

LETTER

Indirect predator effects on clutch size and the cost of egg production

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Abstract

Predator-induced changes in physiology and behaviour may negatively affect a prey's birth rate. Evidence of such indirect predator effects on prey demography remains scarce in birds and mammals despite invertebrate and aquatic studies that suggest ignoring such effects risks profoundly underestimating the total impact of predators. We report the first experimental demonstration of indirect predator effects on the annual 'birth' rate resulting from negative effects on the size of subsequent clutches laid by birds. We manipulated the probability of nest predation and measured the size of subsequent clutches and multiple indices of the mother's physiological condition, while controlling for food availability, date and stage of breeding. Females subject to frequent experimental nest predation laid smaller subsequent clutches and were in poorer physiological condition, particularly regarding non-resource-based indices (e.g. oxidative stress and glucocorticoid mobilization) consistent with both a response to the threat of predation and an increased cost of egg production.

Keywords

Clutch size, cost of reproduction, egg production, nest predation, non-consumptive effects, oxidative stress, predator–prey ecology.

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INTRODUCTION

Predator effects on prey demography have traditionally been ascribed solely to direct killing (Preisser *et al.* 2005). Recent research indicates that predators may also indirectly affect prey demography by other means than direct killing (Creel *et al.* 2007). Predator-induced changes in prey physiology and behaviour may negatively affect the birth rate and increase deaths from other causes (Creel & Christianson 2008). Negative effects on the birth rate provide clearer evidence of indirect predator effects because diagnosing the cause of death of wild animals is often problematic, though it must be established that effects on the birth rate are not attributable to resource limitation (Creel *et al.* 2007; Creel & Christianson 2008). Few experiments have yet tested for indirect predator effects in birds and mammals despite numerous experiments on invertebrate and aquatic species having demonstrated that indirect predator effects may be as, or more, important to prey demography than direct killing (Preisser *et al.* 2005, 2009; Creel & Christianson

2008). Only two experiments, for example, have tested for indirect predator effects on the annual 'birth' rate in birds (total number of eggs laid; Martin 1995; Zanette *et al.* 2006b) and these found opposing results (reviewed in Lima 2009). Eggers *et al.* (2006) reported that recorded calls of nest predators caused Siberian jays to lay almost one less egg per clutch, whereas Fontaine & Martin (2006) found no effect of nest predator removal on the size of the first clutch of the season in any of eight songbird species.

Eggers *et al.* (2006) observed that the birds they studied do not normally re-lay after nest failure, whereas most birds, including most of those Fontaine & Martin (2006) monitored, can expect to lose one or more nests, and consequently lay more than one clutch each season, given typical levels of nest predation (Lima 2009). Modelling suggests subsequent clutches rather than the first of the season may be more likely to show indirect predator effects (Slagsvold 1982; Farnsworth & Simons 2001; Lima 2009). Various authors have argued that in species that lay more than one clutch, a higher probability of nest predation may

not only increase the number of clutches, but also decrease the average clutch size either or both because birds strategically reduce the size of subsequent clutches as a proactive response to avoid future predation based on prior direct experience of nest predation or there are physiological constraints on re-laying (Slagsvold 1982; Martin 1995; Farnsworth & Simons 2001; Lima 2009). Consistent with this, Martin (1995) reported that, across songbird species, not only was the probability of nest predation positively correlated with clutch number, clutch number was negatively correlated with clutch size, and Zanette *et al.* (2006b) found the same within adjacent (<20 km) song sparrow (*Melospiza melodia*) populations. Zanette *et al.* (2006b) further reported that clutch number and size were negatively correlated even in experimentally food supplemented populations, in accord with Williams (2005) proposal that physiological constraints on egg production may stem from non-resource-based mechanisms (e.g. oxidative stress) rather than an “energy trade-off” (Martin 1995). Whether the mechanism involves changes in physiology (e.g. non-resource or resource-based constraints on egg production) or behaviour, or both, a lower average clutch size resulting from a reduction in the size of subsequent clutches constitutes a negative effect on the birth rate, and thus an indirect predator effect on prey demography. That such a reduction in the size of subsequent clutches occurs remains untested experimentally (Lima 2009).

Here, we experimentally test whether wild female song sparrows lay smaller subsequent clutches when subject to a higher probability of nest predation. All subjects were provided with *ad libitum* supplemental food to ensure effects were not attributable to resource limitation, and all were captured before and after the treatment period to assess their physiological condition using a multivariate approach employing 15 physiological indices. We report a significant reduction in the size of subsequent clutches laid by females subject to frequent nest predation, of a magnitude (almost one less egg) comparable to the reduction in the size of first clutches reported by Eggers *et al.* (2006). The physiology of females subject to frequent nest predation differed significantly from those not subject to nest predation in accord with the former being in poorer physiological condition notably with respect to non-resource-based indices, consistent with both a response to the threat of predation and an increased cost of egg production. We conclude that our results, together with Eggers *et al.*'s (2006) concerning first clutches, provide clear experimental evidence of indirect predator effects on the annual ‘birth’ rate in birds. We suggest this reinforces Creel & Christianson’s (2008) concern that the total effect of predators on prey populations may be routinely underestimated, particularly in conservation and management plans for birds and mammals, because indirect predator effects on prey

demography have yet to be fully incorporated into general ecological theory.

MATERIAL AND METHODS

Study area and species

We studied wild song sparrows resident on several small (<200 ha) Gulf Islands, in BC, Canada, from March to July 2007, as part of a long-term project. Details regarding sites and sparrows can be found elsewhere (Zanette *et al.* 2006a,b). Briefly, egg-laying occurs from April to July, the maximum clutch size is 5 eggs, incubation normally lasts 13 days, females may lay 2–8 clutches depending primarily on the probability of nest predation, and provided they suffer no nest predation females may fledge up to three broods. Based on continuous video recordings of 225 nests, predation is the sole cause of the disappearance of a nest’s contents. In 38% of 115 videoed nest predation events, the parents were absent and the disappearance of the nest’s contents presumably provided the principal cue that the nest had been preyed upon.

Field procedures

Nests were located using behavioural cues from the mother (Zanette *et al.* 2006b). A clutch was considered complete if no new eggs were laid for > 24 h. Eggs were weighed to 0.05 g. Inter-nest interval refers to the number of days between the abandonment (see below) of a given nest and the start of incubation of the subsequent nest (Zanette *et al.* 2006b). We are confident we found every nest begun by every experimental subject because every subject was individually colour-banded and there were no inter-nest intervals long enough to suggest we missed a nest.

Experimental design

We experimentally manipulated the probability of nest predation and measured the effects on, the size of subsequent clutches laid by, and physiological condition of, females subjected to either, frequent artificial nest predation (FNP), or no nest predation (NNP). We experimentally controlled for between-treatment differences in the stages of breeding experienced (incubation vs. brood-rearing), developmental period (day of incubation), initiation date (date first egg of the season was laid), duration of the experimental period, food availability and natural nest predation; and we statistically verified that there were no between-treatment differences in the size of the first clutch, initial physiological condition or the dates when condition was assessed. In addition, to control for individual variation we employed a repeated-measures approach of testing for effects on the

before-treatment vs. after-treatment change in each subject's physiological condition (Williams 2005).

In both experimental treatments, we removed all of the eggs from each female's first and subsequent nests on about the sixth day of incubation (Fig. 1). In the FNP treatment ($n = 14$ females), every nest was left empty to simulate natural nest predation, causing the female to initiate a subsequent breeding attempt. In the NNP treatment ($n = 11$ females), incubation of an unviable clutch was simulated by substituting artificial eggs for the female's own eggs which she then incubated for a further 11.5 ± 0.8 (mean \pm SE) days, after which she abandoned and initiated a subsequent breeding attempt. This ensured there was no between-treatment difference in the stages of breeding experienced since both treatments experienced nest-building, egg-laying and incubation, while neither experienced brood-rearing. Incubating an unviable clutch is a natural occurrence (affecting 4% of nests) and females have done so for up to 24 days.

Figure 1 illustrates the timeline of the experiment. Before breeding began, we captured, individually colour-banded, and assessed the physiological condition of every subject, from 6 to 28 March. First clutches were laid between 4 April and 3 May. We paired females with similar initiation dates and randomly-assigned one to each treatment, thereby ensuring there was no between-treatment difference in initiation date ($t_{23} = 0.23$, $P = 0.83$). For each pair, we then used the NNP female's breeding phenology

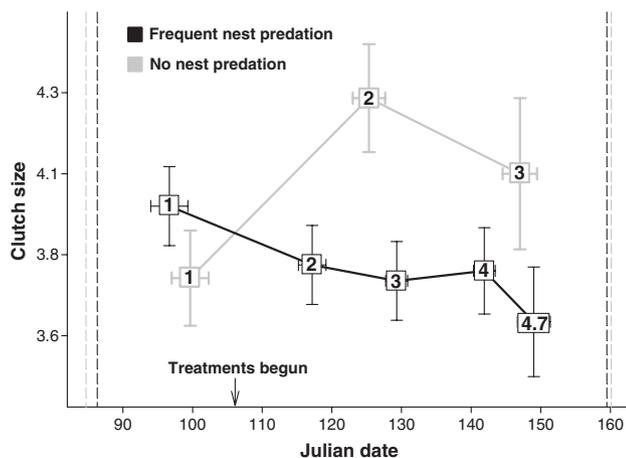


Figure 1 Change in clutch size with date compared between females subject to frequent experimental nest predation (black) and those not subject to nest predation (grey). Numerals signify clutch number. The symbol labelled 4.7 identifies the mean: number, size and lay date, of the clutch (4th or 5th) females in the frequent nest predation treatment were incubating when their after-treatment condition was assessed. Vertical dashed lines indicate the mean date physiological condition was assessed in each treatment, before and after the experimental period. Values are mean \pm SE.

to determine the duration of the experimental period, capturing and assessing her condition on the 8–10th day of incubation of her third clutch, and on as close to the same date as possible, capturing and assessing the FNP female's condition on the 8–10th day of incubation of whichever clutch she was incubating (4th or 5th), thereby ensuring there was no between-treatment difference in the date on which each female's final condition was assessed ($t_{18} = 0.03$, $P = 0.97$). Statistical analyses (details below) verified that there were no between-treatment differences in: the size of the first clutch ($t_{16} = 0.83$, $P = 0.42$), its total mass ($t_8 = 0.63$, $P = 0.55$) or average egg mass ($t_8 = 0.25$, $P = 0.80$); pre-breeding physiological condition (DFA eigen-value = 2.82, Wilk's $\lambda = 0.26$, pDFA $P = 0.40$); or the date pre-breeding condition was assessed ($t_{18} = 0.33$, $P = 0.75$).

Zanette *et al.* (2006b) and Clinchy *et al.* (2004) reported that food supplementation significantly increased egg production and improved adult physiological condition in these populations. To ensure effects in this experiment were not attributable to resource limitation, we provided supplemental food *ad libitum* from 31 March on, to all subjects, from feeders located at the centres of their territories. Carotenoids and egg proteins have been shown to be resources that may limit egg production (Ramsay & Houston 1997; Williams 2005). Consequently, we included 2.6 g of Oro Glo® (containing 0.04 g xanthophyll carotenoids; Kemin Industries, Des Moines, IA, USA) and 60 g of whole egg powder (equivalent to 240 g of wet egg; Travers 2009) in every kilo of feed, otherwise composed of the same high-protein, -fat, -carbohydrate and -calcium mixture (equal parts white proso millet and Purina Mills Aquamax Grower 400, Purina Mills, St Louis, MO, USA; plus 20 g of crushed oyster shell) used by Zanette *et al.* (2006b) and Clinchy *et al.* (2004). Plasma carotenoid levels were significantly higher ($t_{41} = 2.93$, $P < 0.01$) in our experimental subjects as compared with a group of non-food-supplemented sparrows sampled at the same time soon after the end of the treatment period, confirming that the food was consumed in significant quantities.

We controlled for natural nest predation by selecting to work on islands with low levels of nest predation (Zanette *et al.* 2006a) and conducting live-trapping to remove the two most important nest predators (raccoons, *Procyon lotor*; and brown-headed cowbirds, *Molothrus ater*; M. Clinchy and L. Zanette, unpublished data). Females in poorer condition may both suffer increased nest predation and lay smaller clutches (Zanette *et al.* 2006a,b). Our objective in randomly-assigning females to treatment and preventing natural nest predation was to ensure that it was our experimental treatments that affected clutch size and condition, rather than condition that affected nest predation and clutch size.

Assessment of physiological condition

Table 1 lists the 15 indices of physiological condition we evaluated, all of which various authors have suggested may be associated with either or both the threat of predation or egg production. Boonstra *et al.* (1998) argued that changes may be expected in multiple physiological measures in response to a higher probability of predation and Clinchy *et al.* (2004) demonstrated that this was the case in the sparrow populations we studied. Williams (2005) and Harshman & Zera (2007) similarly argued for a multivariate approach to evaluating the costs of reproduction since the underlying mechanisms most likely involve pleiotropic physiological effects. Consistent with the effects of either or both the threat of predation or egg production being pleiotropic and hence complex, contrary examples and rationales exist regarding the expected direction of change in virtually every index, considered singly. Consequently, we used multivariate statistics (details below) to identify, first, whether the physiology of the females in the two treatments differed independent of any assumptions about how they 'should', and second, whether the differences were largely consistent with expectations from the literature.

Costantini (2008) recently argued that ecological studies should measure oxidative stress, in addition to glucocortic-

oids, to better evaluate environmental stressors. Oxidative stress results from the imbalance between damaging reactive oxygen species produced as a by-product of aerobic metabolism and endogenous and exogenous antioxidants that neutralize their toxic effect (Alonso-Alvarez *et al.* 2004b; Monaghan *et al.* 2009). Although some studies have addressed the effect of predators on oxidative stress (Slos & Stoks 2008; Semeniuk *et al.* 2009) most have focused on reproduction (Alonso-Alvarez *et al.* 2004b; Costantini 2008; Monaghan *et al.* 2009). Alonso-Alvarez *et al.* (2006), for example, showed that birds that laid more clutches had lower antioxidant capacity. Carotenoids serve as antioxidants, although a recent meta-analysis suggests their role may be minor (Costantini & Møller 2008), whereas they are clearly utilized in egg production (Williams 2005). Clinchy *et al.* (2004) reported elevated corticosterone (CORT) in response to increased threat of predation in the sparrow populations we studied, and others have found comparable results (Bonier *et al.* 2009). Egg production may also elevate CORT (Williams *et al.* 2008). Corticosteroid binding globulin (CBG) regulates the function of CORT (Breuner *et al.* 2006). Aggression has been shown to elevate CBG in sparrows (Charlier *et al.* 2009) whereas handling stress has been shown to reduce CBG (Breuner *et al.* 2006). As anti-predator behaviour may involve both aggression and stress

Table 1 Physiological indices assessed, the mean for each index in the after-treatment minus before-treatment per cent change per female, the between treatment difference in this change, standardized effect sizes of the DFA factor scores, and the expected change in FNP relative to NNP females consistent with physiological dysregulation

| Scale | Index | After minus before treatment % change | | Between treatment % difference (FNP – NNP) | Standardized effect size of DFA factor score | Expected change in FNP vs. NNP if physiological dysregulation* |
|--------------------|----------------------|---|-------|---|---|---|
| | | FNP | NNP | | | |
| Oxidative stress | Oxidative status | 23.1 | -3.3 | 26.5 | 0.34 | Higher |
| | Antioxidant capacity | 1.4 | 61.2 | -59.8 | -0.63 | Lower |
| | Carotenoids | 22.9 | 73.8 | -50.9 | -0.52 | Lower |
| Glucocorticoids | CORT | 38.7 | 12.3 | 26.4 | 0.46 | Higher |
| | CBG | 19.1 | 5.6 | 13.5 | 0.37 | Higher/lower† |
| Immune function | Immunoglobulin | -0.2 | 17.9 | -18.0 | -0.37 | Lower |
| Haematology | Polychromasia | 45.6 | -2.9 | 48.4 | 0.63 | Higher |
| | Haematocrit | 7.8 | 6.9 | 0.9 | 0.09 | Lower |
| | Haemoglobin | 7.2 | 8.4 | -1.2 | -0.12 | Lower |
| Plasma metabolites | Free fatty acids | -14.7 | -16.4 | 1.6 | 0.09 | Higher |
| | Triglycerides | -14.6 | -20.8 | 6.2 | 0.18 | Lower |
| | Glucose | -0.5 | -8.5 | 7.9 | 0.19 | Higher/lower† |
| | Uric acid | 146.9 | 148.2 | -1.3 | -0.11 | Lower/higher† |
| Visible condition | Mass | 2.2 | -0.4 | 2.6 | 0.18 | Lower |
| | Fat score | 139.4 | 297.2 | -157.8 | -0.89 | Lower |

Numbers in bold indicate meaningful (Cohen 1992) effect sizes.

*Also indicates the quartile (highest or lowest) used to gauge physiological dysregulation.

†Indices with the least clear-cut expectations. In testing for physiological dysregulation we first used the left-hand expectations and then re-tested for physiological dysregulation using the right-hand expectations.

it is unclear whether the threat of predation may be expected to increase or decrease CBG. Egg production may also elevate CBG (Love *et al.* 2004; Williams *et al.* 2008).

Both the threat of predation (Clinchy *et al.* 2004) and reproductive effort (Knowles *et al.* 2009) have been proposed to suppress immune function, leading to lower immunoglobulin levels, and egg production may directly lower immunoglobulin (Boulinier & Staszewski 2008). Threat of predation (Clinchy *et al.* 2004) and egg production (Wagner *et al.* 2008) may both increase the proportion of immature red blood cells (of which polychromasia is a measure) and decrease haematocrit, and egg production has been shown to reduce haemoglobin (Wagner *et al.* 2008). Threat of predation (Clinchy *et al.* 2004) and reproductive effort (Kern *et al.* 2005, 2007) have also both been proposed to mobilize fat, as reflected by increased free fatty acids and decreased triglycerides. Glucose should also be affected, Clinchy *et al.* (2004) suggesting it should be elevated, whereas Kern *et al.* (2007) argued it should decrease. As an antioxidant uric acid may be expected to decrease in response to both predation threat and reproductive effort (Cohen *et al.* 2007; Costantini 2008), though Kern *et al.* (2005) proposed it may increase as a result of the catabolism of protein to produce eggs. The mobilization of free fatty acids in response to predation threat in these sparrow populations shown by Clinchy *et al.* (2004) might be expected to result in decreased mass and fat stores, and a decrease in mass and subcutaneous fat may also be expected as a result of the transfer of proteins and lipids to the egg (Williams 2005).

Details regarding the procedures used to assess physiological condition can be found elsewhere (Travers 2009). Briefly, upon capture, up to 300 μL of blood was collected from the brachial vein, subcutaneous fat was scored using a 7-point scale (Travers 2009), and mass was measured to 0.1 g. Blood used to assay corticosterone was collected within 3 min of the bird being mist-netted. Glucose was measured using a glucose meter (Clinchy *et al.* 2004) and blood smears were prepared within 2 min of the first bleed. All remaining blood was stored on ice for transport. Whole blood, and plasma extracted following centrifugation and measurement for haematocrit, was frozen at $-20\text{ }^{\circ}\text{C}$, within 10 h. Oxidative status and antioxidant capacity were determined as described by Erel (2004) and Erel (2005), respectively (see also Semeniuk *et al.* 2009), and carotenoids were quantified following Alonso-Alvarez *et al.* (2004a). Corticosterone was evaluated following Clinchy *et al.* (2004) and CBG was determined as described Boonstra *et al.* (2001). Immunoglobulins were determined using the ELISA method described by Martinez *et al.* (2003). Polychromasia was evaluated following Clinchy *et al.* (2004) and haemoglobin as described by Wagner *et al.* (2008). Free fatty acid, triglyceride and uric acid concentrations were determined

using enzymatic colorimetric kits, following Kern *et al.* (2007).

Statistical analyses

We tested for treatment effects on egg-related variables using ANOVAs with female identity as a random effect. To test for treatment effects on changes over the season in egg-related variables, we conducted ANCOVAs with both date and date² as covariates and female identity as a random effect. We used discriminant function analysis (DFA) to test for treatment effects on the set of physiological variables considered as a whole, independent of any assumptions about the expected change in any index. Model significance was determined using a permutation test of the DFA's eigen-value, following Mundry & Sommer (2007). We first conducted a DFA of the values from our pre-breeding assessment to ensure there was no initial difference, and then conducted a second DFA on the, after-treatment minus before-treatment change in the value of each index for each female. Table 1 presents the result from the latter analysis, reporting standardized effect sizes for each variable (Cohen 1992). Tabachnick & Fidell (2007) recommend against calculating *P*-values for each variable's contribution in a DFA so we adopted the conservative approach of using each variable's effect size solely as a guide to whether it was worth noting in the Results and Discussion. Finally, to test if the changes in physiology were consistent with females in one treatment being in poorer condition based on expectations from the literature, we used the multivariate test developed by Seeman *et al.* (2001, 2004) to assess what they termed "physiological dysregulation" (or "allostatic load", Seeman *et al.* 2001), calculated as the sum per subject of the number of physiological variables for which that subject's score falls within a specified quartile. Table 1 specifies for each index whether a score in the highest or lowest quartile was considered indicative of poorer condition. Prior to parametric analyses, all data were tested for normality and homogeneity of variances. Degrees of freedom vary because of occasional sample loss (e.g. egg breakage during weighing). We report only values for statistical terms that were significant or where non-significant terms are relevant to the Discussion. The descriptive statistics reported are mean \pm SE.

RESULTS

Females subject to frequent nest predation (FNP) laid significantly smaller subsequent clutches than those (NNP) not subject to nest predation (Fig. 2a; $F_{1,64} = 10.70$, $P = 0.002$). FNP females laid 0.52 fewer eggs than NNP females in the second (first subsequent) clutch each laid (Fig. 1), and 0.33 fewer eggs in the third clutch (Fig. 1), the

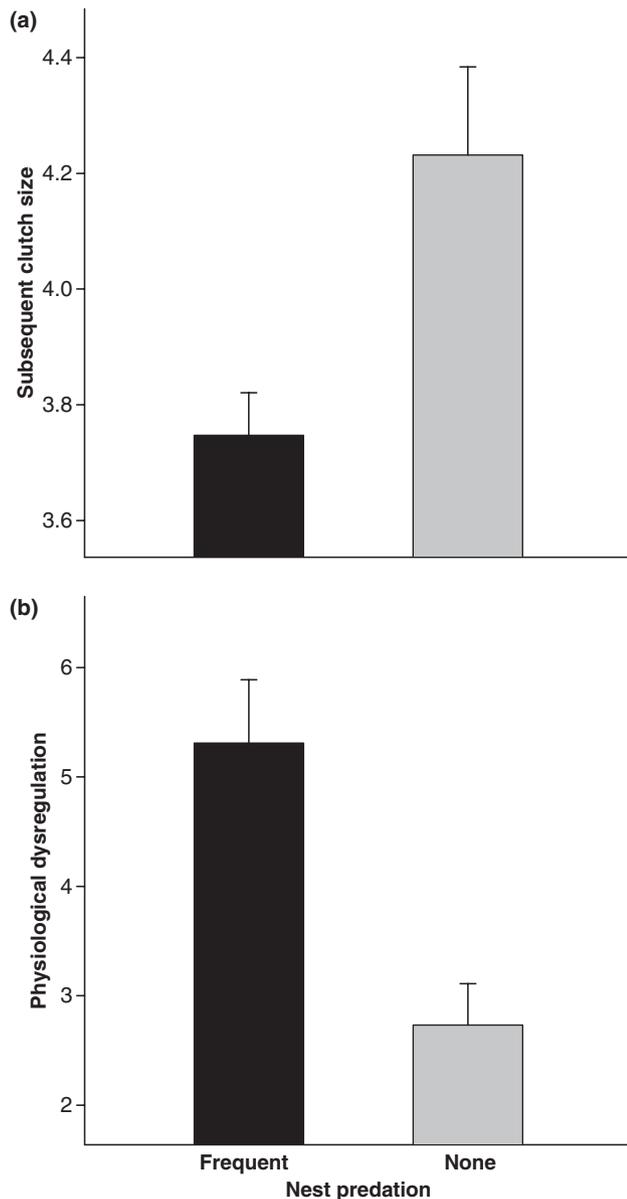


Figure 2 Subsequent clutch size (a) and physiological dysregulation (b) compared between females subject to frequent experimental nest predation (black) and those not subject to nest predation (grey). Values are mean \pm SE.

difference diminishing because clutch size peaked and declined over the experimental period in NNP females (Fig. 1), whereas it declined fairly steadily with each subsequent clutch laid by FNP females (Fig. 1). This difference in the pattern of egg laying across the experimental period was significant (treatment \times date $F_{1,78} = 8.01$, $P < 0.01$; treatment \times date² $F_{1,78} = 7.21$, $P < 0.01$).

The higher probability of nest predation experienced by FNP females caused them to lay significantly more clutches (4.7 ± 0.5) over the experimental period (Fig. 1) than NNP

females (3.0 ± 0.0 ; $t_{18} = 10.00$, $P < 0.001$). As a consequence of laying more clutches, FNP females laid significantly more eggs over the experimental period (FNP = 17.33 ± 0.50 ; NNP = 12.25 ± 0.37 ; $t_{15} = 8.02$, $P < 0.001$), although the proportionate difference in the total number of eggs laid (41%) was notably less than the difference in the number of clutches (53%; i.e. <1 : 1) illustrating in a different fashion the suppressive effect of a higher probability of nest predation on the 'birth' rate (number of eggs laid), demonstrated above with respect to subsequent clutches (Fig. 2a). There was no significant treatment effect on the average inter-nest interval (FNP = 8.82 ± 1.50 days; NNP = 9.50 ± 1.01 ; $t_{23} = 0.74$, $P = 0.47$).

There were no significant treatment effects on average egg mass (FNP = 2.92 ± 0.04 g; NNP = 2.88 ± 0.05 ; $F_{1,56} = 0.24$, $P = 0.63$), total clutch mass (FNP = 11.24 ± 0.28 g; NNP = 12.04 ± 0.42 ; $F_{1,55} = 2.46$, $P = 0.12$), or the change over the experimental period in either average egg mass (treatment \times date $F_{1,62} = 0.05$, $P = 0.81$; treatment \times date² $F_{1,62} = 0.01$, $P = 0.91$) or total clutch mass (treatment \times date $F_{1,61} = 2.77$, $P = 0.10$; treatment \times date² $F_{1,61} = 3.01$, $P = 0.09$).

Females subject to frequent nest predation (FNP) underwent a significantly different set of physiological changes than those (NNP) not subject to nest predation (Table 1; DFA eigen-value = 47.5, Wilk's $\lambda = 0.02$, pDFA $P = 0.01$). Considering just those changes with meaningful (Cohen 1992) effect sizes (Table 1), FNP females had higher oxidative status and lower antioxidant capacity, consistent with oxidative stress. Also consistent with oxidative stress, and greater egg production, FNP females had lower carotenoid levels. In addition, FNP females had higher CORT, lower immunoglobulin levels, higher polychromasia and lower subcutaneous fat scores, all of which accords with their being in poorer condition. FNP females also had higher CBG levels consistent with greater egg production and possibly predator-induced stress. In total, females subject to frequent nest predation (FNP) showed significantly more evidence of physiological dysregulation than those (NNP) not subject to nest predation (Fig. 2b; $t_{18} = 3.39$, $P = 0.003$), even when the assumptions concerning the three indices with the least clear-cut expectations were inverted (Table 1; $t_{18} = 2.81$, $P = 0.012$).

DISCUSSION

Our experimental results indicate that predator-induced changes in prey physiology or behaviour may negatively affect the annual 'birth' rate in birds, irrespective of resource limitation. To our knowledge, this is the first experimental demonstration that a higher probability of nest predation may cause a reduction in the size of subsequent clutches

(Fig. 2a), and this experiment and Eggers *et al.*'s (2006) concerning first clutches, are the first to show indirect predator effects on the annual 'birth' rate in birds (Lima 2009). Considering the first two subsequent clutches together (clutches 2 and 3 in Fig. 1), females subject to frequent nest predation laid 0.85 fewer eggs than those not subject to nest predation, which is comparable in magnitude to the indirect predator effect on first clutches (0.7 fewer eggs) reported by Eggers *et al.* (2006). Experiencing a higher probability of nest predation induced a significant change in physiology, independent of any assumptions regarding the expected direction (Table 1). The physiological changes seen in females subject to frequent nest predation were consistent with their demonstrating oxidative stress, glucocorticoid mobilization, immunosuppression, reticulocytosis and reduced fat storage; and in sum they showed significantly more evidence of physiological dysregulation (Fig. 2b).

The significant quadratic difference (treatment \times date²) in the variation in the size of subsequent clutches across the experimental period (Fig. 1) is also unique, in experimentally demonstrating that the often-observed contrasting seasonal patterns of a peak and decline in clutch size in one group of birds, and steady decline in others, can stem from a difference in the probability of nest predation (Slagsvold 1982; Farnsworth & Simons 2001; Lima 2009). This contrasting seasonal pattern of egg-laying has, among other things, been ascribed to variation in food availability (supply; e.g. Crick *et al.* 1993), which seems unlikely to explain our results because subjects were randomly-assigned to treatment and every female was provided with *ad libitum* supplemental food throughout the experiment.

Experiencing a higher probability of nest predation caused females to lay more clutches (Fig. 1) which were significantly smaller on average (Fig. 2a), consistent with both the positive correlation between the probability of nest predation and clutch number, and negative correlation between clutch number and average clutch size, reported both across and within songbird species by Martin (1995) and Zanette *et al.* (2006b). Textbook logistic, or density-dependent, population growth models assume that the number of births in a population will increase to compensate for an increased number of deaths (Creel & Christianson 2008). Consequently, that FNP females laid more clutches and thus more eggs in total over the experimental period to compensate for the greater number of clutches lost is not unexpected. What has yet to be integrated into population ecology textbooks is that predators may have a negative or suppressive effect on the birth rate (Creel & Christianson 2008), as demonstrated by the reduction in the size of subsequent clutches (Fig. 2a) and resulting negative relationship between clutch number and average clutch size (Fig. 1).

In their test of indirect predator effects on first clutches, Fontaine & Martin (2006) found no effect on clutch size but did find significant effects on average egg and total clutch mass, whereas we found the opposite. Rather than being contradictory, we suggest these contrasting results are consistent with modelling showing that variation in first clutches may be more conservative than variation in subsequent clutches, and subsequent clutches may thus be more likely to reflect indirect predator effects (Slagsvold 1982; Farnsworth & Simons 2001).

Changes in physiology or behaviour or both may have been responsible for FNP females laying smaller subsequent clutches. Our experimental design ensured that the physiological differences between FNP and NNP females (Table 1) could only have resulted from differences in nest predation. There were no initial differences, differences in the experimental period or differences in the day of incubation or date when each female's final condition was assessed (Fig. 1). The physiological differences may have resulted from either: the experience, rate (frequency), or extent (total number) of nest predation events. That the physiological differences we observed could stem wholly from FNP females having experienced nest predation, whereas NNP females did not, is illustrated by recent laboratory work by Willie *et al.* (2010) showing that certain physiological measures may be affected by the experience of egg-laying, but not the rate or number of times females are forced to re-lay. Alternatively, the physiological differences between FNP and NNP females could have been cumulative, reflecting the difference in the total extent of re-nesting, or total number of eggs laid. However, the fact that the largest difference in clutch size was evident in the first subsequent clutch (clutch 2 in Fig. 1) suggests the experience or rate of nest predation is likely to have affected many of the physiological indices we measured, otherwise the largest difference in clutch size must have been associated with the smallest difference in physiology.

Evidence of oxidative stress, glucocorticoid mobilization, immunosuppression and reticulocytosis, as seen in FNP females (Table 1), points to there being non-resource-based constraints on egg production (Williams 2005), on top of the fact that all females were food supplemented *ad libitum*. All of the meaningful (Cohen 1992) physiological differences observed (Table 1) were also consistent with a response to the threat of predation, as outlined in the Methods. Creel *et al.* (2009) recently proposed that indirect predator effects on reproduction could result wholly from the suppression of foraging as opposed to "predation stress", in which case changes in mass and plasma metabolites should have been more prominent rather than the non-resource-based changes seen (e.g. in oxidative stress and glucocorticoid mobilization; Table 1), that may be

considered more indicative of a response to an environmental stressor (Costantini 2008). We concur with Creel *et al.*'s (2009) assessment that responding to the threat of predation involves multiple pathways, and as outlined in the Methods, reproduction similarly involves pleiotropic physiological effects (Williams 2005; Harshman & Zera 2007), which is why we employed a multivariate approach and why we recommend this approach to others.

Conceivably, the strategic adjustment of clutch size could involve a purely cognitive process independent of any physiological changes, although this may be challenging to evaluate in the field. Eggers *et al.* (2006), for example, reported that in response to the threat of predation the birds they studied not only reduced the size of their first clutch but also nested in more concealed locations that were significantly colder, which presumably would have affected the metabolism of the incubating mothers. If we had captured and assessed every female after they laid their second clutch (Fig. 1) and found that FNP females were in better condition this would be consistent with a cognitive process of strategically reducing the size of subsequent clutches, and this would be a productive subject for a future study. We did not do this in the present experiment because our primary objective was to determine the demographic consequences of an indirect predator effect on the average size of all subsequent clutches (Fig. 2a), and capture and assessment entails some risk of unintentionally causing nest abandonment.

Our results, together with Eggers *et al.*'s (2006) concerning first clutches, provide clear experimental evidence of indirect predator effects on clutch size. A recent meta-analysis by Preisser *et al.* (2009) indicated that indirect predator effects may be greater in systems subject to variation in food availability, hence, the magnitude of the effect we report may be considered conservative since we controlled for food availability. Similarly, the magnitude of the effect may be greater given multiple cues concerning the threat of predation rather than just the disappearance of the nest's contents (Lima 2009). Thus, we suggest that additional experiments will likely provide even further support for Creel & Christianson's (2008) contention that indirect predator effects ought to be more fully incorporated into general ecological theory.

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