



## Endocrine consequences of an acute stress under different thermal conditions: A study of corticosterone, prolactin, and thyroid hormones in the pigeon (*Columbia livia*)



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### ABSTRACT

In the context of global change, the physiological and hormonal stress responses have received much attention because of their implications in terms of allostasis. However, most studies have focused on glucocorticoids only as the “common” response to stressors while neglecting other endocrine axes and hormones (e.g. prolactin, thyroid hormones) that play a crucial role in metabolic adjustments. Interestingly, the responsiveness of all these endocrine axes to stress may depend on the energetic context and this context-dependent stress response has been overlooked so far. In the wild, temperature can vary to a large extent within a short time window and ambient temperature may affect these metabolic-related endocrine axes, and potentially, their responsiveness to an acute stressor. Here, we explicitly tested this hypothesis by examining the effect of a standardized stress protocol on multiple hormonal responses in the rock pigeon (*Columbia livia*). We tested the effect of an acute restraint stress on (1) corticosterone levels, (2) prolactin levels, and (3) thyroid hormone levels (triiodothyronine, thyroxine) in pigeons that were held either at cool temperature (experimental birds) or at room temperature (control birds) during the stress protocol. Although we found a significant influence of restraint stress on most hormone levels (corticosterone, prolactin, and thyroxine), triiodothyronine levels were not affected by the restraint stress. This demonstrates that stressors can have significant impact on multiple endocrine mechanisms. Importantly, all of these hormonal responses to stress were not affected by temperature, demonstrating that the exposure to cold temperature does not affect the way these hormone levels change in response to handling stress. This suggests that some endocrine responses to temperature decreases may be overridden by the endocrine responses to an acute restraint stress.

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### 1. Introduction

In the context of global change, there is growing interest in understanding how free-living vertebrates can cope with environmental stressors. In that respect, stress-related physiological mechanisms have received much attention because they are thought to both facilitate and to constrain the ability of individuals, populations, and species to adjust to our changing world (Wingfield, 2008; Wingfield et al., 2011). Specifically, the hypothalamo–pituitary–adrenal (HPA) axis appears relevant because this endocrine axis is thought to help vertebrates to cope with stressors in general (Wingfield et al., 1998; Wingfield, 2003; Romero et al., 2009). When a stressor is perceived by a vertebrate, this axis is activated, which results in a rapid increase of glucocorticosteroid secretion (corticosterone in birds, Sapolsky et al., 2000; Romero, 2004). This increase is important because it allows the individual to restore homeostasis through behavioural and physiological adjustments (McEwen

and Wingfield, 2003; Romero et al., 2009). Importantly, this glucocorticoid (GC) stress response varies between individuals, populations and species (Hau et al., 2010; Addis et al., 2011; Lendvai et al., 2013; Cockrem, 2013) and investigating the proximate basis of this variability is essential to understanding whether vertebrates will be able to adjust to new stressful situations or not (Angelier and Wingfield, 2013; Wingfield et al., 2015).

Research on the endocrine response to stress in wild vertebrates has been mainly limited to GC although other hormonal axes are certainly essential to the ability of individuals to cope with stress. Indeed, multiple stressors can occur in the wild (ex: food shortage, inclement weather, predator attack) and they may require specific behavioural and physiological adjustments that are under control of not only the HPA axis but also other endocrine mechanisms (Romero et al., 2009). Other hormones are known to regulate specific physiological or behavioural processes that may help an individual to cope with stress. For example, prolactin, a pleiotropic hormone, is involved in the regulation of parental behavior (Angelier and Chastel, 2009; Angelier et al., 2016), food intake (Buntin and Tesch, 1985; Koch et al., 2004), body mass regulation (Boswell et al., 1995; Holberton et al., 2008) and lipid

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metabolism (Ben-Jonathan et al., 2006). Similarly, thyroid hormones (triiodothyronine and thyroxine) are involved in metabolic processes (Vézina et al., 2009; Welcker et al., 2013; Elliot et al., 2013) and thermoregulation (McNabb, 2007) in birds. Modifications of all these physiological and behavioural processes are classically involved in the organismal response to stress in vertebrates (Sapolsky et al., 2000), and therefore, studying how prolactin and thyroid hormones levels change in response to stress could help us better understand how individuals cope with stress. A few studies have found that these hormones can be affected by stressors in captive laboratory animals but the results often appear inconsistent (prolactin: Angelier and Chastel, 2009; Angelier et al., 2016; thyroid hormones: Wodzicka-Tomaszewska et al., 1982; Williamson et al., 1985; Hangalapura et al., 2004; Etches et al., 2008). Moreover, we currently lack detailed data on these hormonal stress responses in wild vertebrates because most studies have been limited to only a few species (Angelier et al., 2013, 2016). Finally, the potential functional connection between the GC stress response and the responses of these other endocrine axes to stress remain overlooked in wild vertebrates. Therefore, it appears crucial to further investigate how these endocrine axes may be concomitantly affected by an acute stressor in wild vertebrates.

Interestingly, there is recent evidence that the levels of circulating prolactin and thyroid hormones may change in response to stressors under some circumstances but not others (prolactin: Angelier and Chastel, 2009; Angelier et al., 2016; thyroid hormones, Wodzicka-Tomaszewska et al., 1982; Williamson et al., 1985; Hangalapura et al., 2004; Etches et al., 2008). This suggests that the responsiveness of all these endocrine axes (GC, prolactin and thyroid hormones) to stress may depend on the environmental or the energetic context. For instance, the GC response to an acute stressor is known to be magnified when individuals are in poor body condition (Angelier et al., 2015), and similarly, several environmental factors can affect the intensity of the GC stress response (e.g., risk of predation, Pakkala et al., 2013). In the wild, temperature can vary to a large extent within a short time window (ex: a drop of several °C within a few hours) and such changes in temperature are probably among the most important factors affecting the energetic balance of wild vertebrates because individuals need to adjust their activity and metabolism to the ambient temperature. Several endocrine pathways are thought to govern the metabolic adjustments to cold temperatures (Hulbert, 2000; Sapolsky et al., 2000; Rozenboim et al., 2004), and importantly these endocrine pathways may also be involved in the metabolic responses to stress (Wingfield et al., 1998; McNabb, 2007; Angelier and Chastel, 2009). First, GC levels increase not only in response to stress (Wingfield et al., 1998, 2015; Romero, 2004; Landys et al., 2006; Angelier and Wingfield, 2013) but also in response to temperature changes in endotherms (de Bruijn and Romero, 2011, 2013; Lynn and Kern, 2014), probably because such increase promotes protein and lipid catabolism that sustain increased metabolism and heat production (Astheimer et al., 1992; Sapolsky et al., 2000; Landys et al., 2006; DuRant et al., 2008; Wack et al., 2012). Second, prolactin levels are also affected by stressors (Angelier and Chastel, 2009; Angelier et al., 2016) and by acute and chronic changes in ambient temperatures (Gahali et al., 2001; Rozenboim et al., 2004; Dawson and Sharp, 2010). The metabolic effects of prolactin remain overlooked in birds but changes in prolactin levels may help individuals coping with cold temperature because prolactin is involved in food intake (Buntin and Tesch, 1985; Koch et al., 2004), body mass regulation (Boswell et al., 1995; Holberton et al., 2008) and lipid metabolism (Ben-Jonathan et al., 2006). Finally, the hypothalamus–pituitary–thyroid (HPT) axis is not only affected by exposure to stressors (such as long-term fast or handling stress, e.g. Groscolas and Leloup, 1989) but also involved in the ability of vertebrates to cope with a change in ambient temperature (McNabb, 2007). Thus, triiodothyronine and thyroxine are known to activate heat production in poultry (Klandorf et al., 1981; Williamson et al., 1985; Collin et al., 2003; McNabb, 2007) and elevated thyroid hormone levels have been associated with increased metabolic expenditures in wild birds (Chastel et al., 2003; Criscuolo

et al., 2003; Vézina et al., 2009; Welcker et al., 2013; Elliot et al., 2013). Although the influence of temperature on the GC stress response has been investigated in a few studies (de Bruijn and Romero, 2011, 2013; Narayan et al., 2013; Dupoué et al., 2013; Lynn and Kern, 2014; Narayan and Hero, 2014), no study has, to our knowledge, examined this question on multiple endocrine pathways simultaneously in wild vertebrates.

In this study, our objective was to better understand how these endocrine pathways (corticosterone, prolactin, and thyroid hormones) are affected by a restraint stress under contrasting thermal conditions in a wild bird species, the rock pigeon (*Columba livia*). Specifically, we used the standardized restraint stress protocol established by Wingfield et al. (1992) and, concomitantly, we experimentally reduced the ambient temperature during the restraint period (experimental birds) or not (controls). Therefore, the experimental group had to cope with an acute stressor in a moderately cold environment (10 °C) whereas the control group had to cope with an acute stressor at ambient temperature (21 °C). In temperate areas, such drops of 11 °C are quite common and are part of the daily or seasonal routines of pigeons. We focused on three endocrine pathways, which are thought to govern the metabolic adjustments that allow individuals to cope with stressors (Wingfield et al., 1998; McNabb, 2007; Angelier and Chastel, 2009), and decreases in temperature (Hulbert, 2000; Sapolsky et al., 2000; Rozenboim et al., 2004). As in many other vertebrate species and as previously found in pigeons, we expected corticosterone levels to increase in response to the standardized stress protocol (Wingfield et al., 1992, 1995; Hau et al., 2010; Lendvai et al., 2013; Pakkala et al., 2013). We also predicted that the corticosterone stress response of experimental birds will be stronger than that of controls (prediction 1) because experimental birds may need to additionally activate corticosterone-related metabolic and behavioral adjustments to cold temperature (de Bruijn and Romero, 2011, 2013; Lynn and Kern, 2014). In addition, we expected prolactin levels to decrease in response to the restraint protocol as previously reported in several bird species (Angelier and Chastel, 2009; Angelier et al., 2016). Although the metabolic role of prolactin has been overlooked in birds, previous studies have reported that prolactin levels usually increase in response to changes in ambient temperatures in birds and laboratory rodents (Fregly, 1989; Gahali et al., 2001; Rozenboim et al., 2004; Dawson and Sharp, 2010). This suggests that increased prolactin levels may mediate a physiological and behavioral adjustment to cold temperature, and therefore, we predict that elevated prolactin levels will be maintained in experimental birds that are exposed to moderately cold temperatures despite the restraint stress (prediction 2). As previously reported in other studies (e.g. Wodzicka-Tomaszewska et al., 1982), we also expected that triiodothyronine and thyroxine levels will decrease in experimental and control pigeons in response to handling stress. Because elevated thyroid hormone levels trigger metabolic activities and heat production (McNabb, 2007), elevated thyroid hormone levels should be maintained when individuals have to cope with cold temperatures (Kühn and Nouwen, 1978; Bobek et al., 1980; Collin et al., 2003). Therefore, we predicted that thyroid hormone stress responses will be attenuated in experimental birds relative to controls (prediction 3). Finally, we also investigated whether corticosterone, prolactin and thyroid hormones levels were correlated in order to shed some light on the potential functional connections between these hormonal responses to stressors.

## 2. Materials & methods

### 2.1. Study model

Fieldwork was carried out in 2013 (1–9 May 2013) in Vallauris, France (43°34'N, 7°02'E). Rock pigeons are medium size birds (from 250 to 350 g) and their usual lifespan is about 4–5 years in the wild. This gregarious species is widespread all over the globe and originated from Europe, Asia, and North Africa. This species initially used cliffs to breed and build their nests but they are now very common in urban

and suburban areas (Del Hoyo et al., 1997). Rock pigeons are a relevant model for our research question because they are regularly confronted to changes in ambient temperature during their daily and seasonal routine. For instance, temperature typically decreases by 10–12 °C within a few hours at our study site in the evening and such a drop in temperature falls within the range of common and predictable environmental circumstances for this population. Finally, rock pigeons are commonly depredated by raptors and are therefore likely to encounter stressful predator attacks throughout their lifetime (Del Hoyo et al., 1997).

## 2.2. Capture, stress protocol, body size and body condition

Breeding pigeons were captured using Potter traps. All Potter traps were baited during the month preceding the onset of trapping with a commercial mix of corn and wheat seeds. The closing mechanism of these traps was blocked during that month to allow pigeons to become accustomed to entering the traps. At the beginning of the capture period, the mechanism was unlocked allowing capture. At capture, all birds were bled according to the standardized technique previously described by Wingfield et al. (1992). Immediately after capture in the Potter trap, an initial blood sample was collected within 3 min of capture from the alar vein with a 1-mL heparinized syringe and a 25-gauge needle (Lynn and Porter, 2008; Angelier et al., 2010). A second sample was taken 75 min after the first. Pigeons were kept in individual opaque cloth bags between the initial and second blood samples. In the field, blood samples were held at 4 °C until they were centrifuged. After this protocol, all birds were weighed to the nearest 1 g with a scale. Skull and tarsus lengths were measured to the nearest 0.1 mm with a caliper while their wing length was measured to the nearest 0.5 mm with a ruler. To assess body condition, we used the “scaled mass index” (hereafter SMI, see Peig and Green (2009) for further details). The SMI adjusts the mass of all individuals to that expected if they had the same body size. We used wing length to calculate the SMI because it was the morphological measure that was the best correlated with body mass ( $r = 0.843$ ,  $p < 0.001$ , Peig and Green (2009)). All pigeons were breeding as confirmed by the presence of a brood patch.

## 2.3. Thermal conditions

Immediately after the initial blood sample, restrained birds were randomly assigned to the experimental ( $n = 13$ ) or to the control group ( $n = 15$ ) for the duration of the standardized protocol (75 min). This specific duration was chosen over the classical 30 min restraint (Wingfield et al., 1992) to ensure that enough time was available to initiate metabolic adjustments to the experimental temperature (Østnes and Bech, 1998). While being kept in their opaque cloth bags, control birds were assigned to a room where the temperature was set at 21 °C which was similar to the outside temperature at the time of capture (range: 20–22 °C) while experimental birds were assigned to a similar room where the temperature was set at 10 °C. This latter temperature was selected because a rapid decrease of approximately 10–12 °C is realistic in a wild environment. Moreover, a temperature of 10 °C is also realistic during early May at our study site. Temperatures of the rooms were checked with a thermometer both when a bird was placed in and removed from the room. Temperatures were always steady during the standardized stress protocol. At the time of capture, experimental and control birds did not differ in body size, date of capture, and SMI (all  $p > 0.250$ ).

## 2.4. Hormone assays and molecular sexing

Blood samples were centrifuged, and plasma was decanted and stored at –20 °C until assayed. Plasma concentrations of corticosterone, triiodothyronine and thyroxine were determined by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé (CEBC), as previously described (corticosterone: Lormée et al., 2003; thyroid hormones:

Chastel et al., 2003). Because of the proteic nature of prolactin, prolactin levels were determined by using a heterologous radioimmunoassay as detailed in Angelier et al. (2007). Pooled plasma samples of rock pigeons produced a dose-response curve that paralleled chicken prolactin standard curves, therefore validating our prolactin assay for rock pigeons. All samples were run in one assay (intra-assay variation; corticosterone: 9.79%, prolactin: 8.56%, triiodothyronine: 10.08%, thyroxine: 3.60%). There was not enough plasma to assay prolactin levels for two pigeons. The sex was determined by polymerase chain reaction (PCR) amplification of part of two highly conserved genes (CHD) genes present on the sex chromosomes at the Centre d'Etudes Biologiques de Chizé (CEBC).

## 2.5. Statistical analyses

All statistical analyses were conducted with SAS software (v. 9.4, SAS Institute). We tested the influence of temperature on (1) the GC stress response; (2) the prolactin stress response; and (3) the triiodothyronine and thyroxine stress response by using mixed models (proc mixed, SAS Institute) with bird identity as a random factor. Specifically, we explained corticosterone, prolactin, triiodothyronine and thyroxine levels (dependent variables) with the treatment (experimental birds, controls), the time of sampling (baseline, stress-induced levels), sex (males, females), and their interactions as independent factors. Thus, we tested for a difference in hormone levels over time (change in hormone levels from baseline to 75 min), and then tested for difference in baseline and 75 min measures. Finally, we also tested whether corticosterone, prolactin, triiodothyronine and thyroxine levels and their changes in response to the standardized stress protocol were correlated by using correlations for baseline, and then, stress-induced levels. For these last analyses, we pooled data from experimental birds and controls because we did not find any evidence that our experimental treatment had any effect on these stress responses (see Results).

## 3. Results

### 3.1. Influence of the treatment on the corticosterone stress response

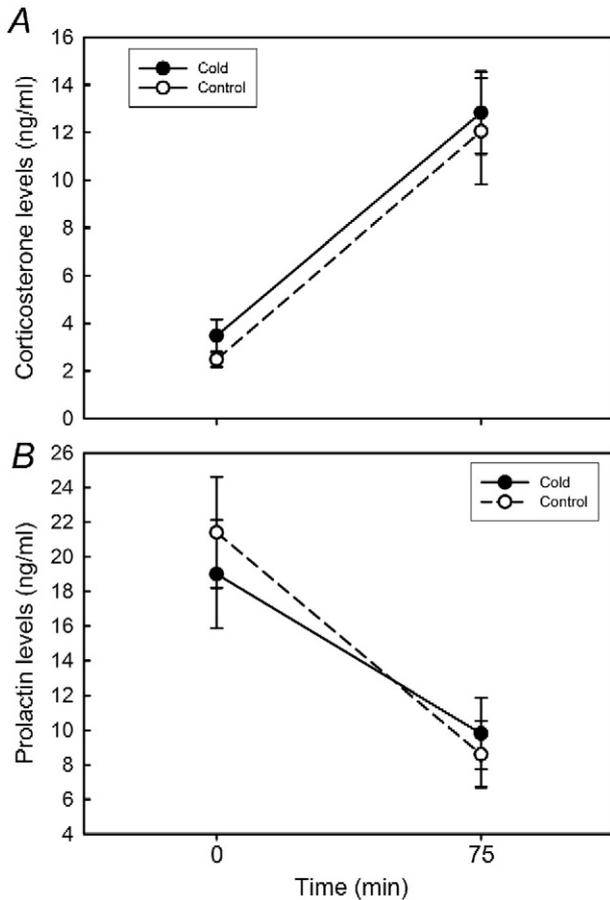
Corticosterone levels were affected by the time of sampling and corticosterone levels significantly increased in response to stress ( $F_{1,24} = 49.79$ ,  $p < 0.001$ , Fig. 1A). However, the corticosterone stress response (change in corticosterone levels in response to the standardized stress protocol) was not affected by the treatment ( $F_{1,24} = 0.04$ ,  $p = 0.837$ , Fig. 1A), the sex of individuals ( $F_{1,24} = 1.33$ ,  $p = 0.260$ ) or their interaction ( $F_{1,24} = 0.04$ ,  $p = 0.837$ ). Moreover, corticosterone levels were not affected by the treatment ( $F_{1,24} = 0.71$ ,  $p = 0.408$ , Fig. 1A), the sex of individuals ( $F_{1,24} = 1.45$ ,  $p = 0.241$ ), or their interaction ( $F_{1,24} = 1.38$ ,  $p = 0.253$ ).

### 3.2. Influence of the treatment on the prolactin stress responses

Prolactin levels were affected by the time of sampling and prolactin levels significantly decreased in response to stress ( $F_{1,22} = 61.68$ ,  $p < 0.001$ , Fig. 1B). However, this prolactin stress response (change in prolactin levels in response to the standardized stress protocol) was not affected by the treatment ( $F_{1,22} = 2.76$ ,  $p = 0.111$ , Fig. 1B), the sex of individuals ( $F_{1,22} = 0.39$ ,  $p = 0.539$ ) or their interaction ( $F_{1,22} = 1.45$ ,  $p = 0.241$ ). Prolactin levels were not affected by the treatment ( $F_{1,22} = 0.19$ ,  $p = 0.667$ , Fig. 1B), the sex of individuals ( $F_{1,22} = 0.11$ ,  $p = 0.747$ ), or their interaction ( $F_{1,22} = 1.21$ ,  $p = 0.283$ ).

### 3.3. Influence of the treatment on the triiodothyronine and thyroxine stress responses

Triiodothyronine levels were not significantly affected by the time of sampling ( $F_{1,24} = 0.23$ ,  $p = 0.636$ , Fig. 2A). Moreover, the triiodothyronine stress response (change in triiodothyronine levels in response to



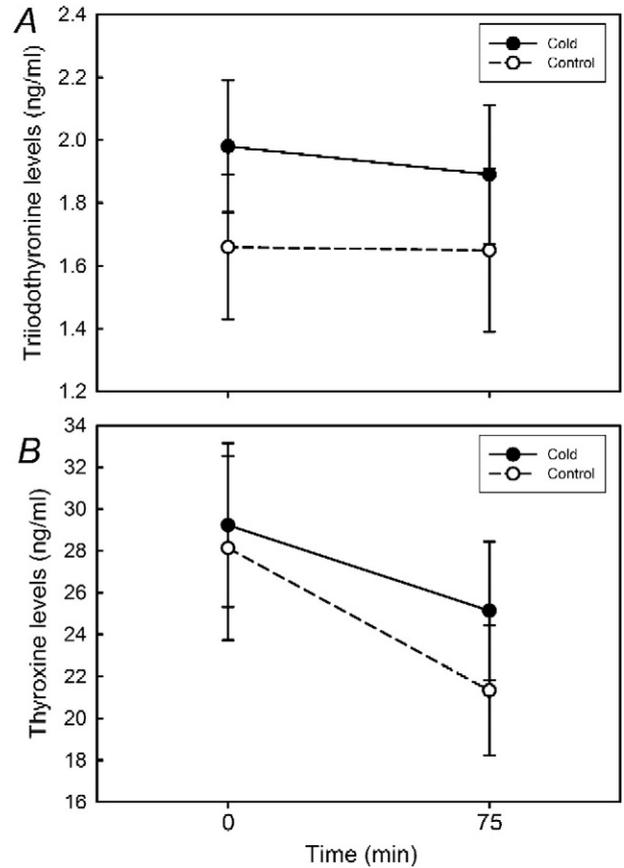
**Fig. 1.** Influence of the experimental treatment on (A) the corticosterone stress response, and (B) the prolactin stress response in rock pigeons. Experimental birds were kept at 10 °C during the 75-min restraint period while controls were kept at 21 °C. Data are expressed as means + standard error.

the standardized stress protocol) was not affected by the treatment ( $F_{1,24} = 0.25$ ,  $p = 0.625$ , Fig. 2A), the sex of individuals ( $F_{1,24} = 0.03$ ,  $p = 0.857$ ), or their interaction ( $F_{1,24} = 0.83$ ,  $p = 0.372$ ). Triiodothyronine levels were not affected by the treatment ( $F_{1,24} = 1.31$ ,  $p = 0.264$ , Fig. 2A), the sex of individuals ( $F_{1,24} = 1.66$ ,  $p = 0.209$ ), or their interaction ( $F_{1,24} = 0.93$ ,  $p = 0.345$ ).

Thyroxine levels were significantly affected by the time of sampling ( $F_{1,24} = 10.60$ ,  $p = 0.003$ , Fig. 2B). The thyroxine stress response (change in thyroxine levels in response to the standardized stress protocol) was not affected by the treatment ( $F_{1,24} = 1.04$ ,  $p = 0.319$ , Fig. 2B), the sex of individuals ( $F_{1,24} = 0.13$ ,  $p = 0.719$ ) or their interaction ( $F_{1,24} = 1.33$ ,  $p = 0.261$ ). Thyroxine levels were not affected by the treatment ( $F_{1,24} = 0.22$ ,  $p = 0.641$ , Fig. 2B), the sex of individuals ( $F_{1,24} = 0.01$ ,  $p = 0.929$ ), or their interaction ( $F_{1,24} < 0.01$ ,  $p = 0.979$ ).

#### 3.4. Correlations between corticosterone, triiodothyronine, thyroxine, and prolactin levels

Regarding baseline hormonal levels, we did not find any significant relationship between corticosterone, triiodothyronine, thyroxine and prolactin levels (all  $p > 0.05$ ). Regarding stress-induced hormonal levels, we did not find any significant relationship between corticosterone, triiodothyronine, thyroxine and prolactin levels (all  $p > 0.05$ ). Finally, we did not find any significant relationship between all the hormonal stress responses (corticosterone, prolactin, triiodothyronine, thyroxine, all  $p > 0.05$ ).



**Fig. 2.** Influence of the experimental treatment on (A) the triiodothyronine stress response, and (B) the thyroxine stress response in rock pigeons. Experimental birds were kept at 10 °C during the 75-min restraint period while controls were kept at 21 °C.

## 4. Discussion

In this study, we found a strong and significant effect of the standardized restraint stress protocol on most hormone levels (corticosterone, prolactin, and thyroxine) although triiodothyronine levels did not vary in response to such stressor. Interestingly and contrary to our predictions, these hormonal responses to stress were not affected by temperature, demonstrating that a moderate drop in temperature (i.e. 10 °C) does not affect the way these hormonal levels change in response to handling stress. This suggests that these hormones might not play a critical role in allowing rock pigeons to adjust to a moderate thermal challenge in a stressful context, although this is quite unlikely given the results from previous studies (corticosterone: de Bruijn and Romero, 2011, 2013; Lynn and Kern, 2014; prolactin: Gahali et al., 2001; Rozenboim et al., 2004; thyroid hormones: Bobek et al., 1980; Pilo et al., 1985; Williamson et al., 1985; Collin et al., 2003). Alternatively, our results may also suggest that some endocrine responses to sudden temperature decreases may be overridden by the endocrine responses to a dramatic life-threatening event (capture and restraint).

#### 4.1. The corticosterone stress response

As previously reported in numerous studies, we found that corticosterone levels increased in response to the standardized stress protocol (restraint; Wingfield et al., 1998; Hau et al., 2010; Lendvai et al., 2013; Pakkala et al., 2013). Contrary to our first prediction, experimental pigeons did not display a stronger corticosterone stress response than controls, demonstrating that the combination of a restraint stressor and an exposure to moderately cold temperature was not associated with a stronger corticosterone stress response compared to a restraint

stressor, alone. This suggests that rock pigeons did not modulate their corticosterone stress response according to the temperature. This may appear surprising because the corticosterone stress response has been shown to be modulated according to the individual state (e.g. age and body condition: Holberton and Wingfield, 2003; Wilcoxon et al., 2011; Goutte et al., 2010; Lynn et al., 2010; Angelier et al., 2015), and even according to the stress context in pigeons (e.g. predation risk, Pakkala et al., 2013). Moreover, the link between temperature and corticosterone secretion has previously been demonstrated in several species. For example, a change in temperature can elicit a rapid secretion of corticosterone in vertebrates such as starlings (de Bruijn and Romero, 2011, 2013), eastern bluebird chicks (Lynn and Kern, 2014) and reptiles and amphibians (Narayan et al., 2013; Dupoué et al., 2013; Narayan and Hero, 2014). Our findings suggest that the corticosterone response to the moderate drop in temperature may have been overridden by the response to handling stress. Indeed, a handling stress is certainly more threatening for individuals than an exposure to a moderate drop in temperature, potentially explaining this lack of influence of exposure to cold temperature on corticosterone levels.

Alternatively, it is possible that this change in temperature was not challenging enough to elicit a further increased secretion of corticosterone in rock pigeons. We exposed pigeons to a realistic but only moderate and transitory decrease in temperature (switch from 21 °C to 10 °C for 75 min). Instead, an intense corticosterone response to cold exposure may only be initiated either when temperature falls below a low threshold or/and when this thermal challenge is maintained for several hours. However, this is unlikely because previous studies have shown that a slight decrease in temperature was important enough to induce an increased secretion of corticosterone in another bird species (de Bruijn and Romero, 2011, 2013). Our results could also mean that the standardized stress protocol triggers a maximal stress response and that individuals could not secrete more corticosterone to cope with the drop on temperature. However, this interpretation is also quite unlikely because a previous study has reported that a predator attack can elicit a stronger corticosterone stress response than a restraint protocol in rock pigeons (Pakkala et al., 2013). Moreover, an ACTH injection (the precursor of corticosterone release) usually triggers an additional secretion of corticosterone in restrained birds (Romero, 2006; Lattin et al., 2012) although this is not always the case (e.g. Romero, 2006). Overall, the hormonal and physiological changes occurring in response to restraint stress are probably quite similar to what an animal needs to maintain body temperature in the face of a moderate drop in temperature (e.g. corticosterone is known to increase metabolic rate, Astheimer et al., 1992; Sapolsky et al., 2000; Landys et al., 2006). Therefore, the restrained pigeons with elevated corticosterone levels are probably already meeting the demands of the drop in temperature, meaning that they do not need to further increase their corticosterone levels to cope with this thermal challenge.

#### 4.2. The prolactin stress response

We found that prolactin levels dramatically and significantly decreased in response to the standardized stress protocol. Although a previous study did not find any significant effect of a 30 min handling stress on prolactin levels in a closely-related species (Miller et al., 2009), our study demonstrates that stressors can result in a sharp decrease in prolactin levels in this species as previously found in many other species (Angelier et al., 2016). Because prolactin is involved in the maintenance of parental care, such decrease in prolactin levels in response to stress means that breeding rock pigeons were likely to decrease, at least transitorily, their parental effort when an acute stressor occurs (Angelier and Chastel, 2009; Angelier et al., 2016). To further highlight the influence of stressors on parental effort, future studies are now required to test whether this prolactin response to stress may vary depending on life-history (breeding vs. non breeding) and breeding (incubating vs. chick-rearing) stages (Angelier et al., 2013).

Contrary to our prediction (prediction 2), this prolactin stress response was not affected by the experimental treatment, suggesting that prolactin may not play a major role in the ability of rock pigeons to cope with a transitory and moderate drop in temperature. Previous studies have found that prolactin levels increase in response to a thermal challenge but the results appear inconsistent (Gahali et al., 2001; Rozenboim et al., 2004; Dawson and Sharp, 2010). Although the metabolic effects of increased prolactin levels are not well-known in birds, prolactin is involved in hyperphagia, food intake (Buntin and Tesch, 1985; Koch et al., 2004) and lipogenesis (Boswell et al., 1995; Holberton et al., 2008), suggesting that changes in prolactin levels could help individuals to cope with changes in temperature. However, our study clearly emphasizes that capture and restraint-related stressors have a more intense effect on prolactin levels in comparison to moderate changes in temperature. Here again, we suggest that the prolactin response to the thermal challenge may have been overridden by the response to handling stress.

#### 4.3. Triiodothyronine, thyroxine, handling stress and thermal challenge

Although we found a significant decrease of thyroxine levels in response to the handling stress, such decrease was not apparent for triiodothyronine. However, a wide range of stressors has been found to induce a decrease in triiodothyronine levels in birds (restraint: Wodzicka-Tomaszewska et al., 1982, fasting: Harvey and Klandorf, 1983; Groscolas and Leloup, 1989; Lea et al., 1992; heat stress: Williamson et al., 1985; Etches et al., 2008). Triiodothyronine levels may only change after a long-term exposure to a stressor (Hangalapura et al., 2004), therefore explaining our results. For instance, triiodothyronine levels only decrease 2 hours after the initiation of the stressor in rats (Helmreich et al., 2006; Helmreich and Tylee, 2011). Alternatively, the energetic or environmental context appears to affect the influence of stress on triiodothyronine levels and may therefore be crucial to consider. For instance, prolonged fasting has been shown to induce a significant decrease in triiodothyronine levels in several species of birds (Harvey and Klandorf, 1983; Lea et al., 1992; Prakash et al., 1998) whereas such a decrease in triiodothyronine levels is not apparent when individuals have accumulated reserves to prepare for such a fasting event (Hohtola et al., 1994; Criscuolo et al., 2003).

Contrary to our third prediction (prediction 3), we found that triiodothyronine and thyroxine levels were not affected by the experimental treatment, suggesting that a drop in temperature does not necessarily affect these hormones in the short-term in rock pigeons (i.e. 75 min). In vertebrates, thyroid hormones are known to sustain metabolic activities and to allow thermoregulation (McNabb, 2007). Indeed, several studies have reported that a cold challenge is associated with increased heat production and metabolic rate (Arens and Cooper, 2005; Swanson and Licknes, 2006; Hawley et al., 2012), and therefore, with increased circulating triiodothyronine (Bobek et al., 1980; Pilo et al., 1985; Williamson et al., 1985; Fregly, 1989; Collin et al., 2003; Hangalapura et al., 2004) or thyroxine levels (Pallazolo and Quadri, 1987). The absence of changes in triiodothyronine and thyroxine levels in response to our experimental drop in temperature is therefore surprising because it suggests that rock pigeons did not adjust their metabolic rate and their heat production to the reduced temperature.

A potential explanation is that our experimental thermal manipulation may not have been intense enough to induce a strong need of thermoregulation. An increase in triiodothyronine level may only be detectable when individuals face an important thermal challenge, and most studies have indeed examined the effect of a drastic reduction in temperature on triiodothyronine levels (Bobek et al., 1980; Pilo et al., 1985; Collin et al., 2003). However, this explanation is quite unlikely because a slight decrease in temperature (from 26.5 to 17.5 °C) can be sufficient to induce an increase in triiodothyronine levels in birds (Kühn and Nouwen, 1978). However, we examined the effect of our thermal challenge on triiodothyronine levels after 75 min of treatment

only whereas the adjustment of thyroid hormone levels to a new thermal condition may require more time. Another possibility is that experimental birds entered torpor instead of increasing their heat production. Heterothermy is indeed known to occur in Columbiformes (Jensen and Bech, 1992; Schleucher, 2001) and individuals may adjust their metabolic rate to the ambient temperature only after a long-term exposure to cold temperatures (Saarela and Vakkuri, 1982; Rintamäki et al., 1986). Such heterothermy is especially obvious when pigeons are fasting (Laurila and Hohtola, 2005), suggesting that stressful conditions may be a prerequisite for heterothermy in this species. Under this scenario, pigeons would face a transient heterothermic phase before adjusting their metabolic rate and heat production to the new thermal conditions. During this phase, thyroid hormone levels would remain unchanged, and this might explain why triiodothyronine and thyroxine levels did not differ between experimental pigeons and controls in our study. Further experimental studies are obviously needed to test the potential interactive effect of cold and stress exposures on thyroid hormones levels in birds. To better understand the influence of cold temperatures on these hormonal responses, it would be interesting in further studies to measure other behavioral and morphological traits (fat content that may buffer heat loss at cold temperatures), shivering behavior (active effort to thermoregulate), body temperature (to measure heterothermy). Moreover, it would have been relevant to manipulate temperature without restraining the pigeons to disentangle more precisely the relative impact of the restraint and the thermal challenge on the triiodothyronine stress response. However, such a manipulation was impossible to conduct in wild birds because experimentally modifying the temperature requires holding them in captivity.

#### 4.4. Links between corticosterone, prolactin and thyroid hormones

Although we did not find any significant relationship between corticosterone and triiodothyronine or thyroxine levels, several studies have shown that the HPA and HPT axes are functionally linked (Decuyper et al., 1983; Kühn et al., 1998). Thus, glucocorticoid, ACTH, and dexamethasone administrations are known to reduce circulating triiodothyronine levels (Mitchell et al., 1986; Darras et al., 1996), suggesting that these endocrine pathways are, at least partly, functionally linked. However, this action of corticosterone on triiodothyronine levels is mediated through the binding of corticosterone on receptors that are located on specific tissues of the HPT axis (Decuyper et al., 1983; Kühn et al., 1998). Therefore, changes in triiodothyronine levels may require the maintenance of elevated corticosterone levels for an extended period of time and this lag between the secretion of corticosterone and its action on triiodothyronine levels could explain why we did not find any relationship between these two hormones in our study.

In this study, we also found no clear connection between corticosterone and prolactin levels. This is not so surprising because most studies failed to find any correlations between these two hormones (Angelier et al., 2013). Although there is strong evidence that corticosterone can affect prolactin secretion (Angelier et al., 2009; Tartu et al., 2015), it appears that the maintenance of elevated corticosterone levels for an extended period of time (i.e. several days) is necessary to impact prolactin levels (Angelier et al., 2016). This certainly explains why most studies, including ours, failed to find significant correlations between prolactin and corticosterone levels (Angelier et al., 2013). Overall, we did not find any clear and significant correlations between any of our hormones of interest (corticosterone, prolactin and thyroid hormones), suggesting that all these endocrine pathways are not tightly and functionally connected when an acute short-term stressor or a thermal challenge occurs. This emphasizes that corticosterone, prolactin, and thyroid hormones may mediate specific physiological and behavioral response to stressors. This also supports the idea that investigating the short-term adjustments of vertebrates to acute stressors may require eco-physiologists to examine multiple endocrine mechanisms (e.g. testosterone and oestradiol, Woodley and Lacy, 2010; Deviche

et al., 2012). Further studies are now needed not only to disentangle the specific physiological and behavioral consequences of these hormonal stress responses but also to determine the adaptive benefits and costs of these multiple endocrine responses to stress in wild birds.

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