	-22.940	60.801
Brood size	0.080	-1.032	0.590

c) Is there a difference in antipredator behaviour between males and females?

Alarm time was not significantly related to sex, although there is a trend with males alarming shorter than females (GLMER1, sex: p= 0.061, **Figure 7a**), independent of EPY status. Threat distance did not show any significant sex differences (**Figure 7b**).
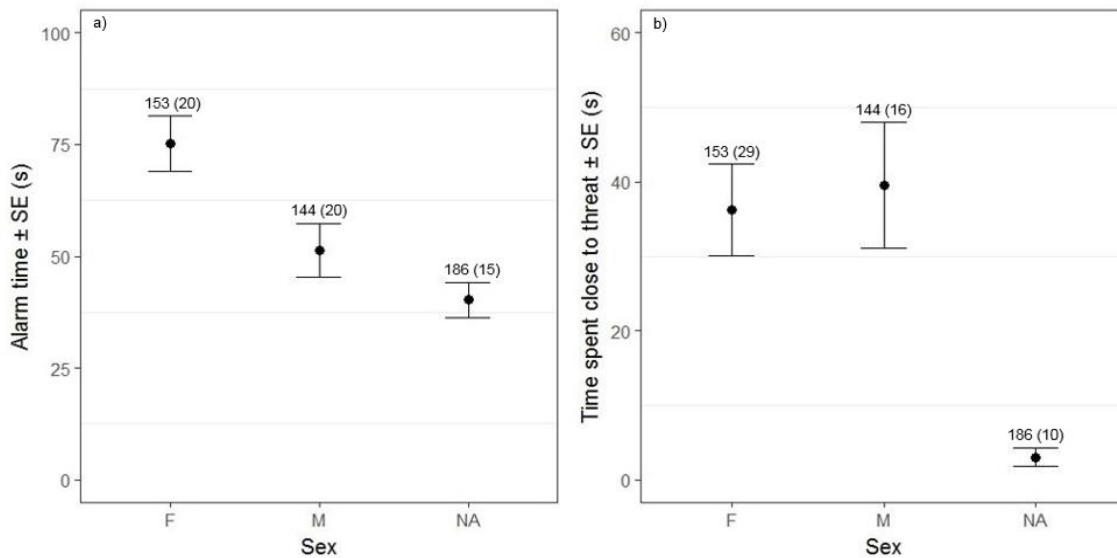
Figure 7: *a) Average alarm time for females (F), males (M) and unidentified (NA) birds. For alarm time there were no significant differences in alarming between females (F) and males (M), but showed a slight trend towards males average alarm time being shorter than what was found for females. b) Average alarm time close to threat (<5m) for females (F), males (M) and unidentified (NA) birds. Sample size is listed as total number of observations first, then total number of individuals in parentheses. NA = represents birds observed by for which sex could not be assigned.*

d) Is antipredator behaviour affected by the social context?

Females more often responded before males to the threat (74 vs 48 occurrences), and more males than females responded second (96 vs 79). However, regardless of sex, alarm time was longer, and distance to the threat shorter for the first bird responding than for the second (GLMER1, responds first: p < 0.001, **Figure 8a**; GLMER2, responds first: p < 0.001, **Figure 8b**). There was a significant effect on the interaction between sex and response order on response time (GLMER1, response order * sex: p= < 0.001). This interaction was due to male second

responder alarming for a shorter time on average (13 s) than female second responders (25 s), while both sexes alarmed for similar durations when they were first responders (males: 129 s; females: 129 s; **Figure 8a**). There was no significant effect on the interaction between sex and response order on threat distance (GLMER2, response order * sex: p= > 0.99; but see trend in **Figure 8b**).
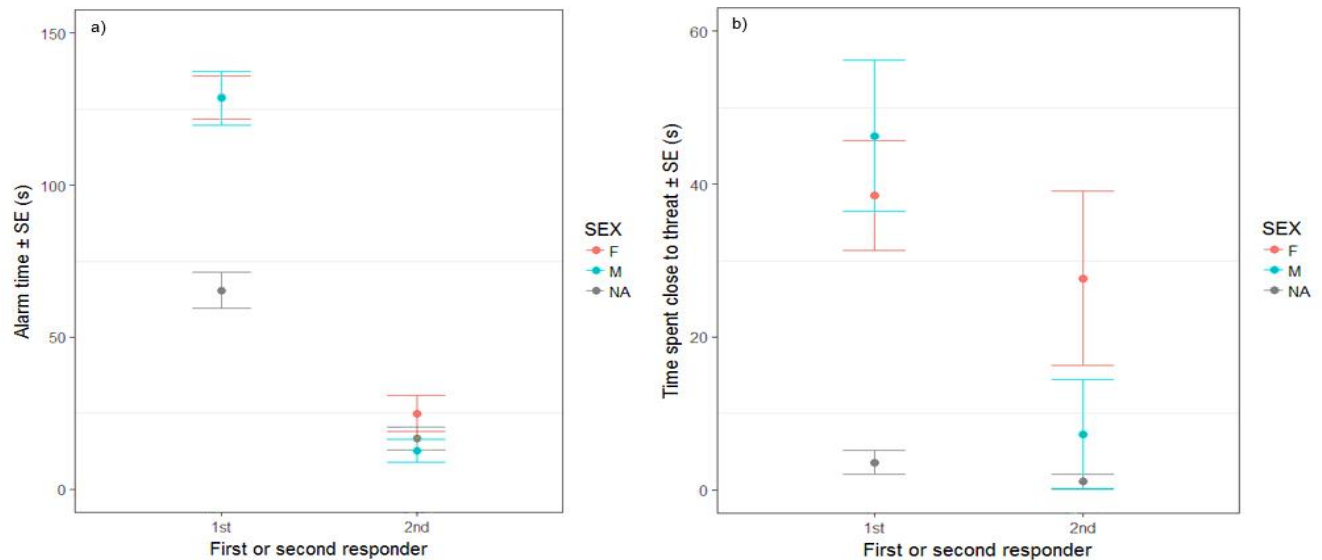
Figure 8: *a) Average alarm time (s) between the first (1st) and second (2nd) bird alarming at the nest, with colour codes for female (F), male (M) and unidentified (NA) birds' average alarm time. The first bird to respond to a threat is also the bird alarming for the longest when compared to the second responder. b) Average alarm time for birds spent at close (<5m) distance to the threat for females (F), males (M), and unidentified (NA) birds. The first bird to respond keep a closer distance to the threat itself than the second responder. There were no sex differences found between the first and second responder neither for alarm time nor for distance. NA= represents birds observed but for which sex could not be assigned.*

The more individuals we heard responding, the longer the alarm time per individual, and the closer the threat distance (GLMER1, number of ind. heard: p= <0.001 (**Figure 9a**, **Table 3a**); GLMER2, number of ind. heard: p= <0.001 (**Figure 9b**, **Table 3b**)[3].

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[3] A similar figure, only showing different sex, is found in the appendix (**Figure A3**).
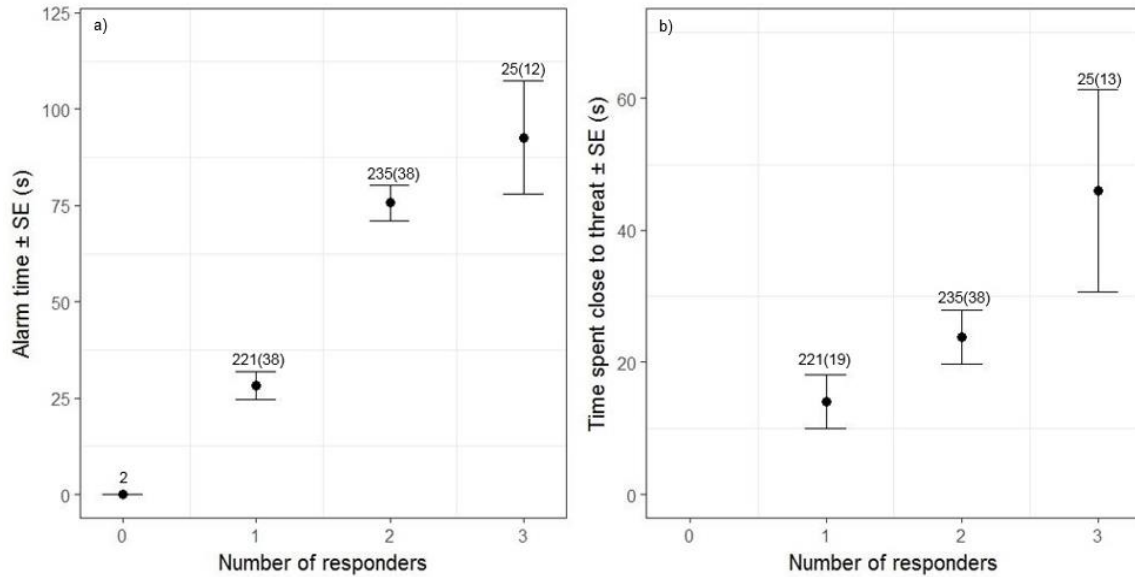
Figure 9*: a) Average alarm time (s) for all birds responding divided into number of responding birds (0-3). The more birds present, the longer birds alarm for. b) Average alarm time spent alarming close to the threat (<5m) in relation to number of birds responding. More birds present meant birds kept at a closer distance for longer than when birds were on their own. Sample size is listed as total number of observations first, then total number of individuals in parentheses.*

Other findings

Birds alarmed for longer later in the day than what was found during early morning observations (GLMER1, time of day: p= 0.019). Age was not included in the final model for alarm time, however older birds kept a farther distance from the threat than younger birds (GLMER2, threat distance: p= 0.042, **Table 3b**). The further away from the nest the threat was, the shorter the alarm time and the farther away from the threat birds kept (GLMER1, nest distance: p= <0.001; GLMER2, nest distance: p= <0.001, **Figure 10a & b**, **Table 3a & b**, respectively). In addition, alarm time was longer, and threat distance shorter when the threat was moving towards the nest than when it was moving away from the nest (GLMER1, direction: p= <0.001; **Figure 10a**, **Table 3a**; GLMER2, direction: p= <0.001; **Appendix, Figure A1**, **Table 3b**). There were no differences in average alarm time between males and females, either towards or away from the nest (**Appendix, Figure A2**).
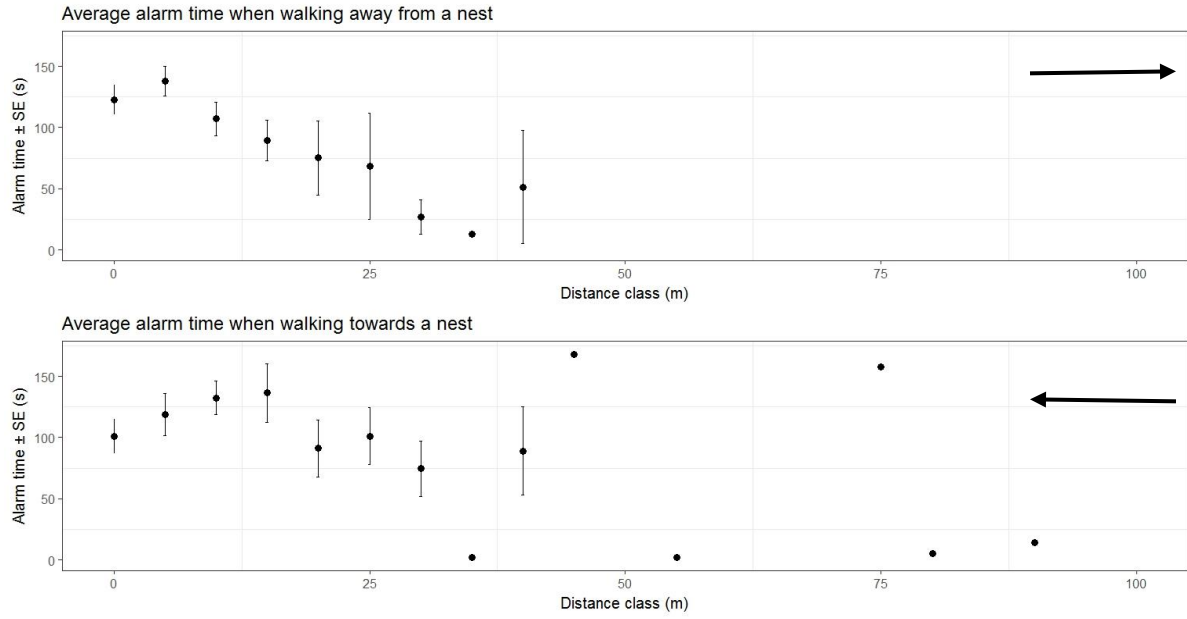
Figure 10: *a) Average alarm time (s) for all responding birds with the threat walking towards a nest, and b) walking away from a nest. Unidentified birds are included. Arrows indicate movement direction.*

4 Discussion

The basis for this MSc was an empirical study addressing some predictions derived from the paper "Extra-pair mating and evolution of cooperative neighbourhoods" by Eliassen & Jørgensen, 2014. We manipulated the perceived predation risk in blue tits (*C. caeruleus*) during the mating stage (before egg laying) to test whether this affected the EPP rates in nests. Later in the season, during chick rearing, we quantified antipredator behaviour (alarm calling) within neighbourhood units (pairs of nests) to explore how it related to EPP rates and whether it had a collective component. Although our findings were not what we first assumed would be the case (*e.g.* no sex differences), we were intrigued by what a single season of sampling could indicate in regards to social context of antipredator behaviour. Our results did not provide evidence that perceived predation risk before egg-laying affected EPP rates. We found little evidence for overall differences in alarm-calling behaviour between sexes, although males tended to alarm for shorter durations than females.

In addition, our results indicate that alarm calling behaviour was influenced by the social context, and more specifically that, regardless of sex, (i) the first bird that started to alarm did so for a longer duration and stayed closer to the threat, and (ii) more birds heard alarming resulted in longer individual alarm calls and a closer approach to the threat. We also found that although alarm calls may be heard by neighbours, they were mostly performed within the vicinity of the nest where individuals were breeding since, (iii) the further away the threat moved from the nest, the less effort was put into alarm calling and (iv) birds would react more strongly to a threat moving towards their nest than a threat moving away from the nest.

a) *Does manipulated early predator exposure affect EPP rates and subsequent antipredator behaviour?*

Delay in egg laying

Contrary to our expectations, we found no evidence that early predator exposure affected EPP rates. Rather unexpectedly, the only significant difference we found when comparing experimental groups was that the egg-laying date was on average three days later for predator-exposed nests than object-exposed. This could be an artefact due to small sample sizes, however we cannot exclude that this also might be an effect of predator exposure, since all nests were at a similar stage (after completion of the nest and before egg laying) during exposure (**Table 1**). Exposure to predation has been shown to affect the egg laying date in other bird species, like the pied flycatcher for which Morosinotto et al., (2010) manipulated the predator density of the pygmy owl (*Glaucidium passerinum)* (80% of diet is songbirds), and Tengmalm's owl (*Aegolius funereus)* (36 % or their diet consists of songbirds). They found that flycatchers strongly avoided pygmy owl territories whilst not obviously responding to Tengmalm's owl territories. Birds distinguished between two potential predators and were also found to show a delay of up to four days in the start of egg laying when nesting near pygmy owls, as well as laying smaller clutches (Morosinotto et al., 2009). As females are dependent on male food provisioning for egg production (Hakkarainen & Korpimäki, 1995), high predation risk could mean that birds are more careful and by so are not able to invest as much in courtship feeding (Korpimäki, 1981), which again will affect the possible investment females can put into their egg laying.

A negative correlation between egg laying date and clutch size, and subsequently brood size, is very common in wild passerines and we also observe it here. Unexposed neighbouring nests show a later egg laying date than experimental nests, which can be explained by the fact that these nests were not ready for egg laying during the first stage of our experiment (exposure) and hence were not matched to the two other groups. Predator-exposed nests were found

to fledge a lower number of young than object-exposed nests. This was associated with smaller clutch size, brood size and number of chicks fledged. These patterns are consistent with the biology of Mediterranean blue tits, where early breeding females produce larger broods (e.g. Marrot et al. 2015). In addition, a delay in egg laying can result in a mismatch between food demand for offspring and caterpillar availability, their preferred prey (Verhulst & Tinbergen, 1991) and hence compromise nestling survival. Blue tits, as many insectivorous birds, depend on a synchronized timing between maximal offspring energy requirements and peak availability of leaf-eating caterpillars (Blondel et al., 1993). Although the average delay was three days for our predator-exposed nests, some nests were delaying their egg laying with up to seven days. The age of highest energetic demands for chicks is 9-11 days post-hatching, and if chicks reach this age after the peak of caterpillar availability it can result in poorer body condition and lower prospects of survival (Blondel & Dias, 1999, 2001; Thomas et al., 2001).

The later egg-laying date and lower number of fledglings in predator-exposed nests could therefore be a combined effect of later breeding (*i.e.* smaller clutches) and lower food availability during the peak of food demand. Delaying egg laying with a few days after encountering a new threat to your nest could be beneficial. Predators that revisit nests may represent a high risk for clutches and one way of reducing this risk is move to a new nest site (Sonerud, 1985). Birds are faced with choosing between possible brood predation or the cost of re-nesting to avoid predation. Therefore, it might be beneficial to wait a few days and see if the same predator reappears at the nest before considering re-nesting. This is something worth looking into at a larger scale as it could show evidence of a trade-off between delay in egg laying when faced with a predator risk and provisioning enough food for the chicks later in the season.

Early experimental exposure and EPP rates

Exposure to either model predators or neutral objects did not affect the frequency of EPP, nor did these two groups together differ from unexposed nests (**Table 2**

& 3). Overall, 57% of all nests, both experimental and unexposed, contained EPP. This percentage is close to that found in older data from the same population (2000-2003, 46%, Mennerat et al. *in revision*, Charmantier & Blondel 2003). Of the 21 nests genotyped, five nests contained unknown fathers of EPY. As most of our nests had neighbours in several directions, the unknown extra-pair fathers could have been nearby, just not part of our sample (**Appendix, Figure A4**). In red-winged blackbirds, 95 % of all EPP were found to be from neighbouring males with adjoining territories (Gray, 1997). Kempenaers et al., (1997) found EPP in blue tits to usually be from close neighbours while Foerster et al., (2003) found that almost 30% of all EPY were due to drifter males passing through the area, which is also supported by earlier research (Leisler et al., 2000). In our study area, Charmantier & Perret (2004) found that the number of neighbours within a 100-meter radius significantly affected the proportion of EPY, and Mennerat et al. (in revision) found that over 90% of EP sires were either first-order or second order neighbours.

Regarding the lack of differences in EPP rates between experimental groups (predator and object exposed), one could argue that the exposure to taxidermy predators before egg laying might not have been "scary" enough. From our own field observations (A. Mennerat pers. obs.), birds seemed to react to taxidermy predators during exposure and in particular, a number of birds were seen simultaneously approaching the squirrel specimen and alarming after detecting it. It could however be that this early season disturbance did not have a long lasting impact on behaviour, or that the disturbance affected behaviour in a way that was not detected in the context of our study. When examining naturally occurring nest predation in the Japanese Great tit, Yuta and Koizumi (2016) found that the frequency of EPP was positively correlated with nest predation rate, with effects only showing in later breeding attempts. This is consistent with other studies (Conrad et al., 1998; Dietrich et al., 2004), although some studies did not find an increase in EPP in the second brood (Rowe & Weatherhead, 2007). However, blue tits rarely have two clutches in one season (Blondel et al. 2006), and so changes in EPP rates might not be detectable before the next breeding

season. For our study population the proportion of nests containing EPY increased to 82% the year after females had experienced predation-related brood failure, as compared to 46% in previously successful females (Mennerat et al. in revision), supporting our idea that we might not see an effect of our predator-exposure treatment, as measured in EPY rates increasing, before the following year.

b) *Is antipredator behaviour related to EPP?*

We found evidence that antipredator behaviour varied according to the presence of EPY in nests. In broods with EPY, adults spent less time alarming than in broods without EPY (**Figure 6a**). Interestingly, the more EPY in the nest, the less time is spent alarming close to the threat, and even though the interaction with sex was not significant, **Figure 6b** is showing that this may apply to males more than females. As presence of EPY in a brood means lower parent-offspring relatedness for the male but not for the female, one could expect that males, but not females, would decrease their investment in nest defence (Lubjuhn et al., 1993; Dixon et al., 1994; Weatherhead, 1989, 1994; Chuang-Dobbs et al., 2001). In addition, males that have offspring in other nests will pay a relatively lower fitness cost than females when their social nest is predated (Trivers, 1972; Rytkönen et al., 1993). The results from our study seem partially consistent with these predictions.

c) *Is there a difference in antipredator behaviour between males and females?*

We found a trend of males alarming for a shorter time than females, but overall no striking sex difference was found in antipredator behaviour of parents. However, it could also be that this population does not exhibit any specific behavioural sex-differences, as no sex-differences was found for a blue tit population in southern Germany by Mutzel et al., (2012).

d) Is antipredator behaviour affected by the social context?

Response to increasing or decreasing danger

Knight & Temple (1986b) pointed out that studies relying solely on alarm calls might not perceive the whole picture as they ignore more energetically demanding and dangerous antipredator behaviours such as diving or approaching the threat closely. Therefore, for our study, both alarm calls and distance to the threat were measured as antipredator defence behaviours. In addition, by measuring the birds' response at various distances from the nest we could also explore how they react to either an increasing or decreasing danger, as well as the spatial range of their response within the neighbourhood (*i.e.* along the walking line between two neighbouring nests).

There is very little previous research to help us interpret the pattern we found here. Most previous research on approaching danger has been with females on nests looking at flushing distances (Burhans et al., 2001; Valcarcel & Fernándes-Juricic, 2009), with species that do not use nest boxes (blackbirds: Kryštofková et al., 2011), or have focused on distraction behaviour (Barash, 1975; Brunton, 1986). Therefore, the following hypotheses are based on logical reasoning and own observations from the field.

There is a distinct difference between the risk posed by a threat suddenly appearing underneath your nest box, moving away from your nest and eventually posing a threat to your neighbour's nest, compared to that of a threat gradually approaching in straight line from a neighbouring nest (**Figure 4**). With a predator appearing directly beneath the nest box, offspring are in immediate danger and so parents face a sudden high risk of brood predation as well as risking injury or death themselves. Parents should therefore defend the nest at high intensity. If, when parents alarm intensely, the predator moves away from the nest (as we did), it gives the impression that alarming has a positive effect, and so alarming should progressively decrease as the danger moves away. This is what seems reflected in **Figure 10a**: average alarm time decreased gradually as the observers were walking away from the nest.

For birds experiencing an approaching predator however, the threat will increase with each observation. Birds will alarm increasingly as the threat is closing up, but with no apparent effect: the threat keeps approaching in straight line. At some point, an increase in alarm time might become too risky as it can convey information to the predator about how close it is getting to its prey (the offspring). Therefore, a more discrete strategy with less alarming could be beneficial. Again, this seems to fit what we observed (**Figure 10b**).

Early warning calls, as was observed with great variance when moving towards the nest (**Figure 10b**), can work as a heads-up call for increased vigilance within the pair. It can also indirectly benefit neighbouring nests, as well as working as signalling to the predator that it has been spotted (Krause & Ruxton, 2002). A vocal warning system is omnidirectional and does not differentiate between species, and so alarming at own nest will also widely broadcast the presence of a threat (Patricelli et al., 2007). We had 11 cases with three individuals responding to the threat where the third individual was identified as a great tit (*Parus major*) (n=4), Eurasian nuthatch (*Sitta europaea*) (n=3) and blue tit (*C. caeruleus*) (n=2), while two alarmers were not identified to species. Blue tits nest in heterospecific neighbourhoods with overlapping territories from other species and so all birds inhabiting an area benefit from neighbouring alarms as an early warning call of possible danger (Lima, 2009).

The further away the threat (*i.e.* observers) was from the nest box, the further away birds were to the threat (**Appendix, Figure A1**). This can be interpreted as parents alarming at safe distance from the threat while assessing the situation, as long as this threat is not too close to their offspring. It is not worth the risk of being caught, injured or killed by defending your offspring in a low-risk situation, and parents keep relatively safe until the predator is within striking distance (Montgomerie & Weatherhead, 1988). We observed that whilst birds would decrease their alarm time, they would still be present higher up in the canopy and quietly observe us.

Response order and number of birds present around the nest

The first bird to alarm at the threat was also the bird that alarmed for longest and approached the threat the closest (**Figure 8a & b**). Rytkönen et al. (1993), hypothesized that a simple explanation to the difference in alarm time for first and second responder could simply be that the parent who invests the most in offsprings stays closest to the nest, and consequently arrives first to defend it. It is argued that, regardless of sex, it will be advantageous for the second bird arriving to stay at a further distance and adjust its defence intensity (*i.e.* alarming) according to the first responder (Weatherhead, 1989). Research has also looked into the personality traits of birds, hypothesizing that differences in antipredator behaviour can be linked to differences in personality (Quinn & Cresswell, 2005). If this is the case, bolder birds might engage in higher levels of nest defence, and so decrease the cost of its social mates' by sharing the risk, regardless of sex (Rytkönen et al., 1993). The distance birds keep to the threat also reflects risk taking. According to parental investment theory (Trivers 1972), the parent who has the most to lose would be the female and should therefore be the parent who takes the highest risks regarding nest defence. However, time spent close to the threat was not found to be significantly different between males and females (**Appendix, Figure A1**). Both parents only approached the threat at close distance (<5m) when the threat was within 10 meters of their nest box (**Appendix, Figure A1**).

There was also an increase in both alarm time and time spent close to the threat (*i.e.* threat distance) when more birds responded to the threat (**Figure 9a & b**). Many studies show that the risk of each individual being injured or killed is greatly reduced in larger groups of prey (Pulliam, 1973; Bertram, 1978; Godin et al., 1988; Cresswell, 1994; Curio, 1978; Bednekoff & Lima, 1998; Krause & Ruxton, 2002; Caro, 2005; Eggers et al., 2008). This type of dilution effect was coined by Hamilton (1971), and explains how cooperation may be beneficial for selfish individuals. More individuals present means lower predation risk as well as increased probability of escaping an attack (Lazarus, 1979; Robert, 1996),

and so more effort can be put into alarm time or approaching the threat (see Lima, 2009). We found that alarm time almost tripled from when two birds were present and alarming compared to that of one bird alarming alone (**Figure 9a**), which could indicate a safety in numbers effect on parents.

My interpretation is that it could be a beneficial strategy for both parents to alter their behaviour according to what the other parent is doing. If, for example, males were to take fewer risks in the form of keeping a further distance, females might benefit from copying their behaviour (Rytkönen et al., 1993), because exposing themselves without their partner would increase the risk on themselves. Equally, if one parent is present at the nest alarming alone, it can be beneficial to alarm less intensely in order to avoid too much attention to oneself and risk being injured or killed. With two parents alarming, there is double the defence but half the risk, although the cost of losing one parent would be detrimental. It might therefore be that we do not see a distinct difference between the sexes if the pair adjusts their antipredator behaviour in response to that of their partner.

Challenges with the experiment and observations in the field

The study area in La Rouvière is a popular hiking area for families, dog walkers and joggers, with birds inhabiting this area experiencing a year-round disturbance. From April, there is performed a weekly nest box-check by the research team of CEFE, which increases to several visits a week during the peak of chick rearing in May (Charmantier & Blondel, 2003). This nonlethal disturbance stimuli by researchers who visit the nest without harming the chicks, gives parents a chance to gradually learn that researchers are not dangerous to them. Fernández-Juricic & Tellería (2000) looked at the effects of human disturbance in urban parks and found that smaller bird species (like the house sparrow, *Passer domesticus*) were more tolerant of human intrusions and had higher tolerance to disturbance than larger species (such as the Eurasian magpie, *Pica pica*). Repeated exposures of researchers may therefore reinforce

parental alarming response as their nest defence always results in departure of the threat without any harm to nestlings or themselves. This effect was coined "positive reinforcement hypothesis" by Knight & Temple (1986 a, b), although research by Rytkönen et al., (1990) found no evidence of a positive reinforcement hypothesis.

For our observations, a distress call from a starling was played before every observation to make sure parents would link approaching researchers (the threat) as a danger, but we cannot be certain that the response measured is what we could expect from an actual predator threat. Studies have previously showed clear evidence of predator discrimination (blackbirds: Krystofkovà et al., 2011, blue tits: Mahr et al., 2014), but these only compare different predator species, not predator versus human threats. As soon as the distress call was played, chicks would stop begging and become silent, indicating it was perceived as a threat to them (pers. Obs. from field, also see Leech & Leonard, 1997). Although we did observe parents alarming towards us throughout most observations, it is however not possible to determine if birds perceived two observers as posing the same level of risk to their nest as a real predator. Interestingly, age played a role in the distance birds kept from the threat (**Table 3b**). In passerine birds, behaviour linked with age are mostly due to age related quality or experience (Weatherhead, 1989). Older birds might keep a distance due to being caught and ringed in a previous year and so are able to distinguish between a real danger to the nest and an intrusive, but not deadly, threat from a researcher.

When female blue tits sound like singing males

*A*s identification of sex became increasingly challenging with distance, most observations after 50 meters are unsexed birds, although those birds whose sex was visually identified tended to be males (**Figure 10**). Some of the identifications performed at distance class >50m were based partially on visual identification but also on vocal identification in the form of song, and for distances above 70 m some birds were assumed to be males by their song, as males are the only sex known to sing during breeding (Mahr et al., 2016). However, as we accumulated

observations, we realized that a number of females were mimicking male song in response to our distress call playback. Mahr et al.,'s paper from later in the same year (2016), reported observations of female blue tits expressing singing behaviour as a response to being presented with a threat, confirming our own observations in the field.

Female song seemed to us to be more frequent when she had to protect the nest on her own, with no other birds present, but this was not recorded systematically. Hinde (1952) suggested that females might gain benefits by calling their mate if there is a particularly threatening situation, and that song could be used to stimulate pair cohesion, defending against a predator as a unit. As we also observed male song during our observations, and so calling for your partner in a threatening situation might be more efficient than just alarming. This behaviour was observed during the nestling stage, when birds already had chicks to take care of and would therefore unlikely serve a purpose as mating call. Thus, singing in response to a predator threat might have a different function than songs in the copulation period, which are primarily used for mate attraction. As the female song very much resembles that of the male (Mahr et al., 2016), it might confuse the predator by creating an illusion of more birds protecting the nest than what is the case, but this is currently only been tested by Mahr et al., (2016). We therefore suggest that mimicking male song may be adaptive for females, in particular those defending their nest alone. Song has been observed to play a role in antipredator behaviour of other passerine species, like the fairy-wren (*Malurus splendens)* and might be more common than first assumed (Greig & Pruett-Jones, 2008; Colombelli-Négrel et al., 2011).

What about the nicer neighbourhood hypothesis?

Regarding the nicer neighbourhood hypothesis, an overlap of alarms between neighbouring nests, males alarming for longer distances or observations of more than two blue tits alarming could be indications supporting the hypothesis. We did not find any direct evidence of such cooperative behaviour amongst our

neighbouring nests, except for a few occasions with three alarming individuals, mostly performed by other bird species in the area (**Figure 9a & b**). Alarm calls from different bird species towards a common threat is interesting when considering the nicer neighbourhood hypothesis, as one would expect a cooperative defence to be within the species not between different species.

We did not measure EPP rates within the whole neighbourhood as this project was conducted within a study site with other projects running simultaneously. EPP data are therefore lacking from most neighbours of our experimental nests (**Appendix, Figure A4**), meaning we do not see the full extent of EPP rates for males in our population. To be able to do, we would ideally want DNA samples from all neighbouring nests, as Eliassen & Jørgensen's theory argues for a neighbourhood cooperation based on EPC between closest neighbours. Research performed in the same area as our experiment found that EPP did not necessarily come from the closest neighbours and argue for an active female choice (Charmantier & Perret, 2004). However, Mennerat et al (in revision) finds contradicting EPP data for the same area suggesting that it is indeed the closest neighbouring males who father the majority of EPY. Other previous studies also show evidence of EPP belonging to neighbouring males (Gray, 1997; Kempenaers et al., 1997). In other words, current data are contradicting or might support multiple interpretations for EPP range. This indicates that it might not be a straightforward answer and that variation could be found both within and between species.

When walking in a line from one nest to the next (*i.e.* within a simplified neighbourhood), alarm calls were rarely recorded all along, and around mid-distance there was often an absence of response (see also **Figure 10a & b**). Mostly alarm calls were brief and feeding resumed once the potential threat was >40 m away (Pers. Obs. from field + **Figure 10**). For 2016, the average distance between occupied nest boxes was approximately 86 meters, giving a halfway-point of 43 meters, with few alarms heard after a 40-meter distance (**Figure 10a**). Alarms after 40 meters mostly consisted of one or two "churrs" (short single alarms), often only lasting for 2-3 seconds. If, following the nicer neighbourhood

hypothesis, relaxed territory defence allowed male territories to overlap more, a greater area within both neighbouring territories should be defended by both males. Males with EPP in a neighbouring nest would be expected to chase the threat even after it is no longer a danger to their social nest, as it could pose a threat to EPY in neighbouring nests. This was not found in our study where both parents resumed feeding behaviours as soon as the threat was far enough away from their own nest to not be perceived a as a threat anymore.

Conclusion

Our study found no solid evidence of cooperative defence between neighbouring EPP nests. Considering the modest sample size, our results should however be considered with caution. A larger sample size, combined with extensive DNA sampling of all neighbouring nests could help highlight the extent of EPP rates within this blue tit population. It could be that some neighbouring nests cooperate more than others (due to higher rates of EPP). Therefore, evidence of neighbourhood cooperation could be present at a smaller scale (*i.e.* between two specific nests, not overall between all nests), and that this cooperation is not clear when comparing several nests with large variation between nest distances. Regarding our experimental predator exposure before egg laying, the small dataset might be the reason why we did not find any significant results, as the effect size (coefficient estimate) was high, suggesting that it might still be important. Adding more years of data to the model might produce a different result.

In any case, this study highlight an area of antipredator behaviour that has received little attention in previous research, namely the relation between EPP rates and antipredator behaviour. It also shows that these complex behavioural strategies and the potential trade-offs between them are yet to be fully understood.

I would suggest this project to be re-ran over several consecutive years, preferably on a blue tit population that are not also part of several other studies, as to see if there is a difference in antipredator behaviour in a blue tit population which have not been exposed to continuous human disturbance. As with most research, a larger sample size and repeated experiments can help determine the impact early predator exposure might have on EPP rates and parental antipredator behaviour. I am also curious as to how much distance between nests affect decision-making, and if there is a threshold for distance between nests or number of surrounding neighbours affecting the EPP rates in a population. Ideally, we would want to test the nicer neighbourhood hypothesis on a blue tit population where all nest boxes are placed with similar distance to all surrounding neighbours. By doing so we would remove the uncertainty of whether or not long-distance neighbours are too far apart for neighbourhood defence to be advantageous, as well as getting a clearer idea of the full extent of EPP in a population. It would also be interesting to test parental antipredator behaviour early in the chick rearing season compared to later on, as sex differences might be clearer in the early stages of chick rearing when re-mating or re-nesting is of a lower costs than later in the season. As we did find predator-exposed nests to show a delay in egg-laying date, it would be interesting to look into the peak of food availability to see if a delay of 3 days on average could make a significant difference on the food availability of delayed chicks. If this were the case, why would such a delay be favoured? It would also be very interesting to focus more directly on the interaction between parent birds when faced with a predator: what is the best defence option if your nest is under attack while your mate is away? The mimicking of male song performed by females was fist discussed by Mahr et al (2016) last year and is an intriguing finding of how antipredator defence might work in ways yet to be investigated. Further studies could therefore focus more directly on how singing behaviour can work as a tool for antipredator behaviour in blue tits.

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Appendix:
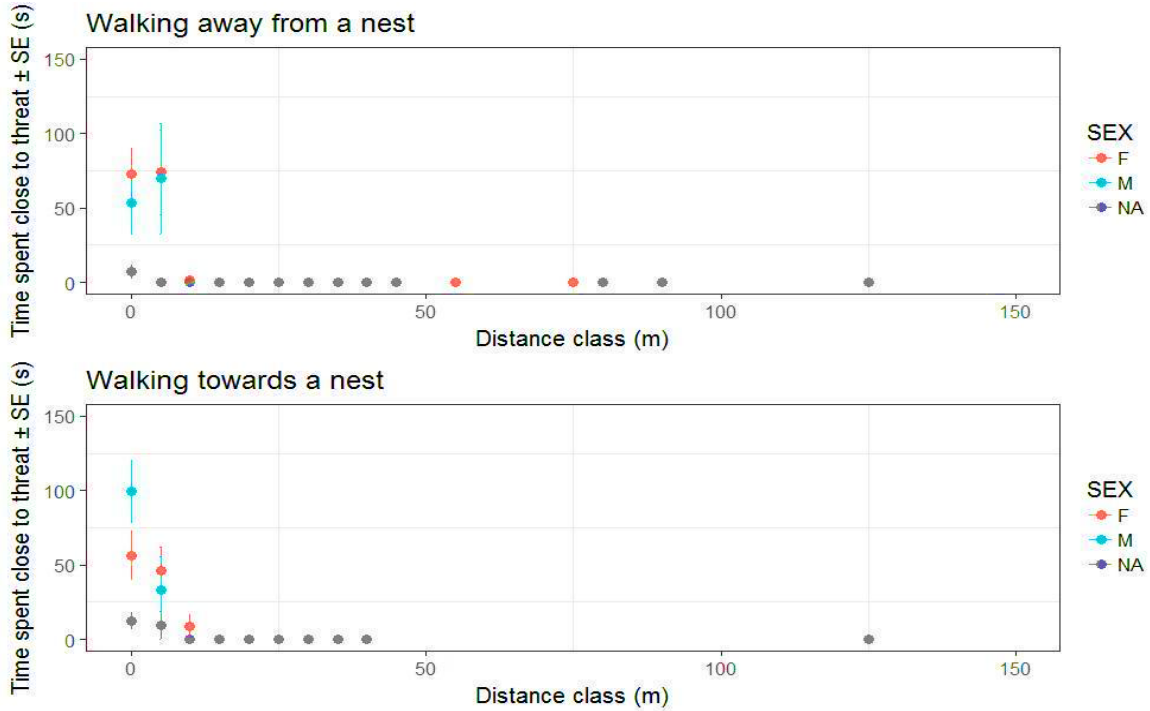
Figure A1: *Time spent close to the threat (i.e. the observers) as the threat is moving away or towards the nest. Here separated into sex. NA= represents birds observed but for which sex could not be assigned.*
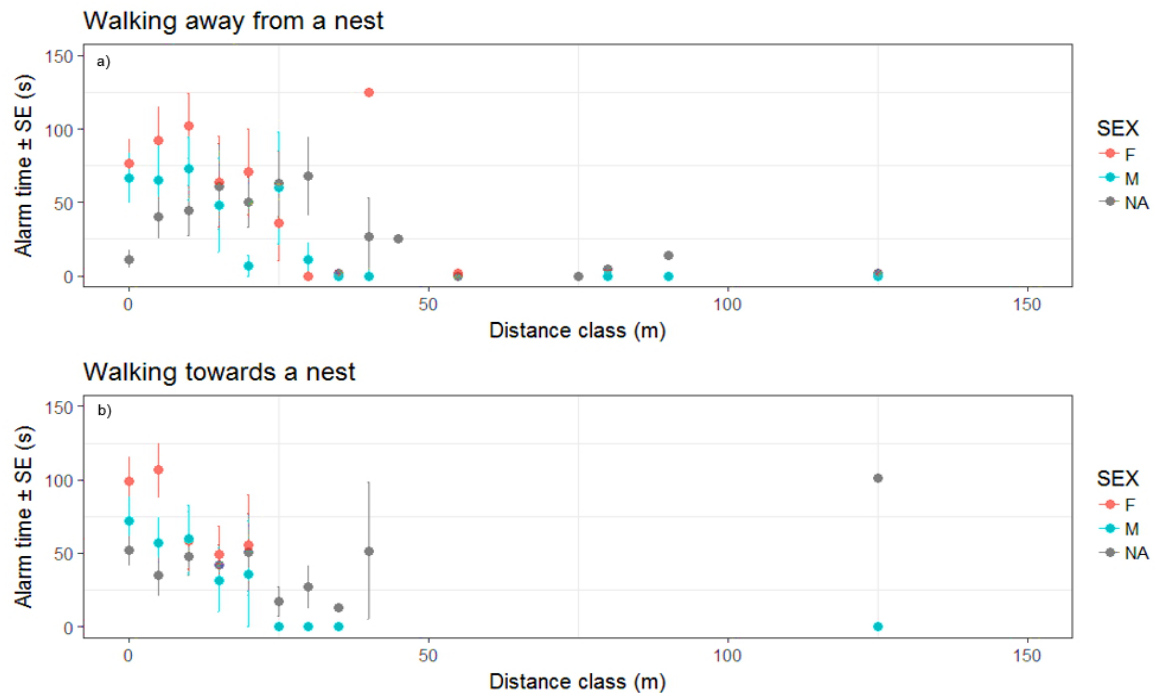
Figure A2: *Average alarm time for all birds observed during all observations, both a) away and b) towards a nest. Sex of responder is indicated by colour. There were no statistical differences in alarm time between males and females, either towards and away from a nest. Na= represents birds observed but for which sex could not be determined.*
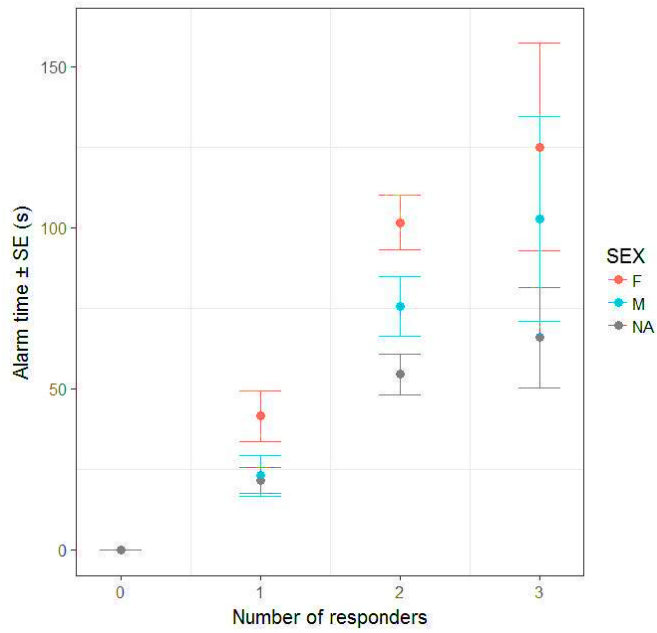
Figure A3: *Total alarm time with different number of responders (0= no alarm- 3= three individuals present and alarming), here separated by sex. NA= represents birds observed but for which sex could not be assigned.*

Figure A4: *Overview of the nest box area in La Rouvière. All nest boxes used in this thesis are circled with colour codes representing the experimental group they were part of.*