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Short communication

Effects of thymol feed supplementation on female Japanese quail (*Coturnix coturnix*) behavioral fear response

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ABSTRACT

Dietary supplementation with thymol has been shown to improve the oxidative stability of eggs and meat during storage. In addition, *in vitro* studies have shown that this compound can act as a positive allosteric modulator of the $GABA_A$ receptor, similarly to its analog phenolic compound propofol. Hence, it is conceivable that thymol could also present anxyolitic and/or fear reducing properties, probably also affecting their locomotor activity. Considering that fear-inducing/stressful situations are practically unavoidable during birds' rearing, the thymol feed supplementation could therefore present beneficial consequences in terms of animal welfare. This study evaluates potential fear reducing properties of thymol feed supplementation and its potential effects on locomotor activity by assessing female Japanese quail behavioral responses during brief mechanical restraint and open-field tests. Birds were evaluated after 2 and 15 days of supplementation. During the brief mechanical restraints, the latencies to struggle were significantly ($P<0.05$) shorter and the number of struggling bouts significantly higher ($P<0.05$) in the thymol group than in their Control counterparts suggesting a fear reducing effect. No effects on locomotor behavior were detected during open-field testing. The results suggest that dietary supplementation with thymol may help reduce female fear responses when birds are exposed to stressful situation without affecting the bird's locomotor activity.

1. Introduction

Diet supplementation with essential oils extracted from herbs and spices has been considered a useful strategy in animal nutrition. These supplements in some cases have been shown to help sustain good health in farm animals and improve their performance. Among the reported effects, it can be mentioned antioxidant, antiparasitic, and antimicrobial properties (see Lee et al., 2004; Windisch et al., 2008; Brenes and Roura, 2010; Hashemi and Davoodi, 2010; Borazjanizadeh et al., 2011 for reviews).

Thymol is one of the main components of the "oregano" essential oil which is extracted from *Origanum vulgare*, an aromatic plant widely used as spice (Abdalla and Roozen, 2001; Sivropoulou et al., 1996; Brenes and Roura, 2010). As an

Abbreviations: CP, crude protein; GABA, gamma-aminobutyric acid; ME, metabolizable energy; SEM, standard error of the mean.

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animal feed supplement, it has been reported in rats that it can help maintain higher levels of polyunsaturated fatty acids in the liver, brain, kidney and heart (Youdim and Deans, 1999a,b, 2000) suggesting that it acts as effective free radicals scavenger. In poultry, thymol has also been shown to improve the oxidative stability of eggs (Botsoglou et al., 1997) and meat during storage (Luna et al., 2010) and specifically in quail, also showed improvements in the hatching success of eggs from thymol supplemented breeders (Luna et al., 2012). In addition, *in vitro* studies have shown that thymol not only may present intrinsic antioxidant properties (Delgado Marín et al., 2011) but also can act as a positive allosteric modulator of the GABAA receptor (Garcia et al., 2006, 2008), similar to its analog phenolic compound propofol (Garcia et al., 2006), but with significantly lower potency (Mohammadi et al., 2001). Propofol has been used as anesthetic in birds (Schumacher et al., 1997; Machin and Caulkett, 1998) and presents sedative properties (Smith et al., 1994; Casati et al., 1999) that is observed in quail during open-field testing (Kembro et al., 2012). Interestingly, at lower doses, propofol has been shown to have fear reducing and/or anxiolytic properties (Pain et al., 1999; Kurt et al., 2003). Taking into account the proposed effects of thymol *in vitro* and the molecular similarities with propofol, it is conceivable that thymol could also present fear reducing properties, and potentially it could also affect the bird's locomotor activity due to sedative properties at higher doses. Thus, considering that fear-inducing/stressful situations are practically unavoidable during birds' rearing (Jones, 1996), the use of thymol feed supplementation to improve the oxidative stability of poultry products could also have beneficial consequences in terms of animal welfare.

This study evaluates the fear reducing properties and effects on locomotor activity of thymol feed supplementation by assessing female Japanese quail behavioral responses during a mechanical restraint and an open-field test. Japanese quail are widely known as a useful model for extrapolation of results to other bird species such as domestic chickens (Baumgartner, 1994; Faure et al., 2003) as well as an important agricultural species for meat and egg production in many countries (Baumgartner, 1994; Jones, 1996). In the first test, while restrained, birds are not able to spread their wings but they can struggle to escape by moving their heads and legs (Hazard et al., 2008). In this context, adoption of immobility is regarded as indicative of intense fear (Jones, 1996; Jones and Satterlee, 1996). The open-field test has been widely used to assess both fear (Jones, 1996) and locomotor activity (Kembro et al., 2008; Satterlee and Marin, 2006). Gallup and Suarez (1980) proposed that open-field behavior in poultry represents a compromise between opposing tendencies to reinstate contact with conspecifics and to minimize detection in the face of possible predation. Open-field tests have also been useful to detect drug sedative effects in poultry. In broilers as well as in quail, an acute administration of the sedative drugs diazepam 1 mg/kg (Marín et al., 1997; Kembro et al., 2007) or propofol 20, 40 and 80 mg/kg (Kembro et al., 2012) significantly decreased locomotion during open-field testing.

To our best knowledge, this is the first study that evaluates the potential fear reducing effects of a dietary supplementation with a single main component of an essential oil in birds, and it's potential concomitant effect on locomotor activity.

2. Materials and methods

2.1. Animals and husbandry

Female Japanese quail (*Coturnix coturnix japonica*) was used in the present study (42 per behavioral trial, see below). The birds' evaluated were taken from a population of a single 240-bird hatch. Egg incubation, chick brooding, and lighting procedures were similar to those described by Nazar and Marin (2011) with the exception that chicks were brooded from day 1 in mixed-sex groups of 40 within each of 6 brooder boxes, each measuring 90 cm × 90 cm × 60 cm (length × width × height). Each box had two feeders covering the front part of the box and 16 automatic nipple drinkers. A wire mesh floor (1 cm grid) was raised 5 cm to allow the passage of excreta and a lid prevented the birds from escaping. Brooding temperature was 37.8 °C during the first week of life, with a weekly decline of 2.8 °C until room temperature (23.9–26.7 °C) was achieved.

At 28 days of age, quail were sexed by plumage coloration, individually weighted and were wing banded to identify each bird. At this age, birds were randomly housed in 28 groups of 1 male and 3 females into cages measuring 20 cm × 45 cm × 25 cm (length × width × height).

From hatch to 28 days of age, birds were fed a starter ration (280 g CP and 11.72 MJ ME/kg) and water *ad libitum*. From this age on and until feed supplementation was initiated (100 days of age), birds were fed with layer ration (210 g CP, 11.51 MJ ME/kg). At 100 days of age, all quail within each cage were assigned to 1 of 2 feed treatments: vehicle (control) or 2 g of thymol (E. Merck, Darmstadt, Germany) per kg of supplemented feed (thymol) which is equivalent to a dose of 80 mg of thymol/day/animal. Thymol was prepared in a 5 mL/L ethanol/distilled water vehicle solution that was uniformly sprayed weekly to fresh commercial feed (Luna et al., 2010, 2012). The chemicals used in this study were reagents grade commercial products. The dose of thymol was selected based on two criteria: (1) previous behavioral studies with lower doses (24–48 mg of thymol/day/animal) produced no detectable behavioral changes in birds treated (Luna, 2012), and (2) the selected dose is significantly lower than the lethal dose 50 of thymol of 980 mg/kg of body weight (Jenner et al., 1964).

Birds were provided a 14-h light (0600–2000 h; approximately 180 lx), 10-h dark cycle. Daily maintenance and feeding chores were done at the same time each day (0900 h).

Prior to initiation of feed supplementation, each cage was randomly assigned to one of four test groups based on the dietary supplement (control or thymol) and the behavioral test protocol (mechanical restraint or open-field, see details below). All birds remained in the same test group throughout the study. Because of the sex ratio within each cage, a low number of male representatives (compared to females) within each test combination was available. Therefore, considering the wide interindividual range of behavioral responses expected, only female representatives were evaluated in this study. Mechanical restraint or open-field behavior of female quail from each treatment were evaluated after 2 and 15 days of thymol dietary supplementation. The testing was conducted during two consecutive days (0800–1400 h). Half of the females were submitted to a mechanical restraint test and the other half to an open field test. Each bird was tested individually. Testing of control and thymol groups were alternated throughout each test day in order to control for a potential confounding time of day effect.

Considering that several studies have reported effects of essential oils supplementation on body weight (Botsoglou et al., 2002; Demir et al., 2003; Sarica et al., 2005) that could also have consequences affecting behavioral performances, birds were also weighed at 0, 7 and 15 days of feed supplementation.

2.2. Mechanical restraint test

The crush cage device had three white wooden walls of 40 cm × 12 cm × 20 cm (length × breadth × height), and a glass front wall, and was placed on the floor of a separate room. As soon as the quail was placed in the device, a movable and close-fitting interior divider made of white wood was pushed up against the bird and then fixed in place; this prevented gross movement without interfering with respiration. The bird could still move its head and legs but could not spread its wings. The experimenter retreated out of the bird's sight as soon as it was effectively restrained and a digital camera placed in front of the apparatus recorded its behavioral responses during 5 min. The latency until the first struggling bout (latency to struggle) and the number of discrete struggling bouts was recorded. Struggling bouts were recorded as discrete events if successive bouts were separated by 5 s or more (Jones et al., 2000). The floor of the apparatus was wiped cleaned after each trial to minimize any effects of soiling and olfactory contamination.

2.3. Open-field test

Three identical open field apparatus were used in this study, each consisting of a white wooden box measuring 60 cm × 90 cm × 60 cm (width × length × height). The test apparatus were maintained at an ambient temperature and light intensity similar to that in the room where the birds' home cages were located. First, the three females housed in the same cage (all exposed to the same dietary supplementation treatment) were placed in a box and transported to a nearby experimental room. Then, to begin a test, each bird was released near the midpoint of one of the three open-field apparatus, and its behavior was recorded onto the computer for 10 min with a video camera suspended approximately 1.8 m directly above the open field. This arrangement made certain that the experimenter was completely hidden from the bird's view during testing. If defecation occurred during a test, after completion of that test the floor was wiped clean before reuse for the next test. We used the ANY-maze computer program (Stoelting Co.,Version 4.96, Wood Dale, IL) to analyze the ambulation of the birds in the open-field apparatus at 0.5 s intervals. The following variables were studied:

- *Latency to ambulate* (s): time from the start of the test until a bird initiates ambulation.
- *Percentage of time spent ambulating*: $t_{\%} = \Sigma t_i / N \times 100$

where t_i is the time interval (s) in which the animal is ambulating and N is the total duration of the test (s).

- *Distance ambulated* (cm): the total (cumulative) distance ambulated by the animal during the test period.
- *Ambulation rate* (cm/s): $TA = N/(t_T - t_{lat})$,

where N is the distance ambulated, t_T is the total duration of the test, and t_{lat} is the latency to initiate ambulation. Due to computer failure during the evaluation of the open-field activity after 15 days of supplementation, the data from one individual could not be processed and was thus lost. Consequently, data from the same individual obtained after 2 days of dietary supplementation was also excluded from the analysis.

2.4. Statistical analyses

A one-way repeated measure ANOVA was used to determine the effects of dietary supplementation (control and thymol), the days of dietary supplementation (2 and 15 days; the repetition factor) and their interaction on the behavioral variables registered during mechanical restraint and open field tests. In order to better fit ANOVA assumptions, all variables were transformed to ranks (Shirley, 1987) with the exception of the number of struggling bouts that was submitted to a log10 transformation. To test the hypothesis, $P<0.05$ was considered significant.

3. Results

Fig. 1 depicts the effect of 2 or 15 days of dietary supplementation on the mechanical restraint parameters. Significant effects of dietary supplementation both on the latency to struggle (Fig. 1a) and the number of struggling bouts (Fig. 1b) ($P=0.02$ and $P=0.05$, respectively) was observed. The latency to struggle was shorter and the number of struggling bouts was

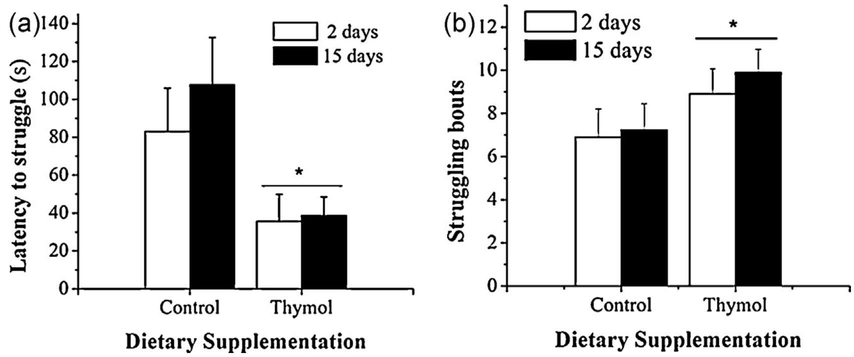

Fig. 1. (a) Latency to initiate struggling and (b) number of struggling bouts during a brief (5 min) mechanical restraint in female Japanese quail that were fed during 2 and 15 consecutive days a thymol supplemented diet and their control. Forty two females were evaluted in this test (21 within each treatment). *P<0.05 compared to their control. Bars represent the mean ± the standar error of the mean.

higher in the thymol than in their control counterparts. No further effects (P>0.05) of the thymol treatment were observed after 15 days of dietary supplementation in comparison to only 2 days of supplementation.

Table 1 depicts the effect of 2 or 15 days of dietary supplementation on the open-field locomotor activity. No significant effects of feed supplementation treatment or days of dietary supplementation on any of the variables evaluated were observed.

No significant body weight changes (P=0.98) were induced by thymol supplementation (244 ± 4 g and 248 ± 4 g, immediately before and 15 days after starting feed supplementation, respectively). Control quail counterparts did not show body weight changes either (257 ± 9 g and 253 ± 5 g, immediately before and 15 days after starting feed supplementation, respectively).

4. Discussion

Dietary supplementation with thymol was able to alter behavior during a mechanical restraint test. The latency to struggle was shorter and the number of struggling bouts was higher in the thymol than in their control counterparts. Adoption of immobility during restraint is considered to be indicative of intense fear (Jones, 1996; Jones and Satterlee, 1996), hence this result suggest a reduced fear response in the thymol supplemented group. To our knowledge, this is the first time a fear reducing effect of a phenol feed supplemented compound is informed in a behavioral test. Previous studies have shown that i.p. injection of propofol (an analog phenolic compound of thymol) has an anxiolytic effect in rats (Pain et al., 1999, 2002; Kurt et al., 2003). In particular, propofol significantly increased the time spent in the open arms of the plus-maze compared to control (Pain et al., 1999; Kurt et al., 2003). During the mechanical restraint trials, no further differences in the fear reducing effect of the thymol treatment were observed after 15 days of dietary supplementation in comparison to only 2 days of supplementation, suggesting that no tolerance to the thymol fear reducing effect were developed during the study. Taken together, these results suggest that thymol supplementation may be considered a management strategy to help reduce bird's fear responses when exposed to a stressful situation.

During open-field testing, no significant effects of feed supplementation treatment or days of dietary supplementation on any of the variables evaluated (latency to ambulate, time spent ambulating, distance ambulated and ambulation rate) were detected. Thus, the fear reducing properties of thymol observed in the mechanical restraint were not evidenced in the open-field test. These results are consistent with previous studies in poultry where testing birds in a T-maze (Marín et al., 1997) or in a brief mechanical restraint (Kembro, 2009) allowed to better detect fear reducing/anxiolytic effects of known neuroactive drugs than the testing in an open-field. For example, broiler chicks treated with a non-sedative dose of diazepam (0.05 mg/kg) showed a faster escape time during a T-maze test, but did not show a reduction in the latency to ambulate during open-field testing (Marín et al., 1997). In Japanese quail, an anxiolytic dose of ondasetron 0.1 mg/kg significantly increased struggling during mechanical restraint, but did not affect ambulation during open-field testing (Kembro, 2009).

Table 1
Open-field behaviors in female Japanese quail ($n = 42$) that were fed during 2 and 15 consecutive days a thymol supplemented diet and in their control.

Behavior	Control		Thymol			P-value			
	2 days	15 days	SEM	2 days	15 days	SEM	Diet	Days	Diet × Days
Latency to ambulate (s)	19.9	29.0	8.7	43.5	17.3	9.9	0.40	0.42	0.15
Time ambulating (%)	12.6	20.0	3.2	12.7	15.9	2.4	0.31	0.09	0.12
Distance ambulated (m)	8.7	15.7	3.1	7.8	9.1	2.1	0.25	0.07	0.19
Ambulation rate (m/s)	0.01	0.03	0.01	0.02	0.02	0.01	0.28	0.09	0.14

Diet, effects of dietary supplementation; Days, effects of days of supplementation; SEM, standard error of the mean.

In all, these results highlight the importance of using a combination of behavioral tests to evaluating the potential fear reducing/anxiolytic effects of new compounds.

Previous studies suggest that a combination of the nature, duration, intensity, and frequency (number of repetitions) of a stressful stimulation can determine whether habituation or sensitization predominate or perhaps whether both processes balance out (Jones et al., 2000). In particular, habituation is thought to be inversely related to stimulus intensity and positively related to the number of exposures (Jones et al., 2000). Quail exposed to repeated mechanical restraint (5 min on each of 4 consecutive days) vocalized and struggled sooner, and showed more struggling bouts after repeated restraint, indicating habituation to this particular stressor (Jones et al., 2000). A separate study in quail immobilized twice daily during 4 consecutive days showed that repeated restraint did not affect latency to struggle (Hazard et al., 2008). In our study, quail did not show signs of habituation nor sensitization in any of the two test situation which could be explained by the limited number of exposures and time between exposures to restraint (13 days).

As mentioned, 2 or 15 days of thymol dietary supplementation induced a behavioral response consistent with a reduced fear response in each of the restraint exposure tests. A reduced latency to struggle and a higher number of struggling bouts during a brief mechanical restraint exposure have been associated with an active coping strategies upon exposure to a threat (showing both behavioral and sympathoadrenal activity) (Jones et al., 2000; Hazard et al., 2008). On the other hand, their passive coping counterparts are characterized by adoption of immobility and an adrenocortical activation. Hence, our birds supplemented with thymol showed during the restraint exposure a behavioral response that is consistent with the adoption of an active stress coping strategy and therefore, it could probably help those birds to better overcome the consequences of the stress exposure. However, further studies are needed to elucidate whether or not the thymol diet supplementation may be used as a protective stress compound.

During open-field testing, quail that were feed with the thymol supplemented diet did not show a decrease in ambulatory activity suggesting that the dose provided has no sedative behavioral effects. In a previous study, no sedative effects were observed in open-field behavior when quail were administered intraperitoneally with a 5 mg/kg dose of thymol (Kembro, 2009). Other compounds that modulate GABAergic activity such as diazepam (Marín et al., 1997) and propofol (Kembro, 2009) at low subsedative doses also show fear reducing effects without decreasing exploratory behavior. While an acute administration of propofol at higher doses ($\geq$20 mg/kg) showed a clear dose dependant decrease in locomotor activity (consistent with a sedative effect) during an open-field test (Kembro et al., 2012). Hence, if higher doses of thymol are used in dietary supplementation, the potential decrease in locomotion due to sedation should be tested.

During this study no body weight changes were induced by thymol supplementation which is consistent with the majority of the studies on essential oils or their main components that show no negative effects on feed intake, body weight gain or final body weight (Windisch et al., 2008).

The reduction of fear and stress in poultry is important because of the deleterious effects of these states on animal performance and welfare (Hemsworth and Barnett, 1989; Mills and Faure, 1990; Jones, 1996). Regardless of the mechanism(s) underlying the behavioral findings of the present study, the current results, coupled with other desirable previously reported physiological and/or product quality consequences of thymol supplementation (Luna et al., 2010; Luna, 2012), suggest that this management procedure could have a beneficial outcome both from the animal well-being and productivity point of view.

In conclusion, thymol dietary supplementation could provide beneficial effects during quail husbandry because it can lower the animals' fear response when exposed to a stressful situation without causing changes in ambulatory activity or a decrease in body weight. Future studies are necessary to further explore the fear reducing properties of thymol, and its usefulness as a dietary supplement for poultry to optimize their efficacy. In addition, due to the importance of male quail for meat production, future studies should also evaluate male responses.

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