Behavioural Brain Research 237 (2013) 124–128

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

# A higher inherent trait for fearfulness is associated with increased anxiety-like behaviours and diazepam sensitivity in Japanese quail

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## HIGHLIGHTS

- Emotionality trait influences exploratory behaviour in Japanese quail.
- Diazepam decreases impact of emotionality on exploratory behaviour.
- Selection on tonic immobility response represents valuable model of bird anxiety.

## ARTICLE INFO

*Article history:*
Received 18 August 2012
Received in revised form
12 September 2012
Accepted 14 September 2012
Available online 20 September 2012

*Keywords:*
Anxiety
Open field
Benzodiazepine
Tonic immobility
*Coturnix japonica*
Bird

## ABSTRACT

This study tested whether lines of Japanese quails divergently selected for a fear response, the tonic immobility, might constitute a reliable bird model for studying anxiety. Previous studies demonstrated that the selection modifies the general underlying emotionality of the birds rather than exerting its effect only on tonic immobility. The behavioural effects of intraperitoneal injections of diazepam, an anxiolytic drug, were assessed in two lines of quail selected either for their short (STI) or long (LTI) duration of tonic immobility. Effects of diazepam were examined in two tests used for measuring emotionality in birds, the open field and the tonic immobility tests. After being placed in the centre of the open field, birds with a high emotionality (LTI quails) stayed longer in the centre of the apparatus than STI quail. Diazepam had anxiolytic effect in LTI birds as it increased the time spent in the outer area. This effect of diazepam appears to be selective because the drug has no effect on other behaviours such as distress calls or escape attempts. The drug has also no effect on the tonic immobility response in any of the two lines. These findings reveal an "anxiogenic" trait of LTI birds in the open field test that can be modulated by the administration of an anxiolytic drug. Therefore quails selected for LTI and STI represent a valuable model to study the mechanisms underlying anxiety in birds.



## 1. Introduction

Studies of transgenic knock-out mice, inbred mice strains differing in emotional behaviours and rodent lines divergently selected for anxiety trait contributed largely to unravel the behavioural, genetical and neurobiological bases of anxiety in mammals [1–8].

The biological substrates of anxiety have been less studied in birds compared with rodents. However, living conditions in wild birds or husbandry practices in farm birds can be a source of anxiety. This study tested whether lines of Japanese quails divergently selected for a typical fear response, the tonic immobility duration, might constitute a valuable animal model to study anxiety in birds. The tonic immobility response is a fear reaction to physical restraint that exists in many species [9] and corresponds to an innate form of temporary motor inhibition characterised by an immobile posture [10]. Tonic immobilty is the last in a series of defensive behaviours displayed by a bird in response to a predator attack [11]. Experimentally, tonic immobility can be induced manually by physically restraining the bird in a V-shaped cradle [12]. Divergent selection for duration of tonic immobility has resulted in two divergent selected lines: the LTI and the STI lines. Quail from

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http://dx.doi.org/10.1016/j.bbr.2012.09.026

the LTI and STI lines display long and short duration of tonic immobility respectively [13]. Previous studies demonstrated that animals from the LTI line not only exhibit long durations of tonic immobility, and also show increased behavioural inhibition when exploring a novel environment during an open field test as well as a higher reluctance to enter a new environment during the emergence test. LTI birds also exhibit a longer latency to approach novel food and are more disturbed by the sudden introduction of a frightening stimulus into their home cage [14,15]. Moreover, in a test where chicks from both lines were presented with a conspicuous coloured bead, LTI chicks showed higher inhibition of bead pecking than STI chicks [16]. Finally, LTI quails exhibited higher reactions to the approach of man than STI quails [17]. Altogether, these findings have led to the assumption that selection for tonic immobility generally affects the emotional reactivity of birds (i.e. the propensity to be more or less easily frightened). Specifically, these findings indicate that unfamiliar environments or objects, that are known to be a potent source of anxiety in many species [18–23], induce stronger reactions in LTI than in STI birds.

To test whether LTI and STI quails might represent a valuable bird model for studying anxiety, we assessed the behavioural effects of intraperitoneal injections of diazepam, a standard anxiolytic drug, to both STI and LTI birds in the open field and the tonic immobility tests. Diazepam is a classical benzodiazepine that binds to a central benzodiazepine receptor increasing the effects of the γ-aminobutiric acid (GABA), with an anxiolytic effect mediated by the enhancement of chloride conductance [24]. Diazepam has been used in many species including birds and shown to reduce fear or anxiety related behaviours [8]. Behavioural effects of diazepam injections were assessed in the open field test because, similarly to mammals, birds exhibit a natural aversion to unfamiliar and exposed environments [18–23]. Effects of diazepam were also assessed in the tonic immobility test because this paradigm was used for the genetic selection of the quail lines and is one of the most common tests for measuring emotionality in poultry [25–29]. We thus tested the hypothesis that anxiolytic effects of diazepam injections in the open field and the tonic immobility tests would be more pronounced in LTI than in STI birds. More specifically, we predicted that diazepam would decrease anxiety related behaviour in LTI birds and would only induce minor or no anxiolytic effects in birds from the STI line considering their very low emotional reactivity.

## 2. Materials and methods

### 2.1. Animals

We used the 50th generation of two lines of Japanese quails selected for their short (STI) or long (LTI) duration of immobility [13]. These lines of quail are selected and maintained at the Pôle d'Expérimentation Avicole de Tours (UE PEAT, INRA, Nouzilly, France) where the experiment was carried out. The two selected lines were hatched and bred under the same conditions. On the day of hatching, chicks from the same line were transferred to communal floor pens. On the 17th day after hatching, sex was determined by feather dimorphism and females were reared in individual cages in a battery (24.5 cm × 35 cm × 20 cm) under a 12:12 h light dark schedule (light on 08:00 h). Water and food were available ad libitum. An ambient temperature of 22 °C was maintained in the animal room.

Animal care procedures were conducted in accordance with the guidelines set by the European Communities Council Directive (86/609/EEC) and with the French legislation on animal research.

### 2.2. Diazepam and injection

Diazepam (Valium, Roche, Neuilly sur Seine, France) was dissolved in saline (0.9%). Injections were given intraperitoneally at a volume of 0.1 ml/100 g. Two doses of diazepam were used: 0.05 mg/kg and 0.5 mg/kg. Control quail received injections of saline. The two doses of diazepam were chosen on the basis of previous studies reporting that a dose of 0.05 mg/kg of diazepam decreased the latency to peck pebbles and to ambulate in the open field test in day-old chicks. The same dose was also shown to decrease the duration of tonic immobility in 15-day-old chicks [30]. Moreover, doses from 0.05 to 1 mg/kg were shown in chicks to be anxiolytic without significant effect on locomotor activity [31]. In pigeons, signs of overdose such drowsiness and sleep-like posture started at doses above 2.5 mg/kg [32].

At the age of two months, 32 female quails from the LTI line and 32 female quails from the STI line were gently handled individually, transported in a transport box (15 cm × 15 cm × 15 cm) made of opaque polycarbonate to a novel room containing no other birds. Each bird received an intraperitoneal injection either of a dose 0.05 mg/kg or 0.5 mg/kg of diazepam as indicated above or with saline. Immediately after the injection, each bird was placed in the closed transport box for 20 min before open field testing. A delay of 20 min between injection of diazepam and open field testing was chosen on the basis of previous studies reporting that a delay of 20 min between injection of diazepam and behavioural testing allow observing significant anxiolytic effect of the drug in chicks [30,31]. Thus, they were 6 treatment groups: LTI-NaCl ($n = 10$), LTI-DZ 0.05 mg/kg ($n = 11$), LTI-DZ 0.5 mg/kg ($n = 11$), STI-NaCl ($n = 10$), STI-DZ 0.05 mg/kg ($n = 11$) and STI-DZ 0.5 mg/kg ($n = 11$).

### 2.3. Open field

Twenty minutes after the injection, reactivity to a new environment was assessed in a square arena (80 cm × 80 cm × 29 cm) made of white wood with a floor made of yellow waterproof plastic surface under dim and dispersed light conditions. Animals were carried individually in the transport box to a room containing no other birds alternating between treatment and line conditions. Each quail was placed in the centre of the open-field arena and allowed to freely explore for 6 min. At the end of the session, birds were returned to the transport box and the arena was cleaned with water. A digital camera was mounted directly above the arena, capturing images at a rate of 5 Hz. The images were transmitted to a PC running the Ethovision tracking system (v XT8.0, Noldus Technology, Wageningen, The Netherlands). The locomotor activity (total distance travelled), the time spent in the inner (a square area of 20 cm × 20 cm at the centre of the arena) and outer (band 40 cm wide from the outer of the arena) areas of the arena was obtained using the Ethovision tracking system. Previous findings indicate that the distribution of activity between the outer and inner areas of the arena may be an important component in the open field behaviour of chicken [18]. The latency to first escape attempts (jumps at the wall), the total number of escape attempts, the latency to first distress call and total number of distress calls were also manually recorded by an experimenter blind to the line or drug treatment. These parameters were previously shown to be behavioural indicators of fear and social motivation in quail or chicks [19,20,33,34].

### 2.4. Tonic immobility

Immediately following the open field test, each animal was individually tested for tonic immobility. The tonic immobility test, a standard and robust test for measuring fearfulness in birds [10,12,15] followed a procedure similar to the one used for the selection [13]. Animals were carried individually to a novel room containing no other birds in the transport box. The quail was placed on its back in a U-shaped wooden cradle covered with cloth and restrained for 10 s (with one hand on the sternum and one lightly cupping the head of the bird). The experimenter remained silent and virtually motionless in the room, out of the bird's sight. If more than 10 s elapsed until the bird righted itself, the duration of tonic immobility was noted. If tonic immobility was not attained after 5 successive attempts, a score of 0 s was given. If the bird failed to right itself after 5 min, the test was terminated and a maximum score of 300 s was given for tonic immobility duration. Two parameters were recorded during the test: the duration of tonic immobility and the number of induction attempts.

### 2.5. Statistical analyses

Kolmogorov–Smirnov tests determined whether the data were normally distributed. Behavioural parameters were compared between treatment and lines by parametric analyses of variance (ANOVA) followed by Fisher LSD tests for post hoc comparisons. Data are presented as means + SE. All statistical analyses were conducted using the statistical software StatView, version 5.0 (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Open field

Analyses of the time spent in the different compartments of the open field revealed that LTI birds stayed longer in the centre compartment of the open field than STI birds ($F(1,58) = 7.96$; $p = 0.006$). Diazepam treatment had no significant effect on this pattern ($F(2,58) = 1.28$; $p = 0.28$) and there was no interaction between lines and treatments ($F(2,58) = 0.24$; $p = 0.78$; Fig. 1A).

In contrast, STI birds spent a significantly higher amount of time at the periphery of the open field (effect of line: $F(1,58) = 26.11$; $p < 0.0001$) but the effect of treatment was dependant on the line