



## Genetic differences in coping strategies in response to prolonged and repeated restraint in Japanese quail divergently selected for long or short tonic immobility

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### ABSTRACT

Exposure to fearful situations elicits behavioral and Hypothalamic–Pituitary–Adrenal (HPA) axis responses characteristic of the coping response of individual animals to counteract environmental challenges. The aim of this study was to investigate behavioral and corticotropic responses concomitantly following prolonged or repeated restraint stress by placing two genotypes of Japanese quail divergently selected for long (LTI) or short (STI) duration of tonic immobility (TI) in a crush cage. In our study, STI quail exhibited higher corticosterone (CORT) levels than LTI quail in response to prolonged restraint. STI quail struggled sooner and much more than LTI quail, and struggling behavior in STI quail progressively decreased during the course of restraint whereas LTI quail displayed very little struggling behavior in the crush cage. LTI quail are thus more likely to adopt a passive behavior coping strategy upon exposure to threat whereas STI quail behave more as active copers. The corticosterone responses shown by LTI and STI quail under restraint stress suggest that adrenocortical correlates of coping behavior in these genotypes of quail may be different from the coping styles previously described in other species. Repeated restraint slightly decreased CORT responses to stress in all experimental groups, but more markedly in male STI quail, whereas adrenal sensitivity and maximum adrenal corticosterone response capacity did not change in any group. On the other hand, neither behavioral habituation nor sensitization processes occurred in the context of repeated restraint in female and male LTI quail and female STI quail, whereas the decreases observed in some behavioral responses were interpreted to be the result of a habituation process in male STI quail.

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### Introduction

The elicitation of instant and adequate fear responses allows animals to cope with fearful situations (Jones et al., 1991). A consistent finding across species is that health and survival can be jeopardized whenever environmental stressors are too demanding. For this reason, and because few studies have paid attention to coping styles in birds, it is important to characterize and understand the mechanisms and factors underlying the strategy adopted by an individual to cope with environmental challenges.

Several studies of the behavioral and physiological responses to fearful situations in mammals to date (Dantzer and Mormède, 1983; Jones and Harvey, 1987) have provided characterization of the type of coping strategy developed by individuals to counteract environmental challenges (Benus et al., 1991; Koolhaas et al., 1999; Koolhaas et al., 2007). Coping response patterns have been classified in two distinct categories based first on behavior observations. One of these styles corresponds to an active response characterized by territorial control

and aggression, originally described by Cannon as the fight–flight response (Cannon, 1929). The second type of coping style, originally described by Engel and Schmale (1972) as the conservation-withdrawal response, is characterized by immobility and low levels of aggression. Since then specific features of active and passive behavioral, physiological and neuroendocrine responses have been characterized further in corresponding proactive and reactive male rats and mice (de Boer et al., 1990a; 1990b; Korte et al., 1992; 1997; van Oortmerssen and Bakker, 1981) and also in rodents exhibiting variations in aggression (Benus et al., 1991; Veenema et al., 2003a; 2003b; Veenema and Neumann, 2007).

In addition to coping strategies, habituation and/or sensitization phenomena can develop in response to prolonged or repeated stress. With habituation (i.e. acclimation), the psychological context of the stressor is changed, and the animal no longer perceives the stressor to be as noxious, resulting for example in lower glucocorticoid responses. However, changes in HPA axis physiology with the acclimation process results in a sensitization process (i.e. facilitation), demonstrated by enhanced glucocorticoid responses to novel stressors compared to the responses of nonacclimated animals (Romero, 2004). Various characteristics such as nature, duration, intensity and frequency of exposure to stressful situations can determine whether habituation

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or sensitization will predominate (Jones et al., 2000). For example, habituation is thought to be inversely related to stimulus intensity and positively related to the number of stress episodes, whereas sensitization and stimulus intensity are considered to be positively related (Servatius et al., 1994). Repeated exposure to stressors can lead to the development of chronic fear, pathological anxiety, depression, gastric disorders or even death (Dantzer and Mormède, 1983; Jones and Harvey, 1987; Jones, 1996; Pitman et al., 1988).

One physiological component of the stress response in vertebrates is the activation of the Hypothalamic–Pituitary–Adrenal (HPA) axis which results in the release of corticosteroids (i.e. corticosterone in birds) from the adrenal glands (Canoine et al., 2002; Harvey and Hall, 1990; Jones et al., 1994; Romero and Wingfield, 2001; Scott et al., 1983; Siegel, 1971). Corticosteroids contribute to the re-establishment of homeostasis via negative feedback mechanisms on hypothalamus and/or pituitary structures decreasing HPA axis activation (Canoine et al., 2002; Schulkin et al., 1994; 1998). They also act to facilitate adaptive behavioral responses by providing the metabolic requirements for flight or fight responses (reviewed in Sapolsky et al., 2000). These changes thus constitute essential components of an animal's adaptive response to environmental challenges (Dantzer and Mormède, 1983; Jones and Harvey, 1987; Pitman et al., 1988).

Genotype is one factor which has been shown to affect the individual's coping capacity in mice and chicks (Korte et al., 1997; van Oortmerssen and Bakker, 1981). Differences in behavioral responses and HPA axis reactivity have previously been reported in two divergent genotypes of Japanese quail selected for long (LTI) or short (STI) duration of tonic immobility (TI) (Mills and Faure, 1991). TI is an unlearned catatonic state and this behavioral response has been shown to be positively correlated with other measurements of fear (e.g. activity in open field test, emergence test) (Jones, 1986; Mills and Faure, 1991). Several behavioral tests conducted in LTI and STI quail have led to the conclusion that LTI quail are more fearful than STI quail (Faure and Mills, 1998). Indeed, STI quail chicks freeze less, vocalize and walk sooner and more in an open field test than LTI quail chicks which show more freezing behavior (Jones et al., 1991). STI quail chicks also emerge sooner from a hole-in-the-wall box than LTI quail chicks (Jones et al., 1991). On the other hand, higher CORT responses following restraint in a crush cage have been reported in STI quail than in LTI quail (Hazard et al., 2005; 2008; Jones et al., 1994; Rémignon et al., 1998). Investigation of the function of the pituitary–adrenal axis with corticosterone measurement suggested that the corticotropin-releasing factor pathway might be involved in the differences in HPA axis reactivity to stress between LTI and STI genotypes, and not the adrenal level (Hazard et al., 2007). However, behavioral and corticosterone responses during restraint in a crush cage have never been investigated concomitantly, either under acute stress conditions or in a chronic stress situation. According to the results described above, LTI and STI quail genotypes are particularly appropriate models for exploring the coping strategies developed in birds submitted to stress because phenotypic differences should tend towards maximum contrast following bidirectional selection, such as the TI response (Ramos and Mormède, 1998).

The aims of the present study were 1) to compare the behavioral responses characterizing LTI and STI genotypes under restraint stress and assess whether the selected quail could be considered to have two different coping strategies, 2) to compare the adrenocortical responses between genotypes under restraint and assess whether the behavior–hormone correlates in LTI and STI genotypes of quail are consistent with previously described coping styles in other avian and mammalian species, and 3) to determine whether habituation or sensitization of behavior and adrenocortical responses occur under repeated restraint stress in these genotypes. These questions were addressed by simultaneously investigating corticotropic and behavioral responses in LTI and STI quail of both sexes submitted to prolonged restraint stress or repeated restraint stress. These two

complementary experimental designs allowed investigation of behavioral and physiological responses to acute (i.e. prolonged restraint) and chronic (i.e. repeated restraint) stress which is essential to poultry well-being and productivity, since chronic stress has been reported to increase fearfulness, reduce disease resistance, and impair egg production, growth and product quality (Jones et al., 1988; Jones, 1996).

## Materials and methods

### *Animals and their management*

Japanese quail (*Coturnix japonica*) from the thirty-sixth generation of two divergent genotypes selected for short (STI) or long (LTI) duration of tonic immobility (TI) were used in the study (Mills and Faure, 1991). Quail were identified by wing banding on the day of hatching. They were exposed to continuous light until 3 weeks of age, then to a 16 h light/8 h dark (16 L/8D) rhythm (light on 6.00 am). Quail from LTI and STI genotypes, and quail used in distinct experiments, were reared separately in collective battery cages. A caretaker checked the quail daily in the morning (from 08.30 h) and refilled the feeders whenever necessary (at least twice a week). No care was provided on the day of the experiment to avoid disturbance. Food and water were provided *ad libitum*. All experiments were carried out according to the legislation governing the ethical treatment of animals according to the European Community's Directive of November 24, 1986 (86/609/EEC), and investigators were authorized by the French governmental authority to carry out these experiments (No. 06255).

### *Experimental procedures*

Groups of 4–6 quail of the same genotype and sex were randomly constituted and placed in wire rearing battery cages one week before the experiments. At the age of 6 weeks, quail were submitted to prolonged restraint (experiment 1), repeated restraint and/or ACTH challenge (experiment 2). Quail from both genotypes and sexes were included in each experiment. All the experiments were performed in a test room close to but separated from the rearing room. Quail were captured in their home cage and immediately submitted to specific treatments (restraint or ACTH challenge) in the test room. Behaviors expressed by restrained quail were video-recorded and/or blood was collected from each quail following each treatment for corticosterone measurement. A group of quail bled immediately after capture and transferred from their home cage to the test room was included in each trial to assess basal CORT concentrations in the specific conditions of each experiment. This brief capture and transfer did not affect basal CORT levels (Hazard et al., 2008).

#### *Experiment 1: prolonged restraint*

A total of 32 six-week-old quail (8 per sex and genotype) were restrained individually in a crush cage (see detailed description below) or a period of 120 min. Behaviors expressed by restrained quail were video-recorded throughout the 120 min experimental period. In order to analyze the evolution of the behaviors expressed during restraint, the 120 min period was subdivided into 7 periods (0 to 10 min, 10 to 20 min, 20 to 40 min, 40 to 60 min, 60 to 80 min, 80 to 100 min and 100 to 120 min). These quail were sacrificed at the end of the 120 min restraint period for CORT measurement. Some additional quail (3 to 4 quail per experimental group) were submitted to the 120 min restraint period, but not video-recorded, in order to increase the number of quail for physiological measurements.

Intermediate CORT measurements were taken by sacrificing a second batch of quail ( $n=120$ ) at 10, 30 and 60 min after the beginning of the restraint period. These quail were not video-recorded. A complementary group of non-restrained quail ( $n=42$ ) was included as

control in order to measure basal CORT concentrations before each experimental protocol.

#### *Experiment 2: repeated restraint*

Experiments were performed on 5 consecutive days. On day 1 (D1), basal CORT levels ( $n=41$ ), CORT levels induced in response to restraint in the crush cage for 10 min ( $n=44$ ), adrenal sensitivity ( $n=45$ ) and maximum CORT adrenal response capacity ( $n=45$ ) in response to ACTH challenge (see below) were measured in naive quail which have not previously been submitted to any treatment.

From day 1 to day 4, each quail from a second batch was subjected to the restraint test 8 times for a 10 min period, twice daily (morning and afternoon). Immediately after each restraint, quail were placed back in their respective home cage. On the morning of the 5th day (D5), basal CORT levels ( $n=43$ ), CORT levels induced in response to the ninth episode of restraint ( $n=45$ ), adrenal sensitivity ( $n=50$ ) and maximum CORT adrenal response capacity ( $n=47$ ) were measured in quail previously submitted to 8 episodes of restraint. Quail used for the ninth episode of restraint were video-recorded during the first (D1) and ninth (D5) restraint episodes in order to monitor the behaviors expressed.

#### *Specific treatments*

##### *Restraint in a crush cage*

Quail were placed individually in a “crush cage” consisting of a wooden box (15 cm long  $\times$  5 to 10 cm wide  $\times$  10 cm high). A movable, close-fitting divider was placed against the quail to fix immobilize it. The walls touched the birds' sides, back and belly. Quail could stand but could not jump or spread their wings. The crush cage was closed at the top by a netting cover which made it possible to video-record behavior during restraint. This restraint test differed from the selection test which consisted of placing each quail on its back in a U-shaped cradle, hand-restraining it for 10 seconds to induce tonic immobility and then releasing it (Mills and Faure, 1991).

##### *ACTH challenge*

Quail were weighed individually in order to adjust the dose injected to body weight. Quail were injected in the *pectoralis major* muscle with mammalian 1–24 ACTH (Immediate Synacthen, Novartis, France; 1 mg = 100 IU) at the dose of 2.5  $\mu$ g/kg of body weight (BW) or 10  $\mu$ g/kg BW diluted in saline solution (0.9% NaCl w/v) in order to test (1) adrenal sensitivity and (2) maximum CORT adrenal response capacity, respectively. Quail were placed back in their home cage for 10 min immediately after the injection and then bled. Control quail received a representative volume of the vehicle (0.9% NaCl; 400 to 700  $\mu$ l per animal) and were also bled 10 min later.

##### *Blood sample collection and corticosterone assay*

Blood samples were collected from each quail directly into a tube containing EDTA (2 mg/ml blood) following sacrifice by decapitation after submission to the different treatments. All samples were temporarily stored on ice. Following centrifugation at 2000 g for 15 min at 4 °C, plasma samples were separated and stored deep frozen at –20 °C until measurement of corticosterone using a specific radio immunoassay (Etches, 1976). Measurements were performed in duplicate through six assays and the intra and inter-assay coefficients of variation (%) were 10.1 and 28.8, respectively. The smallest amount of corticosterone which could be detected using this method varied between 50 and 100 pg.

Sacrifice by decapitation was used in this study since we have previously shown this sampling procedure affects basal CORT levels least compared to venipuncture, cardiac or jugular puncture (Hazard et al., 2004). Using this procedure, we were able to observe even very small amplitude responses. Birds were never blood sampled on more

than one occasion. Serial bleedings were not considered since several studies in avian species have reported that serial bleedings increase CORT levels (Beuving and Vonder, 1986; Harvey et al., 1980; Johnson and van Tienhoven, 1981).

#### *Behavior video-recording*

Behaviors expressed by restrained quail were video-recorded through the top of the crush cage throughout the 120-minute long experimental periods and analyzed by focal sampling using The Observer 3.0 software (Noldus Information Technology, The Netherlands, 1993). The behavior features analyzed in both experiments (1 and 2) were latency to the first head movement and body struggling, time spent struggling and moving the head, number of struggling bouts and head movements as well as number of pecks at the walls of the crush cage. Head movements were recorded when quail moved to both sides successively at regular intervals.

#### *Statistical analysis*

Statistical analysis was performed using the Statview™ V program (Abacus Concept Inc. Brekeley, USA).

CORT values were subjected to multifactorial ANOVA to assess the effects of genotype, sex, treatment (prolonged restraint or repeated restraint) and their interactions. Normality of corticosterone data and equality of the variances of the different groups were checked before performing ANOVA. Whenever specific factor and interaction effects reached significance ( $p < 0.05$ ), *post hoc* tests were performed using the Fisher test (PLSD).

Prior to statistical analysis, behavioral data were log-transformed in order to achieve normality. In experiment 1, repeated multifactorial ANOVA were performed to assess the significance of genotype, sex and restraint duration effect, as well as their interactions. Point to point comparison of behavioral responses expressed during the different successive periods was performed using *t*-tests for repeated measures. In experiment 2, multifactorial ANOVA was performed to assess the significance of genotype, sex, repeated restraint effect and their interactions. CORT values and behavioral data are expressed as means  $\pm$  standard error. The alpha level of significance was  $p < 0.05$ .

## **Results**

### *Experiment 1: prolonged restraint*

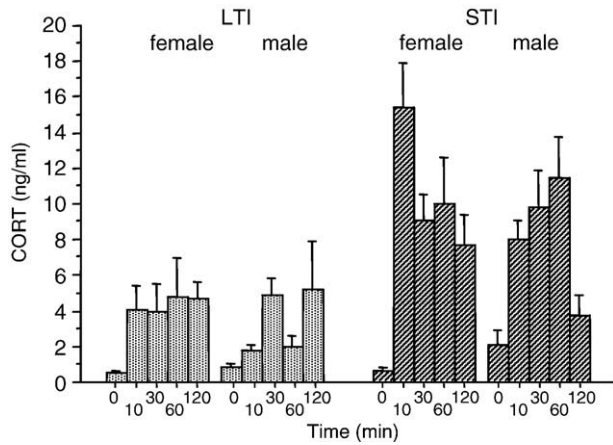
#### *Corticosterone*

Placement in the crush cage induced significant increases in CORT levels in quail according to genotype ( $F_{1, 185} = 38.63$ ,  $p < 0.0001$ ) equally in both sexes ( $F_{1, 185} = 2.30$ ,  $p = 0.1$ ) (Fig. 1). CORT levels induced by restraint were significantly higher in STI quail (8.6 times basal level) than in LTI quail (4.7 times basal level) whereas basal CORT levels measured in control quail did not differ significantly between the 2 genotypes. In LTI quail, restraint-induced CORT levels remained at high levels from 10 (3.1  $\pm$  0.8 ng/ml) to 120 min (5.0  $\pm$  1.3 ng/ml). In STI quail, restraint-induced CORT levels were maintained at higher levels from 10 (11.4  $\pm$  1.5 ng/ml) to 60 min (10.7  $\pm$  1.7 ng/ml) and then reached intermediate values (5.7  $\pm$  1.0 ng/ml) 120 min after the beginning of the restraint period.

#### *Behaviors*

*Struggling.* Duration of latency to the first struggling bout was significantly longer in LTI quail (514.3  $\pm$  125.0 s) than in STI quail (17.5  $\pm$  5.5 s) ( $F_{1, 26} = 53.81$ ,  $p < 0.0001$ ) but did not differ significantly between sexes ( $F_{1, 26} = 1.35$ ,  $p = 0.3$ ) (Fig. 2A).

Durations of each struggling bout did not change throughout prolonged restraint in Experiment 1. Therefore, only frequency of



**Fig. 1.** Changes in corticosterone concentrations over time (CORT, ng/ml plasma) measured in LTI and STI quail following restraint periods in a crush cage of varying durations (0, 10, 30, 60 or 120 min) ( $8 \leq n \leq 12$  quail per experimental point,  $n_{\text{total}}=207$ ) (means  $\pm$  SE) (ANOVA, genotype effect:  $F_{1, 185}=38.63$ ,  $p<0.0001$ , time effect:  $F_{4, 185}=10.68$ ,  $p<0.0001$ , sex effect:  $F_{1, 185}=2.30$ ,  $p=0.13$ , genotype  $\times$  time effect:  $F_{4, 185}=5.25$ ,  $p=0.0005$ , genotype  $\times$  sex effect:  $F_{1, 185}=0.36$ ,  $p=0.55$ , time  $\times$  sex effect:  $F_{4, 185}=2.0$ ,  $p=0.098$ ).

struggling bouts and not time spent expressing these behaviors was reported in Experiment 1 in order to avoid redundancy. The number of struggling bouts was significantly affected by genotype factor ( $F_{1, 28}=46.20$ ,  $p<0.0001$ ), duration of restraint ( $F_{6, 168}=21.22$ ,  $p<0.0001$ ), their respective interaction (time  $\times$  genotype,  $F_{6, 168}=10.04$ ,  $p<0.0001$ ) and sex ( $F_{1, 28}=5.52$ ,  $p=0.03$ ) (Fig. 2B). At the onset of restraint, significantly more struggling bouts occurred in STI quail than in LTI quail, as well as in male quail compared to female quail in both genotypes. In STI quail, the number of struggling bouts significantly decreased with time during the restraint period (from  $6.0 \pm 1.4$  and  $7.4 \pm 0.9$  bouts per min in female and male quail during the first period of restraint (i.e. 0 to 10 min) to  $0.9 \pm 0.3$  and  $2.3 \pm 0.9$  bouts per min by the end of the restraint period, respectively,  $F_{6, 90}=27.11$ ,  $p<0.0001$ ). In LTI quail, the number of struggling bouts slightly increased throughout the first 40 min of restraint and then decreased slightly but significantly ( $F_{6, 90}=3.38$ ,  $p=0.005$ ) and remained at low levels thereafter (in average  $0.6 \pm 0.3$  bout/min).

**Head movement** (see Supplementary file 1). The duration of latency to the first head movement was significantly longer in LTI quail ( $348.3 \pm 90.7$  s) than in STI quail ( $78.0 \pm 34.6$  s) ( $F_{1, 26}=8.30$ ,  $p=0.008$ ) and did not differ significantly between sexes ( $F_{1, 26}=0.5$ ,  $p=0.5$ ) (Supplementary file 1A).

Duration of restraint significantly affected ( $F_{6, 168}=4.49$ ,  $p=0.0003$ ) the number of head movements according to genotype (genotype  $\times$  time effect:  $F_{6, 168}=4.29$ ,  $p=0.0005$ ) and sex (sex  $\times$  time effect:  $F_{6, 168}=2.15$ ,  $p=0.05$ ) (Supplementary file 1B). The number of head movements during restraint was similar in male and female quail of the LTI genotype ( $F_{1, 14}=1.08$ ,  $p=0.3$ ) whereas it was significantly higher in male quail than in female quail of STI genotype ( $F_{1, 14}=13.83$ ,  $p=0.002$ ). In LTI quail of both sexes, the number of head movements during the 10 to 40 min period of restraint was significantly higher than the number measured during the first period of restraint (i.e. 0 to 10 min) and over 40 min of restraint ( $F_{6, 90}=4.39$ ,  $p=0.0006$ ). In male STI quail, the number of head movements gradually and significantly decreased with the increase in duration of restraint (from  $8.5 \pm 1.9$  to  $3.0 \pm 1.0$  bouts per min,  $F_{6, 42}=5.36$ ,  $p=0.0003$ ) whereas in female STI quail it remained fairly stable at a low level (i.e.  $1.6 \pm 0.3$  bouts per min) throughout restraint ( $F_{6, 42}=0.94$ ,  $p=0.5$ ).

**Number of pecks** (see Supplementary file 2). The number of pecks at the walls of the restraint cage was significantly affected by genotype ( $F_{1, 28}=8.84$ ,  $p=0.006$ ) and sex ( $F_{1, 28}=14.32$ ,  $p=0.0007$ ) factors and by

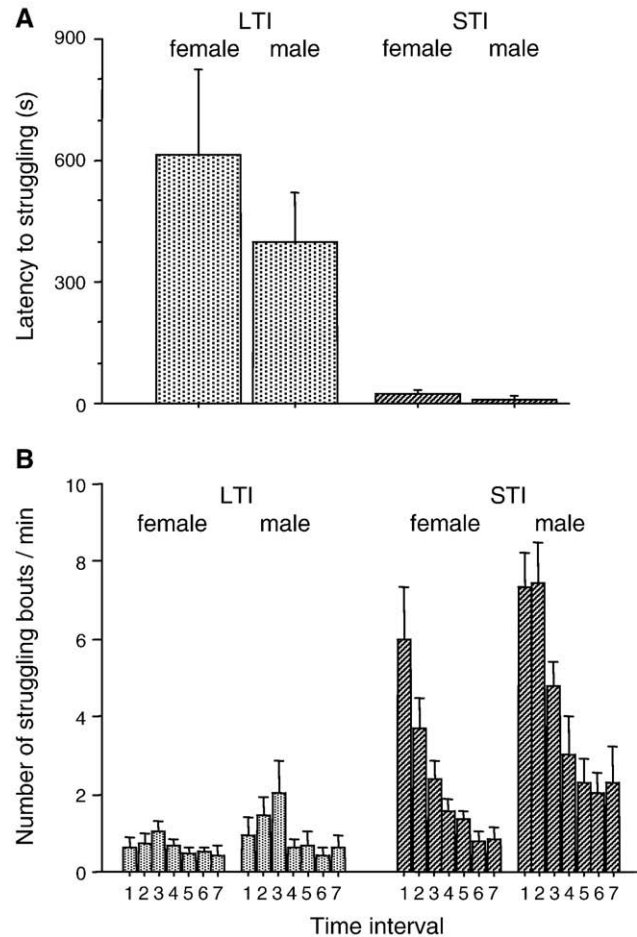
their interaction ( $F_{1, 28}=7.63$ ,  $p=0.01$ ) (Supplementary file 2) but it was not affected by the duration of restraint ( $F_{6, 168}=1.52$ ,  $p=0.2$ ). Male STI quail gave significantly more pecks than male LTI quail throughout the restraint period ( $F_{1, 14}=8.46$ ,  $p=0.01$ ) whereas the numbers of pecks were similar in females of both genotypes ( $F_{1, 14}=0.40$ ,  $p=0.5$ ). In STI quail, males gave significantly more pecks than females ( $F_{1, 14}=12.05$ ,  $p=0.004$ ) while no significant difference was observed between sexes in LTI quail ( $F_{1, 14}=2.36$ ,  $p=0.15$ ).

#### Experiment 2: repeated restraint

##### Corticosterone

Basal CORT levels were low in all groups of quail and did not differ significantly between genotypes ( $F_{1, 76}=0.54$ ,  $p=0.5$ ) or sexes ( $F_{1, 76}=0.74$ ,  $p=0.4$ ) or after repeated restraint ( $F_{1, 76}=2.11$ ,  $p=0.1$ ) (Fig. 3A).

Being placed in the crush cage for the first time was associated with significant increases in CORT levels compared to basal CORT levels in both LTI quail ( $F_{1, 43}=7.05$ ,  $p=0.01$ ) and STI quail ( $F_{1, 38}=33.22$ ,  $p<0.0001$ ) (Day 1, Fig. 3A vs Fig. 3B) while being placed in the crush cage for the ninth time induced significant increases in CORT levels in



**Fig. 2.** Latency to struggling (s) (A) (ANOVA (log), genotype effect:  $F_{1, 26}=53.81$ ,  $p<0.0001$ , sex effect:  $F_{1, 26}=1.35$ ,  $p=0.3$ , genotype  $\times$  sex effect:  $F_{1, 26}=1.14$ ,  $p=0.3$ ) and number of struggling bouts per min measured in LTI and STI quail following restraint in a crush cage for periods of 0–10 (time interval 1), 10–20 (time interval 2), 20–40 (time interval 3), 40–60 (time interval 4), 60–80 (time interval 5), 80–100 (time interval 6) and 100–120 min (time interval 7) (B) (Repeated ANOVA (log), genotype effect:  $F_{1, 28}=46.20$ ,  $p<0.0001$ , sex effect:  $F_{1, 28}=5.52$ ,  $p=0.03$ , genotype  $\times$  sex effect:  $F_{1, 28}=1.67$ ,  $p=0.2$ , time effect:  $F_{6, 168}=21.22$ ,  $p<0.0001$ , genotype  $\times$  time effect:  $F_{6, 168}=10.04$ ,  $p<0.0001$ , sex  $\times$  time effect:  $F_{6, 168}=1.15$ ,  $p=0.3$ ) ( $n=8$  quail per experimental point) (means  $\pm$  SE).

STI quail ( $F_{1, 41}=35.77, p<0.001$ ) but not in LTI quail ( $F_{1, 43}=2.49, p=0.1$ ) (Day 5, Fig. 3A vs Fig. 3B). CORT levels induced in response to restraint were significantly higher in STI quail than in LTI quail ( $F_{1, 81}=98.99, p<0.0001$ ) and CORT levels induced in male STI quail were slightly lower than those induced in female STI quail (interaction genotype and sex:  $F_{1, 81}=5.05, p=0.03$ ) (Fig. 3B). Observation of a significant sex effect ( $F_{1, 81}=11.03, p=0.002$ ) and a significant interaction between sex and genotype ( $F_{1, 81}=5.05, p=0.03$ ) indicated that CORT levels induced in response to restraint in the crush cage were significantly higher in female STI quail than in male STI quail,

whereas CORT levels did not differ between sexes in LTI quail. Repeated restraint did not significantly affect restraint-induced CORT levels ( $F_{1, 81}=3.23, p=0.08$ ).

Injection of 1–24 ACTH at a dose of 2.5  $\mu\text{g}/\text{kg}$  BW to check for adrenal sensitivity induced significant increases in CORT levels in all groups of quail ( $F_{2, 132}=25.93, p<0.0001$ ) compared to saline-injected quail, and increases were significantly higher in STI quail than in LTI quail ( $F_{1, 87}=6.82, p=0.01$ ) (Fig. 3C). Repeated restraint ( $F_{1, 87}=0.05, p=0.8$ ) and sex ( $F_{1, 87}=0.06, p=0.8$ ) did not significantly affect adrenal sensitivity.

Injection of 1–24 ACTH at a dose of 10  $\mu\text{g}/\text{kg}$  BW to investigate maximum CORT adrenal response capacity induced significant increases in CORT levels in all groups of quail (Fig. 3D) ( $F_{2, 129}=58.75, p<0.0001$ ) compared to saline-injected quail. CORT responses were not significantly affected by repeated restraint ( $F_{1, 84}=0.52, p=0.5$ ) and were similar between both genotypes ( $F_{1, 84}=1.58, p=0.2$ ), but significantly higher in female quail than in male quail ( $F_{1, 84}=9.57, p=0.003$ ).

### Behavior

**Struggling.** Latency to struggling was significantly longer in LTI quail than in STI quail ( $F_{1, 82}=116.58, p<0.0001$ ) but remained similar between the first and the ninth episodes of restraint ( $F_{1, 82}=2.37, p=0.13$ ) (Fig. 4A). Significant interaction between sex and repeated restraint factors ( $F_{1, 82}=6.88, p=0.01$ ) indicated that females showed significantly longer latency than males ( $F_{1, 82}=22.06, p<0.0001$ ) during the first episode of restraint but not during the ninth episode.

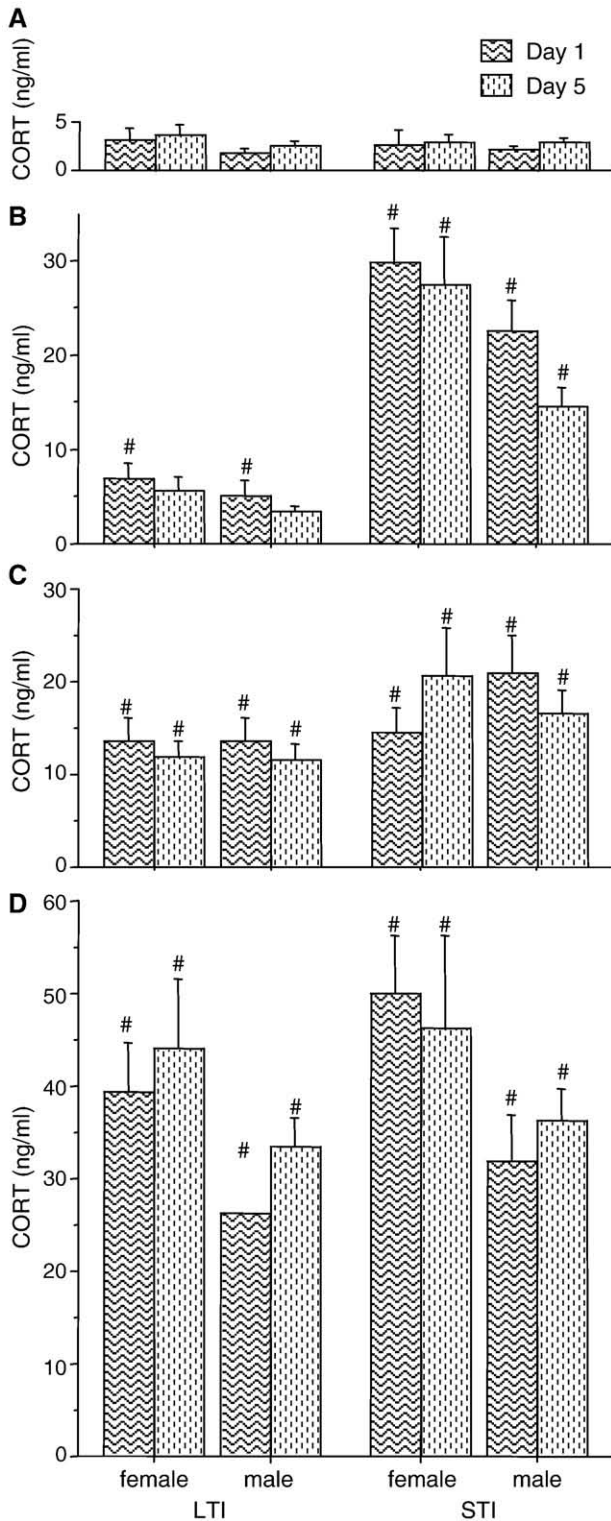
The numbers of struggling bouts were also significantly higher in STI than in LTI quail ( $F_{1, 82}=115.04, p<0.0001$ ) and in male than in female quail ( $F_{1, 82}=15.51, p=0.0002$ ) (Fig. 4B). However, repeated restraint did not affect the number of struggling bouts ( $F_{1, 82}=0.54, p=0.5$ ).

Time spent struggling was significantly higher in STI quail than in LTI quail ( $F_{1, 82}=78.39, p<0.0001$ ) and was also significantly higher in male than in female quail ( $F_{1, 82}=32.08, p<0.0001$ ) (Fig. 4C). Time spent struggling decreased significantly with repeated restraint ( $F_{1, 82}=5.67, p=0.02$ ) and the greatest decrease was observed in male STI quail.

### Head movement

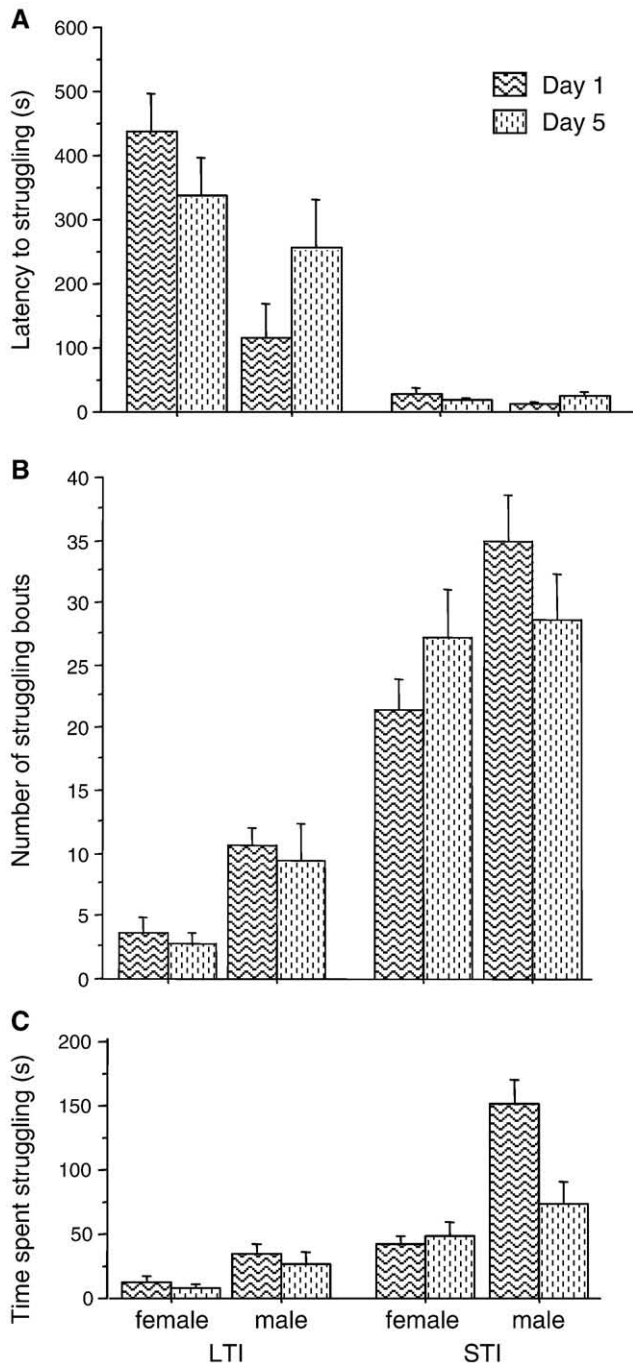
Latency to the first head movement was similar between both genotypes ( $F_{1, 82}=0.57, p=0.45$ ) and sexes ( $F_{1, 82}=3.0, p=0.09$ ) (Fig. 5A). Repeated restraint did not significantly affect latency to the first head movement ( $F_{1, 82}<0.0001, p=0.99$ ) although latencies decreased in female LTI quail and increased slightly in male quail.

Numbers of head movements were not affected by genotype of quail ( $F_{1, 82}=0.45, p=0.5$ ) or by repeated restraint ( $F_{1, 82}=0.18, p=0.7$ ) (Fig. 5B). On the other hand, male quail of both genotypes moved their heads during restraint significantly more often than female quail ( $F_{1, 82}=9.96, p=0.002$ ).

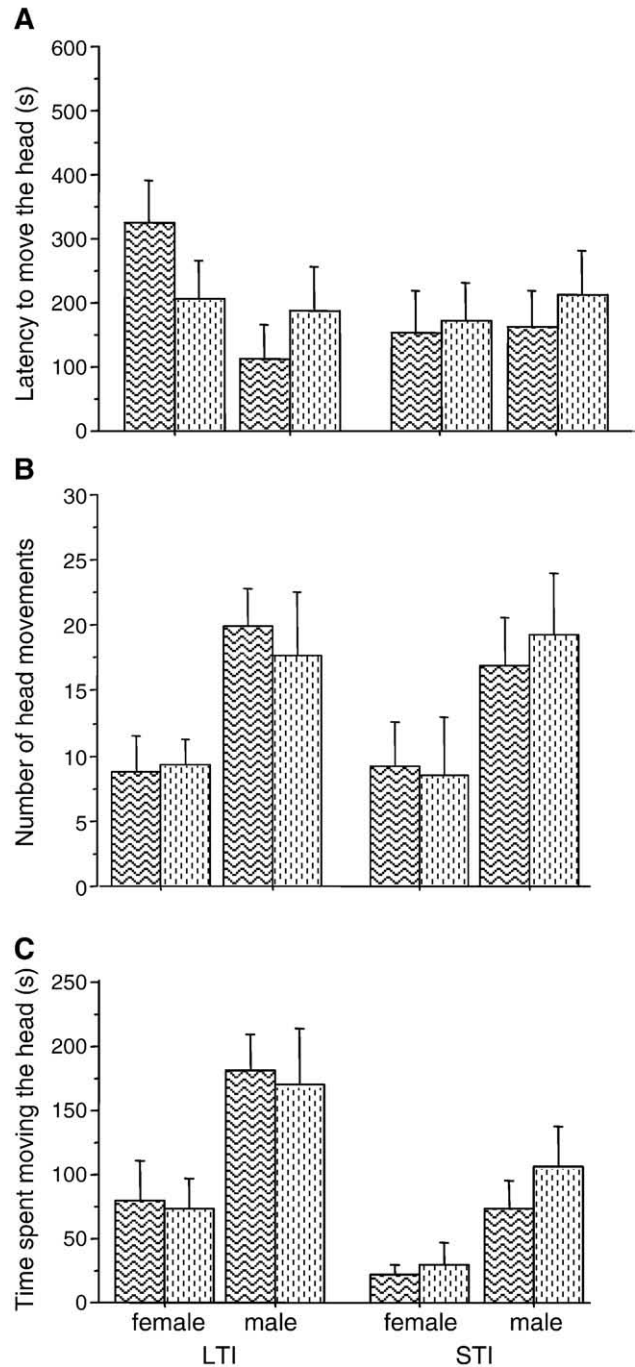


**Fig. 3.** Corticosterone concentrations (CORT, ng/ml plasma) measured (A) in LTI and STI quail undisturbed ( $9 \leq n \leq 12$  quail per experimental point) (ANOVA, genotype effect:  $F_{1, 76}=0.54, p=0.5$ , sex effect:  $F_{1, 76}=0.74, p=0.4$ , repeated restraint effect:  $F_{1, 76}=2.11, p=0.1$ , interaction: NS), (B) following 10 min of restraint in a crush cage ( $10 \leq n \leq 12$ ) (ANOVA, genotype effect:  $F_{1, 81}=98.99, p<0.0001$ , sex effect:  $F_{1, 81}=11.03, p=0.002$ , repeated restraint effect:  $F_{1, 81}=3.23, p=0.08$ , genotype  $\times$  sex effect:  $F_{1, 81}=5.05, p=0.03$ , genotype  $\times$  repeated restraint effect: NS, sex  $\times$  repeated restraint effect: NS), (C) 10 min after injection of 1–24 ACTH at doses of 2.5  $\mu\text{g}/\text{kg}$  body wt ( $11 \leq n \leq 14$ ) (ANOVA, genotype effect:  $F_{1, 87}=6.82, p=0.01$ , sex effect:  $F_{1, 87}=0.06, p=0.8$ , repeated restraint effect:  $F_{1, 87}=0.05, p=0.8$ , interaction: NS) or (D) 10  $\mu\text{g}/\text{kg}$  body wt ( $9 \leq n \leq 13$ ) (ANOVA, genotype effect:  $F_{1, 84}=1.58, p=0.2$ , sex effect:  $F_{1, 84}=9.57, p=0.003$ , repeated restraint effect:  $F_{1, 84}=0.52, p=0.5$ , interaction: NS). Each measurement was performed on the first day (Day 1) prior to any treatment and on the fifth day (Day 5) following eight 10 min periods of restraint in a crush cage (twice daily for four consecutive days). (means  $\pm$  SE) # indicates significant differences between CORT levels induced by restraint (B) and basal CORT levels (A) or between CORT levels induced by injection of 1–24 ACTH (C, D) and CORT levels in quail receiving vehicle injection (data not shown).

LTI quail spent significantly more time moving their heads than STI quail ( $F_{1, 82}=9.48, p=0.003$ ) (Fig. 5C). The time spent moving the head was also significantly higher in male quail than in female quail for



**Fig. 4.** Latency to struggling (s) (A) (ANOVA (log), genotype effect:  $F_{1, 82}=116.58, p<0.0001$ , sex effect:  $F_{1, 82}=22.06, p<0.0001$ , repeated restraint effect:  $F_{1, 82}=2.37, p=0.1$ , sex $\times$ repeated restraint effect:  $F_{1, 82}=6.88, p=0.01$ , genotype $\times$ repeated restraint effect: NS, genotype $\times$ sex effect: NS), total number of struggling bouts (B) (ANOVA (log), genotype effect:  $F_{1, 82}=115.04, p<0.0001$ , sex effect:  $F_{1, 82}=15.51, p=0.0002$ , repeated restraint effect:  $F_{1, 82}=0.54, p=0.5$ , genotype $\times$ sex effect:  $F_{1, 82}=5.47, p=0.02$ , genotype $\times$ repeated restraint effect: NS, sex $\times$ repeated restraint effect: NS) and time spent struggling (s) (C) (ANOVA (square roots), genotype effect:  $F_{1, 82}=78.39, p<0.0001$ , sex effect:  $F_{1, 82}=32.08, p<0.0001$ , repeated restraint effect:  $F_{1, 82}=5.67, p=0.02$ , sex $\times$ repeated restraint effect:  $F_{1, 82}=5.33, p=0.02$ , genotype $\times$ sex effect: NS, genotype $\times$ repeated restraint effect: NS) measured during a restraint period of 10 min in LTI and STI quail. Each of these measurements was performed on the first day (Day 1) prior to any treatment and on the fifth day (Day 5) following eight 10 min periods of restraint in a crush cage (twice daily for four consecutive days). ( $10\leq n\leq 12$  quail per experimental point) (means $\pm$ SE).



**Fig. 5.** Latency to the first head movement (s) (A) (ANOVA (log), genotype effect:  $F_{1, 82}=0.57, p=0.45$ , sex effect:  $F_{1, 82}=3.00, p=0.09$ , repeated restraint effect:  $F_{1, 82}<0.0001, p=0.9$ , interaction: NS), total number of head movements (B) (ANOVA (log), genotype effect:  $F_{1, 82}=0.45, p=0.5$ , sex effect:  $F_{1, 82}=9.96, p=0.002$ , repeated restraint effect:  $F_{1, 82}=0.18, p=0.7$ , interaction: NS) and time spent moving the head (s) (C) (ANOVA (square roots), genotype effect:  $F_{1, 82}=9.48, p=0.003$ , sex effect:  $F_{1, 82}=15.63, p=0.0002$ , repeated restraint effect:  $F_{1, 82}<0.001, p=0.9$ , interaction: NS) measured during a restraint period of 10 min in LTI and STI quail. Each of these measurements was performed on the first day (Day 1) prior to any treatment and on the fifth day (Day 5) following eight 10 min periods of restraint in a crush cage (twice daily during four consecutive days). ( $10\leq n\leq 12$  quail per experimental point) (means $\pm$ SE).

both genotypes ( $F_{1, 82}=15.63, p=0.0002$ ). Repeated restraint did not significantly affect time spent moving the head ( $F_{1, 82}<0.001, p=0.9$ ).

Numbers of pecks were not reported in Experiment 2 because Experiment 1 indicated that only STI male quail exhibited this behavior.

## Discussion

While quail are restrained in a crush cage they cannot spread their wings, but can still move their heads and legs, and thus making them struggle. We observed that STI quail struggled much more frequently than LTI quail, specially at the beginning of restraint. In addition, latency to struggling was longer in LTI quail and they rarely showed struggling behavior during the restraint period. Adoption of immobility during restraint, as well as in other unfamiliar and potentially threatening situations, is generally considered to be indicative of intense fear (Faure et al., 1983; Gallup, 1979; Jones, 1996; Jones and Satterlee, 1996). The present behavioral findings therefore suggested that LTI quail have greater underlying fearfulness under this experimental situation of restraint. This hypothesis is consistent with the selection criteria (i.e. tonic immobility) since previous studies have reported that LTI quail show more pronounced behavior inhibition upon exposure to alarming stimulation than STI quail (Jones et al., 1991; 1994).

A positive relationship between fear-induced behavior inhibition and circulating levels of glucocorticoids have been hypothesized in several avian and mammalian species (Carli et al., 1979; Coordimas et al., 1994; Jones et al., 1988; 1992; Kalin et al., 1998). However, the present findings regarding corticosterone responses to restraint indicated the opposite relationship in our genotypes of quail with STI quail showing higher CORT levels throughout the restraint period. Our present results concur with previous findings indicating no positive relationship between CORT levels following TI and duration of the fear-induced response of TI in LTI and STI quail (Hazard et al., 2008).

We hypothesize from these results that differences in CORT responses to restraint stress between LTI and STI quail may partly result from differences in the behavior being expressed by quail during restraint. Indeed, high CORT levels may result from physical activity of quail during restraint since such increases in CORT levels have been reported during physical exercise in ducks (Rees et al., 1985), probably to provide energy to support the physical response. STI quail, that were physically more active during restraint, may thus have required higher CORT levels to support struggling behavior. However, the struggling behavior of STI quail progressively decreased during the course of restraint and reached low levels of activity, whereas CORT levels remained high throughout the restraint period. Consequently, although higher levels of physical activity can explain higher levels of CORT at the onset of restraint in STI quail, it cannot explain the maintenance of high CORT levels throughout the restraint period. Although we have no scientific evidence to support this hypothesis, we cannot exclude the possibility that high CORT levels were maintained during restraint, either because STI quail remained muscularly contracted throughout restraint while quail were immobile, or to maintain high levels of available energy to cope physically (i.e. by escaping) with the stressful situation as soon as possible.

The decrease in struggling behavior with the increase in restraint duration in STI quail may indicate that behavioral adjustment takes place in STI quail during prolonged restraint. We have no scientific evidence to confirm that these changes result from exhaustion with the length of restraint duration, although the overall duration of time spent struggling was very limited since each bout lasted a few seconds, or are an adaptative strategy.

Whatever the underlying cause, both the corticotropic and behavioral responses observed in the present study provide scientific evidence indicating that different coping strategies are developed by the two genotypes of quail during restraint in the crush cage. The greater number of struggling bouts in STI quail compared to LTI quail may be interpreted as more frequent flight attempts from restraint in STI quail, as previously suggested by Faure et al. (1996). We can thus hypothesize that LTI quail are more likely to adopt a passive coping

strategy upon exposure to threat whereas STI quail appear to be more active copers. This hypothesis is consistent with the higher fear-induced behavior inhibition in LTI quail than in STI quail previously reported by Jones et al. (1994). Similarly, studies in pigs have indicated that interindividual differences in TI responses may reflect coping behavior strategies in response to stress (Erhard et al., 1999). In agreement with the present findings, they showed that pigs with short or long duration of TI adopted active or passive behavior strategies, respectively. In rodents, proactive (active) male rats and mice exhibiting lower attack latency, higher active avoidance and lower conditioned immobility have also been characterized as having lower HPA axis activity and reactivity than reactive (passive) animals (de Boer et al., 1990a; 1990b; Korte et al., 1992; 1997; van Oortmerssen and Bakker, 1981). Interestingly, genotypes of Japanese quail selected for high (HS, High Stress) or low (LS, Low Stress) CORT responses to immobilization (Satterlee and Johnson, 1988) correspond to behaviorally passive and active animals, respectively (Jones and Satterlee, 1996). In the light of the present results, the corticosterone responses shown by LTI and STI quail under restraint stress suggest that adrenocortical correlates of coping behavior in these genotypes of quail may be different from the coping styles previously described in other species. Adoption of these coping strategies in LTI and STI quail could be viewed as a mechanism developed to maximize fitness through stress (Blas et al., 2007).

Head movement may also be part of the difference in coping strategies developed in LTI and STI quail, and could be interpreted as an exploratory behavior, as reported by Murphy and Wood Gush (1978) in restrained hens. Both genotypes differed in the course of this exploratory behavior, and male STI quail exhibited much more of this exploratory behavior than female STI quail, which exhibited very low levels of this behavior throughout the restraint period (Supplementary file 1). Interestingly, the high levels of exploratory behavior in male STI quail decreased progressively with restraint duration, as reported for struggling behavior. Thus, while LTI quail are more likely to adopt exploratory behavior (as suggested by the higher number of head movements) and female STI quail attempted preferentially to escape the stressful situation in an active way (as shown by the increased struggling behavior), male STI quail exhibited both behaviors in the restraint stress. In addition, male STI quail were also observed to give a large number of pecks at the wall of the restraint cage whereas this behavior was not expressed in the other groups of quail (Supplementary file 2). The expression of this behavior during restraint has previously been interpreted to be characteristic of a conflict between flight and exploratory behavior (Murphy and Wood Gush, 1978). We therefore hypothesize that male STI quail have a conflicting profile between escaping from the stressful situation in an active way (i.e. pecking and struggling) and performing exploratory behavior (i.e. head movements) or that they adopt a more complex mixed coping strategy.

Whereas repeated restraint did not affect latency to struggling, latency to the first head movement or time spent moving the head in any of the experimental groups of quail, repeated restraint was associated with a decrease in the time spent struggling in STI male quail while struggling behavior remained similar between the first and ninth episodes of restraint in the other 3 groups of quail. The present results suggest that a habituation process occurred in the behavioral response to repeated restraint in male STI quail but not in the other groups. On the other hand, we observed a tendency ( $p=0.08$ ) to a decrease in CORT responses following repeated restraint in all experimental groups, but more markedly in male STI quail. A decrease in the corticotropic response to repeated restraint (Guémené et al., 2001; Guémené and Guy, 2004) or to daily treadmill exercise (Rees et al., 1983) in ducks has been interpreted as the consequence of a habituation process. The slight decrease in CORT response observed therefore suggests that a slight habituation may occur in all genotypes and sexes in the corticotropic response to repeated restraint. On the

other hand, we observed that repeated activation of the HPA axis, and consequently repeated stimulation of the adrenal gland did not induce any change in the functioning of the adrenal gland. Indeed, neither adrenal sensitivity nor maximum adrenal capacity in response to ACTH challenge was affected by repeated restraint. This result does not support previous findings indicating that repeated acute stress can induce initial hypersensitivity of the adrenal glands and then exhaustion (Brodish and Odio, 1989; Harbuz and Lightman, 1992). The slight habituation process observed in the corticotropin response to repeated restraint might therefore not be due to changes in adrenal functioning but might involve changes in the perception of the experimental context.

Habituation and sensitization are thought to be positively related to the amount (number and duration) of exposures to stressful situations (Servatius et al., 1994) and it may be that the experimental procedure was not long enough in the present study to allow such processes in quail. However, the effects of repeated restraint have previously been investigated in LS and HS Japanese quail selected for divergent CORT responses to immobilization (Satterlee and Johnson, 1988). The phenomenon of sensitization, characterized by an increase in the struggling behavior in HS and LS quail and an increase in the CORT response in HS quail, was observed after only 5 repeated restraint episodes (i.e. 5 min daily on consecutive days) (Jones et al., 2000). Habituation was also observed very rapidly in ducks following twice daily manipulations of very short duration (Guémené et al., 2001). We would therefore have expected that nine repeated restraint episodes (i.e. 10 min twice daily on consecutive days) should have been sufficient to affect HPA axis and/or behavioral responses through habituation or sensitization in LTI and STI quail. Interestingly, a recent study provided evidence that repeated stress-induced HPA axis activity depends on the physical context in which the stressors occur (Grissom et al., 2007). Since they showed that habituation was greater in animals housed and repeatedly stressed in the same room, we cannot exclude the possibility that marked habituation could have occurred if the repeated restraint had been undertaken in the rearing room and not in the test room used throughout the repeated restraint procedures.

The present findings also indicate sex effects upon behavioral and corticotropin responses. Male quail undertook more struggling bouts, which contradicts previous reports regarding the absence of sex differences in fear-related behavior in Japanese quail selected for TI duration (Jones et al., 1994; Launay et al., 1993; Mills and Faure, 1986) or selected for adrenal responses (Jones et al., 2000). Nevertheless, such sex effects have been reported in rats where males and females showed different behavioral responses to a restraint test (Albonetti and Farabollini, 1992). Moreover, Boissy and Bouissou (1994) reported that androgens inhibited fear behavior responses in cows. The apparently slightly lower degree of fearfulness in male quail could thus be related to the effects of sex steroids in the present study since quail of both genotypes were sexually mature at the time of testing (Hazard et al., 2005). The effects of sex steroids could also be involved in the greater CORT responses to restraint stress in STI than in LTI quail (found in Experiment 2), as reported in rats (Handa et al., 1994).

In conclusion, the present findings suggest that divergent selection for tonic immobility in quail results in the development of different coping strategies in response to restraint stress between LTI and STI quail. LTI quail are more likely to adopt a passive behavior coping strategy upon exposure to threat whereas STI quail behave more as active copers. The corticosterone responses shown by LTI and STI quail under restraint stress suggest that adrenocortical correlates of coping behavior in these genotypes of quail may be different from the coping styles previously described in other species, with LTI quail showing lower (and not higher) CORT responses. On the other hand, neither behavioral habituation nor sensitization processes occurred in the context of repeated restraint

in female and male LTI quail and female STI quail, whereas the decreases observed in some behavioral responses were interpreted to be the result of a habituation process in male STI quail. These results may have important implications for poultry well-being and productivity. Indeed, chronic stress can cause increased fearfulness, reduced disease resistance, and impaired egg production, growth and product quality (Jones et al., 1988; Jones, 1996). Thus, since LTI and STI quail did not show sensitization, but some habituation was detected in male STI quail, artificial selection for short duration of TI may increase the likelihood of adaptation to chronic stress, while providing an interesting model to investigate further the behavioral and corticotropin responses to chronic stress.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2008.07.002.

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