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1 **Innate immunity, assessed by plasma NO measurements, is not suppressed**
2 **during the incubation fast in eiders.**

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13 Running title: Innate immunity in incubating eiders

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1 **ABSTRACT: Innate immunity, assessed by plasma NO measurements, is not suppressed**
2 **during the incubation fast in eiders.**

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4 Immunity is hypothesized to share limited resources with other physiological functions and
5 may mediate life history trade-offs, for example between reproduction and survival. However,
6 vertebrate immune defence is a complex system that consists of three components. To date,
7 no study has assessed all of these components for the same animal model and within a given
8 situation. Previous studies have determined that the acquired immunity of common eiders
9 (*Somateria mollissima*) is suppressed during incubation. The present paper aims to assess the
10 innate immune response in fasting eiders in relation to their initial body condition. Innate
11 immunity was assessed by measuring plasma nitric oxide levels (NO), prior to and after
12 injection of lipopolysaccharides (LPS), a method which is easily applicable to many wild
13 animals. Body condition index and corticosterone levels were subsequently determined as
14 indicators of body condition and stress level prior to LPS injection. The innate immune
15 response in eiders did not vary significantly throughout the incubation period. The innate
16 immune response of eiders did not vary significantly in relation to their initial body condition
17 but decreased significantly when corticosterone levels increased. However, NO levels after
18 LPS injection were significantly and positively related to initial body condition, while there
19 was a significant negative relationship with plasma corticosterone levels. Our study suggests
20 that female eiders preserve an effective innate immune response during incubation and this
21 response might be partially determined by the initial body condition.

22
23 Key-words: Body condition; corticosterone; fasting; innate immune response;
24 lipopolysaccharides; nitric oxide; reproductive effort; trade-off

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1 INTRODUCTION

2

3 Trade-offs between life history components are a central concept in evolutionary ecology.
4 The publication of a paper by Hamilton and Zuk in 1982 [1] generated a considerable interest
5 in the role that parasites might play in the evolution of reproductive strategies. This stimulated
6 the emergence of the field of ecological immunology, which investigates the role of immune
7 effector systems in determining host fitness in the wild [2]. The observation that a heavy
8 parasite load during reproduction is associated with a reduced immune response [3] and a
9 decreased adult survival rate [4], led to the hypothesis that immunity must share limited
10 resources with other physiological functions and this may to some extent underpin the costs of
11 reproduction [5].

12 Recent experiments have indicated that high reproductive effort increases metabolic
13 rate, leading to oxidative stress [6,7] due to increased generation of oxidative metabolites and
14 free radicals [8]. Immunosuppression during reproduction has been viewed as an undesirable
15 consequence of a high metabolic rate [9]. However, even if a high metabolic rate during
16 reproduction might contribute to immunosuppression, it is most likely not the only factor
17 involved [9]. As recently outlined by Viney et al. [10], a “maximum immune response” does
18 not necessarily mean that the response is “optimal”, since an immunosuppression might be of
19 adaptive value [9] and then accepted as a cost by the organism. Firstly, since an activated
20 immunity enhances oxidative stress [8], it carries a non-negligible risk of autoimmune
21 pathology [11,12]. Immunosuppression could therefore be explained by the
22 immunopathology-avoidance hypothesis [9]. In this context, the control of the production of
23 nitric oxide (NO) by immune cells is primordial since overproduction of NO is involved in
24 sepsis shock [13]. Secondly, the resource-limitation hypothesis suggests that different
25 functions compete for limited resources and that investment in costly behaviours, such as

1 reproduction, reduces the amount of resources available to immune defense [9]. This second
2 hypothesis assumes that there is an energetic or nutritional cost associated with the immune
3 system [9,14,15]. However, evidence for an energetically costly immune response is still
4 equivocal [9].

5 Vertebrate immune defense is one of the most complex biological phenomena. It
6 consists of three components (innate immunity, acquired humoral immunity, and acquired
7 cellular immunity) between which trade-offs may appear [5,10,16-18]. Consequently,
8 multiple immune assays, which challenge different components of the immune system, should
9 be used to gain a better understanding of overall immunocompetence [19]. Since assessment
10 of acquired immunity has proven insufficient for ecological immune studies [16,20], assaying
11 the innate component is of primary importance.

12 Eider ducks are a useful model for studying the intensity of the immune response in relation
13 to body condition, since females rely solely on their stored body fat during incubation [21].
14 Previous studies have shown that the acquired immunity of common eider ducks (*Somateria*
15 *mollissima*) is suppressed during the incubation fast [18,22], and that its experimental
16 activation has strong negative effects on the fitness of female eiders [22]. In fact,
17 immunocompetence may be fixed by fat stores, the main energetic reserves of the organism
18 that determine individual quality of reproductive adults [5,23]. Thus, the decrease in acquired
19 immune response of female eiders can be understood as a consequence of fat stores depletion.
20 However, whether and to what extent eider's whole immune response during reproduction
21 depends on individual quality and is suppressed when body conditions deteriorate is still
22 under debate [24].

23 Based on these data, we tested whether during the incubation fast, female eiders preserve or
24 not an impenetrable first line of defense. In other words, do eiders maintain a highly effective
25 innate immune response to compensate for the loss of their acquired immune components?

1 To date, only one study has assessed the constitutive innate immunity by using an *in*
2 *vitro* assay [25]. We used here an alternative method, commonly used in immunology studies,
3 which is easily applicable to animals in the wild. The innate immune response consists of the
4 activation of phagocyte cells as heterophils and macrophages [26]. Destruction of recognized
5 pathogens by those cells is mainly driven by the production of NO [27], which can be
6 experimentally stimulated by injection of lipopolysaccharides (LPS) [28]. We measured
7 plasma NO concentrations in breeding female eider ducks before and after LPS injection at
8 various stages during their incubation period, while also considering their initial body
9 condition. We assessed body condition by measuring corticosterone levels, an increase in one
10 component being strongly associated with a decline in the other [29,30], and also by
11 calculating a body mass index (mass/tarsus length³).

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1 MATERIAL AND METHODS

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3 The study was conducted in a common eider colony on Storholmen Island, Kongsfjorden,
4 Svalbard Archipelago (78°55' N, 20°07' E) from June to July 2005. The breeding population
5 of eiders in the study site consisted of about 900 nests. Females in the study area laid between
6 1 and 6 eggs, but a clutch size of 4 to 5 eggs was most common (49.35 % and 24.62 %
7 respectively, N=845). Eider ducklings are precocial and are cared for by the female only.
8 Incubation lasts between 24 and 26 days [21]. All birds started their incubation between June
9 07 and June 13, the main laying period for the colony. Ducks that laid their eggs after this
10 period were not considered in this study. Ambient temperatures in June and July ranged from
11 2 to 10°C.

12

13 Groups and sampling protocol

14 Nests were checked at least every two days throughout the study period. This was done to
15 determine initial clutch size but also to investigate the rate of egg predation and nest
16 desertion. A clutch of eggs was considered complete when no additional egg was laid during a
17 two-day period [31]. Female eiders were caught on their nests using a bamboo pole with a
18 nylon snare. Blood (1- 2 ml) was collected from the brachial vein within three minutes of
19 capture. It was stored in tubes containing EDTA (an anticoagulant agent) and kept on ice until
20 being centrifuged at 10, 000 rpm for five minutes at 4°C in the laboratory. Plasma samples
21 and blood cells were separately stored at -20°C and plasma was subsequently used to measure
22 corticosterone and nitric oxide levels. After blood sampling, body size was recorded (wing
23 and tarsus lengths) and birds were weighed with a portable electronic balance (± 2 g). Initial
24 body mass (after laying the last egg) was extrapolated from body mass at sampling as
25 described by Criscuolo et al. [32]. Finally, to measure the innate immune response, each

1 female was injected with a solution of lipopolysaccharides (LPS) intra-peritoneally (see
2 below). To assess female body condition, the following index was used: female initial body
3 mass was divided by (tarsus length)³, as reported by MacColl and Hatchwela [33]. Mean body
4 condition of 51 females was 8.80 ± 0.10 (mean \pm SE, N=51). Consequently, we classified
5 females with an index ≤ 8.80 as being in bad condition, while females with an index > 8.80 as
6 being in good condition.

7 A total of 51 females with clutch sizes ranging from 3 to 6 eggs (4.06 ± 0.09 eggs, N=51)
8 (mean \pm SE) was used in this study. All birds were caught twice. To prevent nest desertion we
9 only caught birds that were already incubating for at least five days. To cover the entire
10 incubation period we caught individuals that were at different stages of their incubation
11 period (17.94 ± 0.77 days into incubation, N=51) (mean \pm SE).

12

13 Innate immune response: LPS injection

14 To evaluate the innate immune response, we first collected blood samples from incubating
15 females and subsequently injected 0.35 mg.kg^{-1} LPS intra-peritoneally (Sigma L-2630, diluted
16 in phosphate-buffered saline). Birds were recaptured 24 h after injection and another blood sample
17 was taken. Nitric oxide levels were subsequently determined (see below) prior to and 24 h after
18 LPS injection. The ‘innate immune response’ was calculated as the increase (in percentage) in
19 nitric oxide production following LPS injection.

20

21 Assessment of the nitric oxide levels

22 There are various assays for the quantification of NO in biological models as reviewed by
23 Archer [34]. The free radical NO has a short half-life *in vivo* since it is quickly oxidized,
24 mainly to the inorganic nitrite (NO_2^-) and nitrate (NO_3^-). Diazotization is a standard technique
25 for indirect quantification of NO production. It has been previously used in chickens [35] and

1 is based on the reaction of NO_2^- with Griess components (sulphanilamide and N-(1-
2 naphthyl)ethylene-diamine, Sigma G4410-10G), and the generation of a purple-azo-dye
3 product, which is measured with a spectrophotometer at 550 nm [35]. This method requires
4 previous enzymatic reduction of NO_3^- to NO_2^- using bacterial nitrate reductase (Sigma N-
5 7265).

6 In our assessment, plasma (10 μl) was diluted 1:2 with sterile distilled water (10 μl) and
7 incubated for two hours at ambient temperature with 80 μl of a medium composed of nitrate
8 reductase (Sigma N-7265, 0.4 u.mL^{-1}), β -NADPH (Nicotinamide adenine di-nucleotide
9 phosphate, Sigma 7505, 1 mg.mL^{-1}) and HEPES (GIBCO 15630-056, 0.1 M). A volume of
10 100 μL of Griess reagent was added to each sample and the optical density was measured at
11 550 nm after 15 minutes. Nitrite levels were determined with a standard curve generated from
12 a NaNO_2 solution at 2.5 mM. Sodium EDTA was used as anticoagulant and no ultrafiltration
13 of plasma was done, as previously recommended, to avoid interference in NO_3^- quantification
14 [36]. The absorbance of background coloured plasma was controlled for the non-specific
15 increase in absorbance after the Griess reaction due to hemolysis or to the presence of blood
16 haemoproteins [36]. No particular elevated backgrounds due to haemolysis were found in the
17 samples.

18

19 Assessment of the corticosterone levels

20 Corticosterone concentration was determined by radioimmunoassay (RIA) in our laboratory
21 using an ^{125}I RIA double antibody kit from ICN Biomedicals (Costa Mesa, CA, USA). The
22 corticosterone RIA had an intraassay variability of 7.1 % (N=10 duplicates) and an interassay
23 variability of 6.5 % (N=15 duplicates).

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1 Statistical analyses

2 Statistical analysis was conducted with SPSS 12.0.1 (SPSS Inc., Chicago, Illinois, USA).
3 Values are means \pm standard error (SE). When data were not normally distributed
4 (Kolmogorov-Smirnov test, $P < 0.05$), values were log transformed before using parametric
5 tests. When transformations were not possible, non parametric tests were used.

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1 **RESULTS**

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3 Table 1 provides details of the female eiders used in this study. Initial body mass, initial
4 clutch size, and wing length were not significantly different between the two groups (good
5 and bad body condition). Body mass (1600 ± 23 g, $N=51$) was inversely related to sampling
6 date during the fast (Spearman rank correlation: $r_s=-0.68$, $N=51$, $P<0.0001$). There was no
7 significant relationship between female initial body mass and initial clutch size (Spearman
8 rank correlation: $r_s=-0.03$, $N=51$, $P=0.81$).

9

10 Nitric oxide production in relation to incubation length

11 As expected, LPS injection stimulated NO production. Indeed, NO levels were significantly
12 increased by 70 % following LPS injection (repeated measures ANOVA: $F_{1,50}=107.02$,
13 $P<0.0001$) (106.82 ± 7.85 μM , $N=51$ before LPS injection versus 179.13 ± 9.64 μM , $N=51$
14 after LPS injection).

15 The percentage of increase in NO production following LPS injection (the ‘innate immune
16 response’) did not vary significantly throughout the incubation period, ranging from 110 to 74
17 % (linear regression: $r^2=0.07$, $N=51$, $P=0.06$). By contrast, NO levels before LPS injection
18 (NO1) increased significantly by about 40 % as the incubation progressed (range: 86.66-
19 120.94 μM ; linear regression: $r^2=0.12$, $N=51$, $P=0.01$) (Figure 1). However, NO levels after
20 LPS injection (NO2) did not vary significantly throughout the incubation period (range:
21 168.30-186.72 μM ; linear regression: $r^2=0.04$, $N=51$, $P=0.15$). Finally, there was a significant
22 negative relationship between the innate immune response and NO1 levels (linear regression:
23 $r^2=0.57$, $N=51$, $P<0.0001$) but not NO2 levels (linear regression: $r^2=0.03$, $N=51$, $P=0.23$)
24 indicating that when NO levels were high before LPS injection, no further increase was
25 observed after LPS injection (Figure 2).

1 Nitric oxide production in relation to female body condition

2 We first assessed female body condition by dividing their initial body mass by their (tarsus
3 length)³. The body condition index was not significantly related to neither innate immune
4 response nor NO1 levels (linear regression: $r^2=0.00$, $N=51$, $P=0.99$; $r^2=0.04$, $N=51$, $P=0.18$,
5 respectively). By contrast, there was a significant positive relationship between body
6 condition index and NO2 levels (linear regression: $r^2=0.12$, $N=51$, $P=0.01$), so that females in
7 good body condition had greater NO2 values than females in bad body condition (Figure 3).

8 We subsequently assessed female body condition by measuring their corticosterone levels.
9 Plasma corticosterone levels did not vary significantly over the incubation period (linear
10 regression: $r^2=0.001$, $N=51$, $P=0.82$). Mean corticosterone level was 28.70 ± 4.66 ng.ml⁻¹
11 (range: 22-33 ng.ml⁻¹; $N=51$). We found a significant negative relationship between body
12 condition index and plasma corticosterone levels (linear regression: $r^2=0.12$, $N=51$, $P=0.01$).
13 Hence, females in good body condition had lower levels of corticosterone than females in bad
14 body condition.

15 There was a significant negative relationship between plasma corticosterone levels and the
16 innate immune response (% increase of NO after LPS injection) (linear regression: $r^2=0.10$,
17 $N=51$, $P=0.02$) (Figure 4), whereby high levels of corticosterone were associated with a low
18 innate immune response. We furthermore found a significant negative relationship between
19 plasma corticosterone and NO2 levels (linear regression: $r^2=0.11$, $N=51$, $P=0.01$) (Figure 5),
20 where high levels of corticosterone were associated with low values of NO2. However, there
21 was no significant relationship between plasma corticosterone and NO1 levels (linear
22 regressions: $r^2=0.04$, $N=51$, $P=0.15$).

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1 **DISCUSSION**

2

3 Our study is the third part of an investigation into the immunocompetence of female eider
4 ducks during the incubation fast, and allows us to give an overview of how the different
5 components of immunity are affected during this phase. Suppression of both T-cell-mediated
6 and humoral acquired immune responses were previously observed in incubating female
7 eiders [18,22,37], but no significant correlation between these two components has been
8 observed [18]. This immunosuppression appears to be adaptive since it enhances the future
9 survival of the females [22]. Roitt et al. [38] suggested that, birds should consequently keep a
10 high innate barrier to compensate for the loss of the acquired immunity, so that pathogens can
11 be eliminated before the acquired components are triggered. Support for this hypothesis
12 comes from indirect measurements of the innate immunity in eiders (i.e.
13 heterophil/lymphocyte counts) by Hanssen et al. [39]. Furthermore, the plasma NO
14 production measured in the present study clearly illustrates that the innate response in our
15 eiders was not suppressed during fasting. Similarly, studies on severely starved chickens
16 showed an enhanced innate immunity while the lymphocyte response (as part of the acquired
17 immune response) was suppressed [40]. A possible explanation for these observations is that
18 the recruitment of phagocyte cells from the innate system is less energy-consuming than the
19 activation of humoral and cellular acquired immunities. Favouring the innate system could
20 also act as a buffer against the fitness costs associated with acquired immunities [22].
21 However, it is still unclear if the activation of an immune response is energetically costly and
22 evidence from a number of immuno-ecological studies is equivocal. Demas et al. [41] showed
23 that oxygen consumption of mice was increased by 15-20 % after an immunoglobulin
24 response was triggered. Svensson et al. [42], however, found only a weak increase in the
25 BMR of blue tits (*Parus caeruleus*) after antibody response challenge. Similarly, Verhulst et

1 al. [15] reported that the metabolic costs of eliciting a humoral immune response are
2 negligible in zebra finches (*Taeniopygia guttata*). Also, Hanssen et al. [22] did not find an
3 accelerated body mass loss in female eiders responding to an injection of antigens. Therefore,
4 the nature of the costs linking immune responses to reproductive effort and overall energy
5 metabolism still escapes our understanding. However, since apoptosis of leukocytes is
6 naturally induced by free radicals [43], oxidative damage could mediate such a cost [8,9].
7 Finally, despite the fact that innate immunity was not suppressed during the incubation fast in
8 eider ducks, it nonetheless seemed to be limited to a certain threshold. In fact, while NO1
9 levels significantly increased as incubation progressed, NO2 levels did not vary significantly
10 over the course of the incubation period. As a consequence, the innate immune response
11 (NO2-NO1/NO1) did not vary throughout incubation. Moreover, we found a negative
12 relationship between NO1 levels and the innate immune response, so that higher NO1 levels
13 were associated with a lower innate immune response. This could be a potential mechanism to
14 reduce the risks of oxidative damage, since this non-specific defense leads to an over-
15 production of free radicals. NO is a potent vasodilator playing a crucial role in sepsis shock
16 [13], and its production must be then carefully controlled. Moreover, NO is produced not only
17 by macrophages but also by endothelial cells and, interestingly, by adipose tissue [44], which
18 may explain the unexpected variations of NO1 levels (before LPS injection and immune
19 system challenge) in eiders. In this context, it would be interesting to test both the
20 immunosuppressive effect of an increased oxidative stress but also changes in basal NO levels
21 during the incubation fast in future experiments.

22 Trade-offs between different components of the immune system were reported in red
23 jungle fowl (*Gallus gallus*) [17]. Before the breeding season cell-mediated immunity and the
24 proportion of lymphocytes of male jungle fowl were positively correlated with comb length.
25 By contrast, during the breeding season, males with large combs had lower levels of

1 lymphocytes but a greater cell-mediated immunity [17]. The underlying mechanisms are still
2 unknown. In a previous study on incubating eiders, humoral immunosuppression was
3 correlated with an increase in plasma concentration of two heat shock proteins (HSP60 and
4 HSP70) [12]. HSP's are known to be immunogenic for innate responses [45] and to promote
5 lysis activity of the innate component members such as natural killer cells (NK) [46]. Hence,
6 they might contribute to the maintenance of the innate immunity during fasting. NK cells are
7 specialized in the destruction of infected host cells, which could partly compensate for
8 lymphocyte deficiency against viral infections [26]. In addition, in previous studies,
9 suppression of the acquired immunity was stronger in female eiders with a low initial body
10 condition [39], while adverse effects on fitness from parasites were also more pronounced in
11 these individuals [47]. We found that NO₂ levels in our eiders were positively correlated with
12 initial body condition. Hence, the immunocompetence of eider ducks might to some degree
13 depend on initial body condition [5]. This idea is supported by the fact that humoral immunity
14 in mammals is controlled by body fat stores [23], which raises the question of the hormonal
15 control of the immune system [48]. The peptide hormone leptin is secreted primarily by
16 adipose tissue and has been shown to enhance a variety of immunological parameters in
17 mammals [49,50] and birds [51]. Since circulating levels of leptin are generally proportional
18 to the amount of body fat [52], decreases in body fat stores may affect immunity via changes
19 in endocrine signaling with the immune system [48]. To examine the role of leptin for the
20 immune function of fasting eiders, it would be useful to manipulate leptin concentrations [51]
21 at different incubation stages. Corticosterone might also be a potential functional link between
22 body condition and immune response. It was namely implicated in the *in vitro* inhibition of
23 chicken lymphocyte proliferation [53] and in decreased phagocyte ability in starved rats [54].
24 Conversely, corticosterone can also promote phagocytosis [55] and NO production in synergy
25 with prolactin [56]. This latter peptide has shown to be involved in incubation behaviour in

1 eiders [57]. In this study, we reported that corticosterone, the primary avian stress hormone,
2 tended to be negatively correlated with the innate immune response, so that high levels of
3 corticosterone were associated with a low innate immune response.

4 In conclusion, we propose that in contrast to the fate of both acquired immune
5 components, the innate immunity is not suppressed during the incubation fast of female
6 eiders. Moreover, the levels of NO₂ seem to depend on the initial amount of body reserves.
7 Females in good body condition showed higher NO₂ levels than birds in bad body condition.
8 Similarly, females with high corticosterone values had low NO₂ values and a decreased
9 innate immune response. Nevertheless, the evolutionary significance of our observations
10 remain to be validated. Finally, despite the fact that hormonal and neuroendocrine systems are
11 important promoters of innate immunity [58], their interactions need to be investigated in
12 further experiments.

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7 Svalbard.

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1 **FIGURE LEGENDS**

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3 **Figure 1.** Nitric oxide levels before LPS injection (NO1) in female eiders versus incubation
4 stage and according to their initial body condition: good (●) or bad (▽). LPS,
5 lipopolysaccharides.

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7 **Figure 2.** Relationship between nitric oxide levels before LPS injection (NO1) and innate
8 immune response (%) in incubating female eiders according to their initial body condition:
9 good (●) or bad (▽).

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11 **Figure 3.** Relationship between body condition index (mass/tarsus length³) and nitric oxide
12 levels after LPS injection (NO2) in incubating female eiders. Females having an index ≤ 8.80
13 or > 8.80 were classified as in bad (▽) or good (●) initial body condition, respectively. LPS,
14 lipopolysaccharides.

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16 **Figure 4.** Relationship between corticosterone levels and innate immune response (%) in
17 incubating female eiders according to their initial body condition: good (●) or bad (▽).

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19 **Figure 5.** Relationship between corticosterone levels and nitric oxide levels after LPS
20 injection (NO2) in incubating female eiders according to their initial body condition: good
21 (●) or bad (▽). LPS, lipopolysaccharides.

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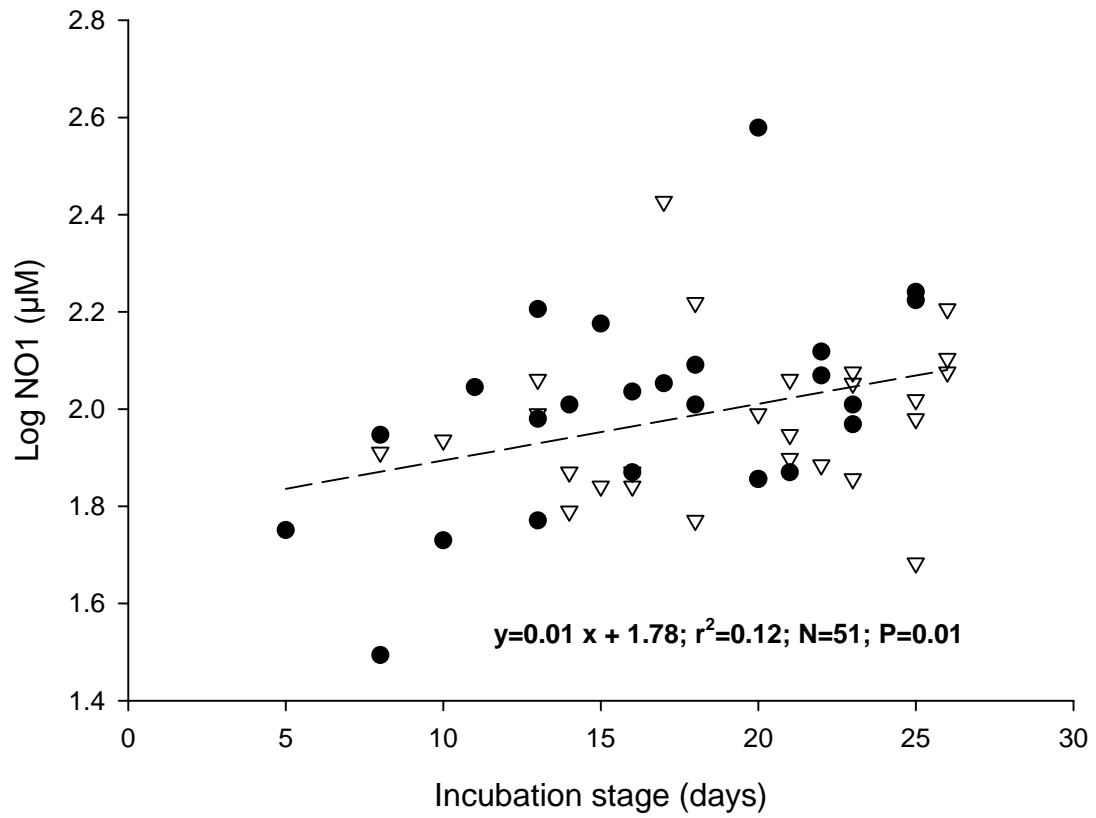
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1 **FIGURE 1.**

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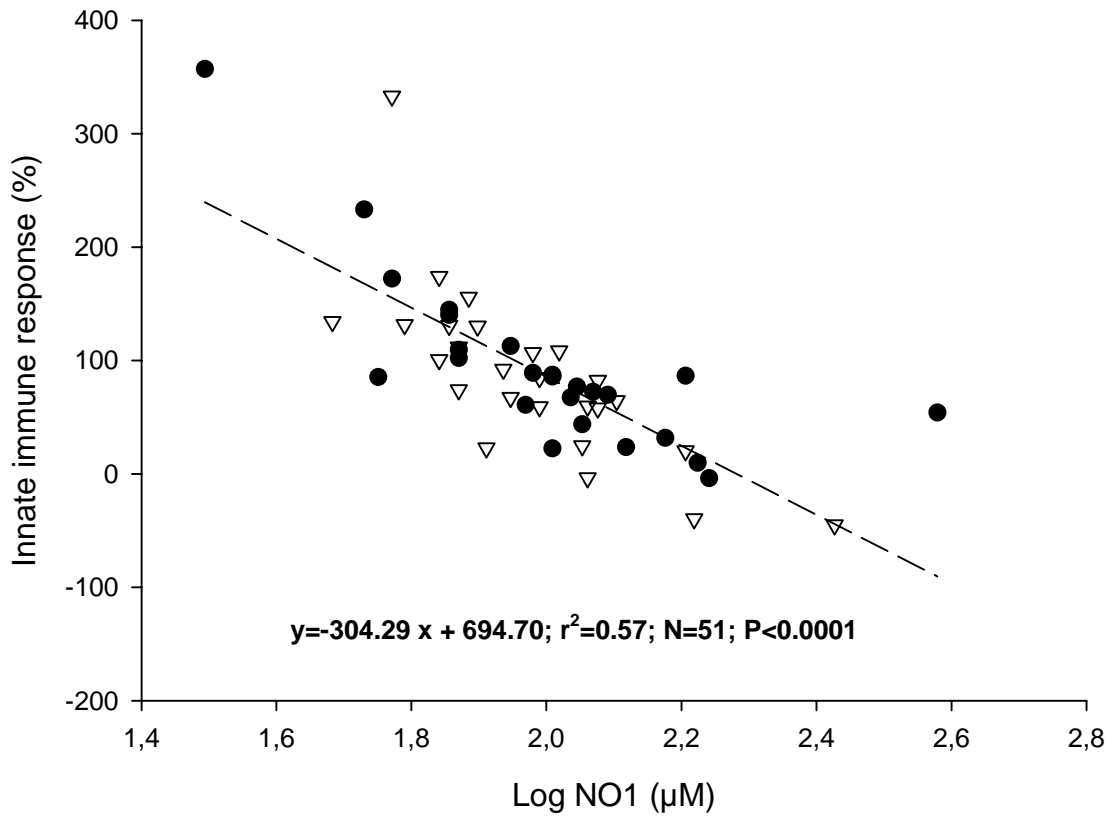
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1 **FIGURE 2.**

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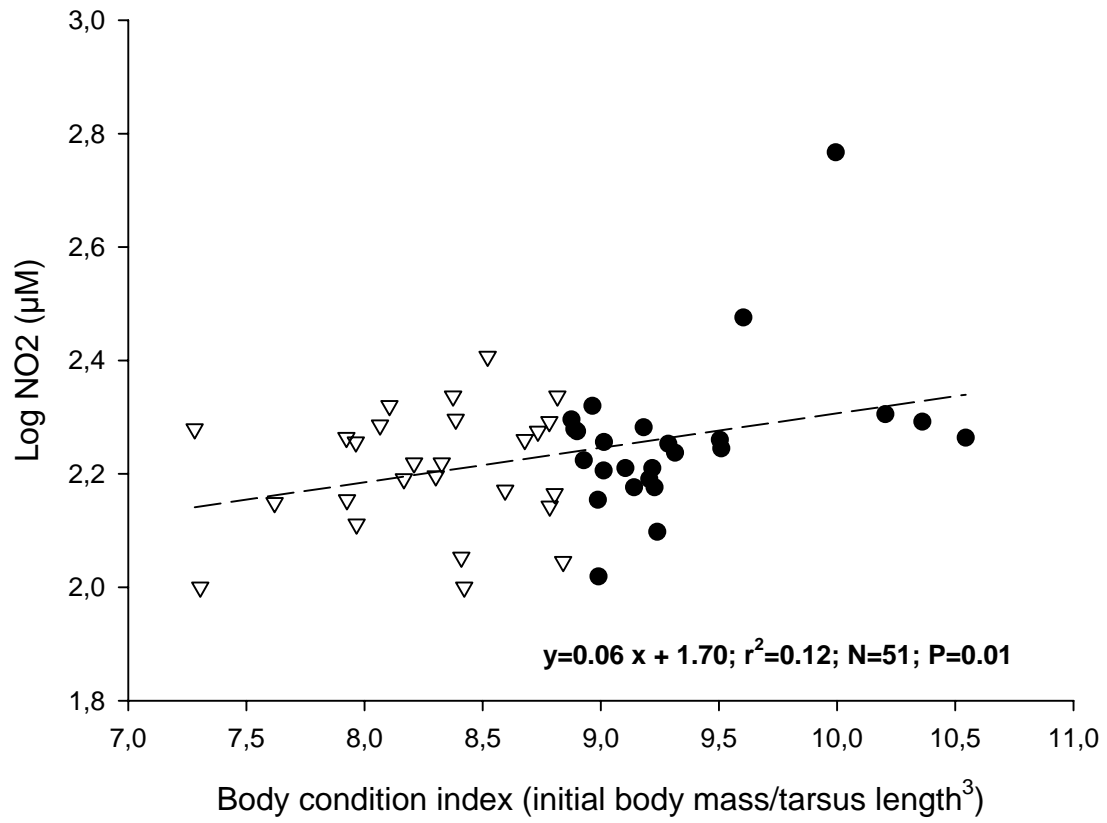
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1 **FIGURE 3.**

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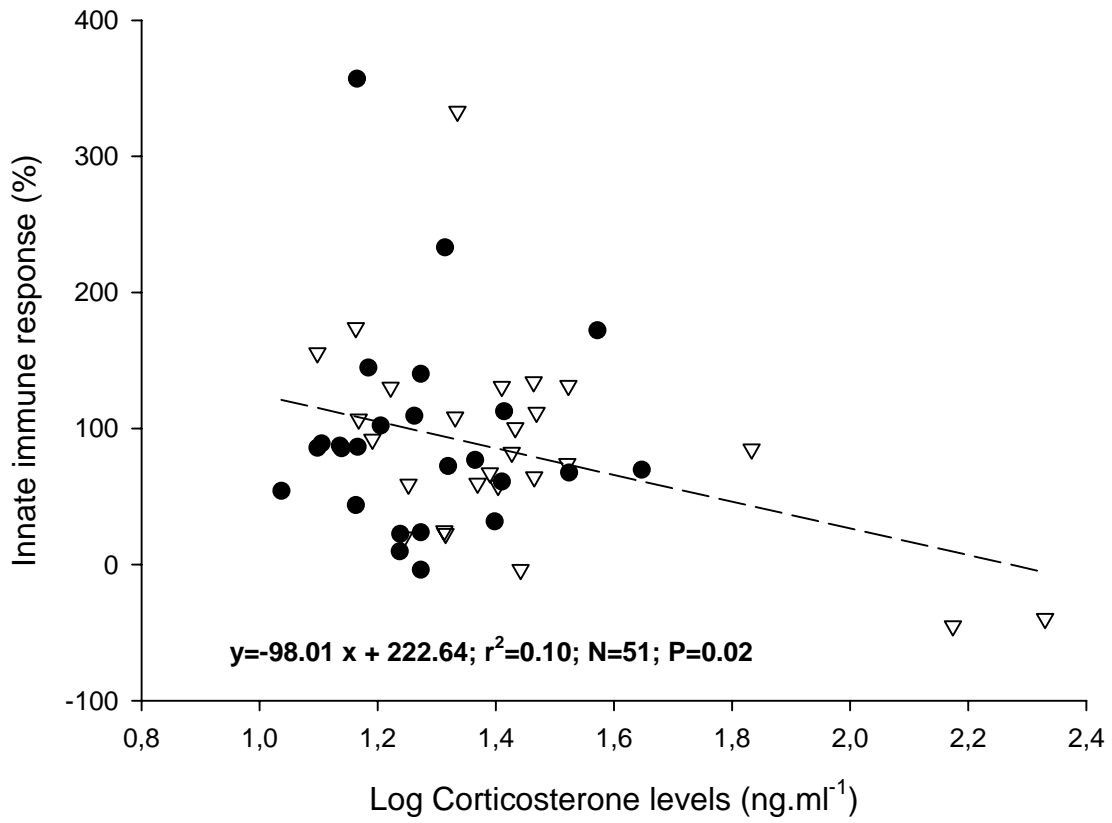
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1 **FIGURE 4.**

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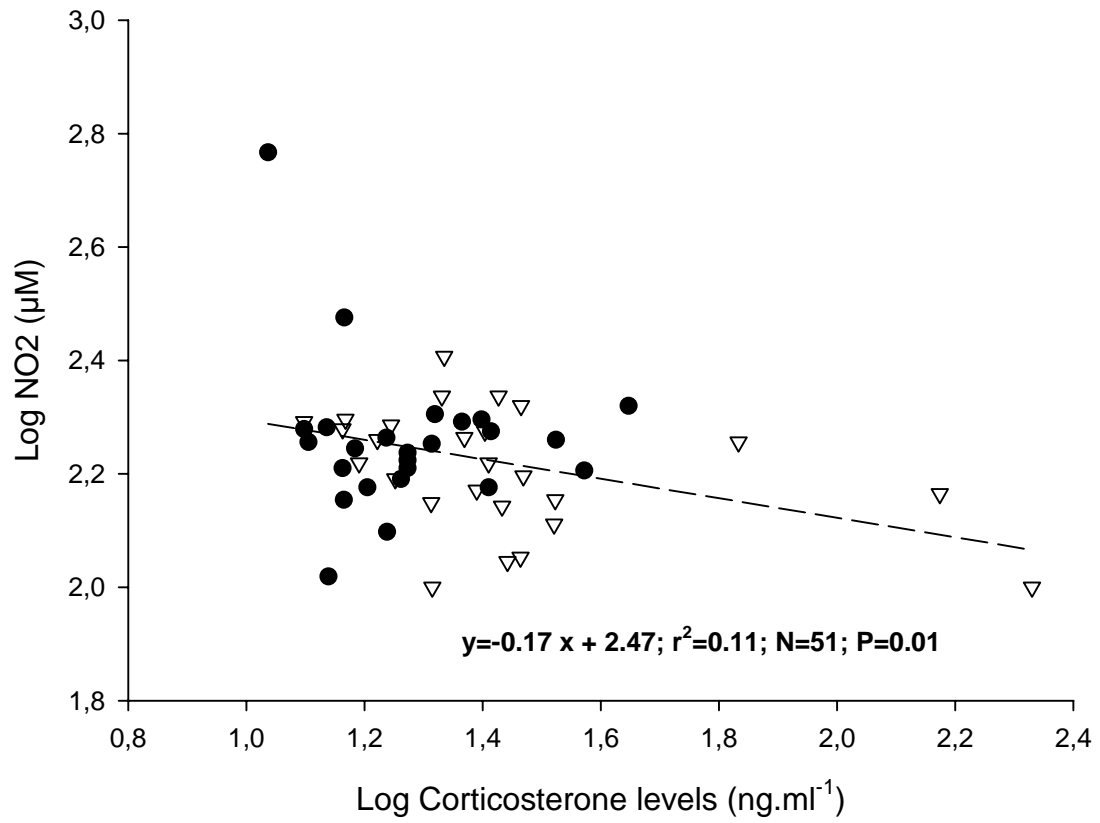
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1 **FIGURE 5.**

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1 **TABLE**

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3 **Table 1.** Profiles of female eiders according to their body condition. Body condition was
 4 calculated by dividing female initial body mass by (tarsus length)³. Females having an index
 5 ≤ 8.80 or > 8.80 were classified as in bad or good body condition, respectively. Values are
 6 means \pm SE.

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	Group 1: females in good body condition N=25	Group 2: females in bad body condition N=26	U-test	P
Initial body mass (g)	2018 \pm 23	1990 \pm 24	-0.48	0.63
Initial clutch size (eggs)	4.00 \pm 0.13	4.11 \pm 0.13	-0.50	0.62
Tarsus length (cm)	6.00 \pm 0.03	6.22 \pm 0.02	-4.48	<0.0001
Wing length (cm)	29.04 \pm 0.10	29.19 \pm 0.13	-0.73	0.47
Time of sampling (days)	16.64 \pm 1.11	19.19 \pm 1.03	-1.65	0.10
Body mass at sampling (g)	1643 \pm 34	1559 \pm 28	-1.87	0.06
Body condition index	9.33 \pm 0.09	8.28 \pm 0.09	-6.12	<0.0001
NO1 (μM)	112.45 \pm 13.29	101.42 \pm 8.72	-0.56	0.58
NO2 (μM)	192.47 \pm 17.79	166.31 \pm 7.69	-0.96	0.34
Innate immune response (%)	93.34 \pm 15.17	86.06 \pm 14.68	-0.09	0.92
Corticosterone (ng.ml⁻¹)	20.17 \pm 1.63	36.91 \pm 8.79	-2.50	0.01

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