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Expression of aggressiveness modulates mesencephalic c-fos activation during a social interaction test in Japanese quail (*Coturnix japonica*)

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Highlights

- Mesencephalic activation is involved in active expression of aggressive behavior.
- Non-aggressive males and the test controls show similar c-Fos labeling.
- In general, mesencephalic c-Fos expression was not influenced by rearing condition.

ABSTRACT

It is well known that during a social conflict, interactions are dependent on the animal's propensity to behave aggressively as well as the behavior of the opponent. However, discriminating between these two confounding factors was difficult. Recently, a Social Interaction (SI) test using photocastrated males as non-aggressive stimuli was proposed as a useful tool to evaluate aggressiveness. The avian Intercollicular- Griseum centralis complex (comparable to mammalian periaqueductal gray) has been reported as a crucial node in the descending pathways that organize behavioral and autonomic aspects of defensive responses and aggressiveness. Herein, using the SI test, we evaluated whether mesencephalic areas are activated (expressed c-fos) when photostimulated adult males are confronted with non-responsive (non-aggressive) opponents. Furthermore, we also examined whether mesencephalic activation is related to male performance during the SI test (i.e., aggressive vs. non-aggressive males) in birds reared in enriched or in standard environments. Five mesencephalic areas at two anatomic levels (intermediate and rostral) and locomotion during SI testing were studied. Aggressive males showed increased c-fos expression in all areas studied, and moved at faster speeds in comparison to their non-aggressive and control counterparts. Non-aggressive males and the test controls showed similar c-fos labeling. In general, rearing condition did not appear to influence c-fos expression nor behavior during the SI test. Findings suggest that mesencephalic activation is involved when males are actively expressing aggressive behaviors. This overall phenomenon is shown regardless of both the environmental stimuli provided during the birds´ rearing and the potentially stressful stimuli during the SI trial.

Keywords: aggression; avian brain; periaqueductal gray; Intercollicular nucleus; Stratum griseum periventriculare.

Abbreviations: Ag: Aggressive; **AHA**: Anterior hypothalamus; **Aq**: Cerebral Aqueduct; **BNST**: Bed nucleus of the stria terminalis; **Cb**: Cerebellum; **CP**: Commissura posterior; **EE**: Enrichment Environment; **GCt** : Griseum centralis; **Ico**: Intercollicular nucleus; **ICol**: Lateral intercollicular nucleus; **ICom**: Medial intercollicular nucleus; **LAS**: Lateral septum; **MEA**: Medial amygdala; **MLd**: Nucleus mesencephalicus lateralis, pars dorsalis; **MPOA**: Medial preoptic area; **nonAg**: non-aggressive; **PAG**: Periaqueductal gray; **PC**: Photocastrated; **POM**: Preoptic medial zone; **SGPd**: Dorsal part of the stratum griseum periventriculare; **SI**: Social Interaction test; **STD**: Standard Environment; **testCON**: Test control; **VMH**: Ventromedial hypothalamus

1. INTRODUCTION

Male aggressiveness during social interactions has been extensively studied and is frequently associated with circulating testosterone level or its metabolites [1–3]. Moreover, whether the male resulted in the winner or loser during previous interactions, or if the interaction occurred in the presence of an audience, have also been reported as influential factors [4]. Aggression has been shown to have a genetic/hereditary component as well as being dependent on interactions between social and physical environmental factors [5–11]. The expression of an aggressive behavior is clearly a matter of both the birds´ underlying aggressiveness (propensity to behave aggressively) and the behavior of the potential opponent. Fighting with an unfamiliar male conspecific can potentially increase testosterone production in quail in a context dependent manner, for example inducing high postconflict testosterone metabolite levels when in the absence of an audience [4]. Testosterone is aromatized in the preoptic medial zone (POM) [12–14]. Beyond this hormone response, the characterization of the neural circuits that control aggression presents difficulties because these circuits also regulate fear responses, as well as other social behaviors. Indeed, it has been suggested that aggressive behaviors are emergent properties of a social behavior network that includes the medial preoptic area (MPOA), lateral septum (LAS), anterior hypothalamus (AHA), ventromedial hypothalamus (VMH), periaqueductal gray (PAG), medial amygdala (MEA) and bed nucleus of the stria terminalis (BNST) both in birds and mammals [15–18].

In mammals, the periaqueductal gray (PAG) is a crucial node in the descending pathways that organize behavioral and autonomic aspects of innate defensive, aggressive, and fear responses [19–21]. Also, it is considered an essential relay of harmful stimuli, and of hypothalamic inputs (related to predators) to limbic, thalamic and cortical circuits involved in fear conditioning and learned aversive responses [22].

In birds, neurochemical, anatomical, hodological and functional evidence suggests that the mesencephalic Intercollicular nucleus and the Griseum centralis (ICo-GCt complex) could be comparable to the mammalian PAG [23,24]. Furthermore, recent functional data obtained from pigeons indicate that other midbrain regions, such as the dorsal part of the stratum griseum periventriculare (SGPd, also identified as the layer 15 of the optic tectum) and the nucleus mesencephalicus lateralis, par dorsalis (MLd), are also part of the midbrain defensive circuitry in birds [25]. Although the association

between these regions of the brain and aggression has received less attention in birds, a study in zebra finches exposed to a resident intruder test showed modulation of c-fos expression in the medial ICo and GCt areas of dominant males (i.e., performed a larger number of aggressive behaviors against its opponent) in comparison to their subordinate counterparts [24]. However, in their study given the nature of the test, the level of underlying aggressiveness observed could be highly influenced by the opponent's behavior.

Recently, a Social Interaction (SI) test has been proposed as a useful tool to evaluate aggressiveness in adult males [26]. This test characterizes the aggressive responsiveness of experimental photostimulated males by confronting them with a photocastrated (PC), and therefore non-aggressive, male counterpart in a novel test environment. During interactions, strong interindividual variations were reported in the photostimulated males. For example, birds that actively peck at PC counterparts as well as those that did not peck at opponent at all were both observed. Interestingly, PC birds neither initiated the aggressive interactions nor defended aggressively against the photostimulated males. Thus, the test can be used to classify males with divergent propensity to behave aggressively.

This study evaluated whether c-fos expression, as an indicator of neural activity [27], can be altered in the mesencephalic defensive circuitry of adult males that showed divergent aggressiveness in the SI test (i.e., showing an aggressive vs. a non-aggressive profile).

Environmental enrichment (EE; i.e. increasing the biological relevance of captive environments by offering variety of stimuli [28]) is a technique that nowadays is widely promoted to improve animal welfare of captive animals [29,30]. It has shown a wide range of positive behavioral and physiological effects, including reduction in aggressive behaviors when birds are reared in enriched environments [31–33]. Environmental enrichment has also been shown to induces visible structural and functional changes in the brain [34]. Furthermore, adult pigeons housed in EE for 40 days, showed increased neurogenesis in limbic structures (e.g. hippocampus), as well as, attenuation of defensive behaviors in response to novel environments [25]. Under this premise, it is conceivable that animals raised in EE, having a greater number of environmental stimuli to process, could potentially show differentiated neurophysiological mechanisms in different areas of the brain, compared to animals living in impoverished environments ("wastelands")

[28,34,35]. This study includes birds that were reared either in enriched (EE) or in standard (STD) environments.

2. MATERIALS AND METHODS

2.1 General procedure

The study was performed with Japanese quail (*Coturnix japonica*) a species widely used for studies covering neuroendocrine and social behaviors [36,37]. Also, they are considered an excellent laboratory model for the extrapolation of data to other poultry species with higher commercial relevance because of their high physiological similarity [37–39]. The animals were bred according to standard laboratory protocols [40,41] and according to the National Institute of Health guide for the care and use of laboratory animals [42]. Experimental protocol was approved by the Institutional Council for the Care of Laboratory Animals (CICUAL, Comite Institutional de Cuidado de Animales de Laboratorio) of the Facultad de Ciencias Exáctas, Físicas y Naturales, Universidad Nacional de Córdoba.

Figure 1 shows a squematic representation of experimental setup. A total of 170 birds were raised under either EE or STD environmental conditions from one week of age until the end of the experiment (see Section 2.3 for details). Morphometric data was montinored throughout rearing. The SI test, as described in Caliva et al [26], was performed between 118 and 130 days of age, except in control animals. Animal behavior during SI testing was recorded onto a computer and was remotely monitored by experimenter. Acording to male aggressive behavior displayed (see Section 2.4) males were clasified as aggresive (Ag) or non-aggressive (nonAg). Two control situations were included in the experimental design. Males exposed to the experimental apparatus without the presence of an opponent (testCON) and non-manipulated males that always remained in the home cage (naïve) with their cagemate were used as control groups. Thus, 32 males (8 from each categorical group associated with the SI test (Ag, nonAg, testCON, and naïve)) were sacrificed for the study, half raised under EE condition and the other half under STD. Locomotor and aggressive behaviors during the SI testing, and the c-fos expression in five neuroanatomical mesencephalic areas of interest were evaluated in those birds (see Sections 2.5 to 2.8 for details).

2.2 Animals and husbandry

Mixed-sex Japanese quail hatchlings were randomly housed in groups of 50-60 in white wooden brood boxes measuring 90 x 80 x 60 cm (length x width x height respectively) with a feeder along one wall, and 16 automatic nipple drinkers. A wire-mesh floor (1 cm grid) was raised 5 cm to allow the passage of excreta to the collection tray to facilitate cleaning and comfort of the animals, and a lid prevented the birds from escaping. Brooding temperature was 37.0 °C during the first week of life, with a weekly decline of 3.0 °C until room temperature (24 to 27 °C) was achieved. Food and water were provided *ad libitum*. The first week of life all animals were raised under the same standard conditions. During this week in all of the boxes corrugated cardboard was placed as flooring. At 7 days of age, the animals were redistributed between the six boxes order to obtain the same density of chicks per box (46 animals per box). The next day, the EE protocol (see below) was applied to half of the boxes while the other half remained without enrichment, under standard (STD) conditions. Quail were subjected to a daily cycle of 14 h light (300 to 320 lx): 10 h dark (long photoperiod; photostimulated) throughout the study, with the exception of PC stimulus birds that were submitted to a short photoperiod light cycle (06 h light: 18 h dark) beginning at 4 weeks of age until testing ended [26].

At 28 days of age, test animals were sexed by plumage coloration, marked with a wing band and weighed. One-hundred seventy birds were randomly housed in pairs of 1 male and 1 female in cages of 20 x 40 x 20 cm (width x length x height respectively). Animals continued in the same rearing condition they were in during the first rearing stage (EE or STD). After reallocation in cages, every two-weeks birds were weighed, male cloacal gland length (mm) and width (mm) were measured using a digital caliper and cloacal gland foam production was assed. Cloacal gland volume was estimated as ($4/3$ x 3.5414 x a x b^2), where a = 0.5 x length, and b =0.5 x width [43]. Cloacal gland foam production was quantified by subjective scaling of the amount of foam ejected upon manual expression (squeezing) of the foam gland, using a scale of 1 (no foam expressed) to 5 (maximum amount of foam expressed).

2.3 Environmental enrichment protocol

The EE protocol is an extended version of the one applied in Nazar et al. [44] in brood boxes of chicks and juvenile quail (Table 1, Brood boxes). Using this protocol, the

authors showed an improved immune response and reduced detrimental effects of a later stressor exposure in juvenile quail housed in an EE in comparison to STD brood boxes [44]. The original protocol (used also herein up to the 28 days of age) included wooden platforms, hanging bottle caps, hanging colored strips, and Velcro cylinders [44] (Table 1). These same elements were used in the adult cages (29-130 days of age), as well as other well-known enrichments including sandboxes and alternate substrates such as synthetic grass and plastic leaves. It should be noted that birds actively interacted with the enrichment elements provided.

Unless stated otherwise, during EE 3 different sets of enrichments were rotated between boxes or cages every 7 days (Table 1). Within each period, EE home boxes/cages received weekly one of the three enrichment sets in a random order in such a manner that all EE animals were exposed to the 3 enrichment sets. The replacement of the enrichment items was done on a fixed day and time every week, and during this procedure all the animals were removed from the box and placed temporarily in a basket. Control animals underwent the same manipulation procedure. During the last month of EE (92-130 days of age) all EE birds were provided the same sequence of enrichment elements to minimize potential variability in the birds´ responses between cages. Additionally, throughout the complete period of SI testing (113 and 130 days of age) sandboxes were added as enrichment.

2.4 Social Interaction (SI) Test.

The SI test was performed in the context of a larger study, where all birds (except control males) between 118 and 130 days of age were evaluated. The SI test is described in detail in Caliva et al [26]. Briefly, the SI test consists in encounters between an unfamiliar test adult male and a PC stimulus adult male, in the presence of the test bird's female cagemate (audience). The use of an audience is based on the work of Hirschenauser et al (2013) who observed that with a familiar audience, winners attacked losers twice as often than during fights without an audience.

First, the test male and its female cagemate were placed in a central compartment separated by an opaque partition from a PC stimulus male. After 2 min, the test male and the PC stimulus male remained in the same compartment, while the female cagemate was placed in a nearby compartment at one side of the apparatus, and used as a social audience. Immediately after, the central opaque partition was removed and the test and PC stimulus

birds were allowed to interact. Direct interaction lasted a maximum of 10 min. However, if during the interaction a quail received more than 5 consecutive aggressive pecks, showed a clear and continued escaping (retrieval) behavior, and/or showed any sign of physical damage, the interaction was immediately interrupted [45]. A video-camera was positioned 1 m above the apparatus and connected to a computer that allowed constant monitoring and recording during the test while out of the sight of the birds. Using a customized code [46] in Matlab R2017a (MathWorks Inc) based on Behavioral Collect [47] the x,y coordinates of the center of the animal at 1s intervals were recorded, The distance ambulated was defined by the distance the animal moved between 2 successive time interval of 1 s. If the distance ambulated in a given time interval exceeded a threshold distance of 1 cm, then the bird was considered mobile [48]. Percent of time mobile was estimated as the total time mobile divided by test duration multiplied by 100. Speed was estimated as total distance ambulated divided by test duration. Using ANY-maze© Behavioural tracking software (Stoelting) the following aggressive behaviors during the third stage of the SI test as defined in Caliva et al [26] were recorded and are publically available at figshare [49]:

Pecks: when one bird raises its head and vigorously pecks the other bird's body (usually on the head).

Grabs: when a bird catches ("grabs") with their beak the neck or head region of the other bird.

Mounts: while performing a grab, the bird approaches the other bird from behind, and places both feet on the dorsal surface of its torso, stepping over the other birds' tail.

Cloacal contacts: during mounting, the bird lifts his tail and tilts his pelvis underneath the other bird and briefly presses its cloaca against the other bird.

Chase: a bird runs after another that is escaping.

Attack with claws: the subject jumps with claws forward impacting directly onto the other bird's body.

Latency to initiate the first aggression: the time from the removal of the central opaque partition to the first aggression towards opponent.

Latency to perform 5 aggressions: the time from the removal of the central opaque partition to the completion of 5 aggressions towards opponent.

Herein, when grabs, mounts or cloacal contacts were performed by one male towards another male, they were considered as aggressive behaviors [50]. Males that performed more than 5 aggressiveness behaviors were considered aggressive (Ag), and

males that did not perform any aggressive behavior towards PC opponent were considered non-aggressive (nonAg).

Males exposed to the experimental apparatus without the presence of an opponent (testCON) and non-manipulated males that always remained in the home cage (naïve) with their cagemate were used as control groups. A total of 8 males from each categorical group associated with the SI test (Ag, nonAg, testCON, and naïve) were sacrificed for study, half raised under STD condition and the other half under EE. Due to technical limitations only 8 perfusions were able to be performed each day, thus one animal of each of these 8 experimental groups were perfused daily. The assignment of birds to test condition (SI testing or testCON, and naïve) was randomized. The order in which the birds were evaluated in SI test and controls was also randomized.

2.5 Perfusion, fixation, cut and storage.

Sixty minutes' post SI test, the quails were anaesthetized intra-peritoneally with 30% chloral hydrate and perfused transcardially with a blood-washing solution consisting of 0.8% sucrose, 0.8% NaCl and 0.4% glucose followed by 4% paraformaldehyde in 0.2M borate buffer pH 7.4. The brains were left in the skull overnight at 4 °C, after being removed from the skull and placed in 30% sucrose [51]. The brains were cut either coronally at 50 μm on a freezing microtome and the sections were collected serially in the same fixative solution into five compartments and stored at -24 C in cryoprotection buffer (propylene glycol 30 % sucrose in 0.2 M PB) until required for the immunohistochemical procedures. Naïve animals were anaesthetized directly in their home cages.

2.6 C-fos Immunohistochemistry

For each experimental group eight mesencephalic sections were studied for each animal. The c-fos label was applied according to the protocol previously described and validated in detail [25]. Unless otherwise indicated, the sections were rinsed four times in in PBS 0.3% Triton-X (PBST) for 5 min before initiating protocol and between incubations. After the first rinse, the sections were placed in a solution of methanol + H_2O_2 0.3% for 30 min for blocking endogenous peroxidase. After a second rinse, the sections were incubated with PBST plus 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 60 min. This was followed by incubation with the anti-Fos primary

antibody (SC-52, rabbit IgG, Santa Cruz, in PBST 1: 4000) overnight (approximately 20 h) at 4 ° C. The sections were incubated for 90 min with a biotinylated secondary antibody (Vectastain Elite ABC Kit – VectorLabs®, in PBST, 1:1000) and then incubated for 2 h with biotinylated Avidin enzyme complex (Vectastain Elite ABC Kit – VectorLabs®, in PBST, 1:500)). For visualization, the sections were incubated 4 min in a solution of 0.05% 3, 30-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, in PBST with 0.03% of H_2O_2), for 10 min. The sections were then washed in PBS 0.01 and mounted on gelatin-coated glass slides, air-dried for 48 h and dehydrated in a graded series of alcohols and xylenes before being covered slipped with DPX mounting medium (Sigma-Aldrich, St. Louis, MO). This procedure yielded a consistent cell nuclei staining pattern similar to the observed in previous studies in mammalian [52–54] and avian species [25, 55, 56]. Furthermore, we performed negative controls by suppressing either the primary or the secondary antibodies from the reaction. This resulted in sections with no observable staining and little background color due to DAB incubation. In addition, midbrain sections of pigeons were incubated together, in the same reaction medium with sections of quail brain as positive controls. In those sections c-fos staining was identical to the observed in previous works from our laboratory [25].

2.7 Definitions of neuroanatomical areas

The mesencephalic regions of interest were defined based on neurochemical and functional data previously published by Melleu et al. [25]. A microphotograph of a transversal plane of the mesencephalon is shown in Figure 1 (right panels). Briefly in this study, two levels (intermediate and rostral, A 2.0 and A 3.0 respectively, of stereotaxis of the brain of the quail [56], in each level, the five neuroanatomical areas of interest were analyzed, the GCt, this is a nucleus that is located at the dorsomedial level of the mesencephalon, dorsally to the nucleus nervi oculomotorii in the intermediate level, and below the posterior commissure at the rostral level. In both anatomical level, the other areas studied were, the nucleus mesencephalicus lateralis pars dorsalis (MLd), above the aqueduct (or tectal ventricle); the Intercollicular nucleus (ICo) which can be further divided into two regions, with respect to their position in relation to the MLd, the medial (ICom) and the lateral (ICol) intercollicular nucleus, and surrounding the lateral aqueductal expansion, a different cell layers can also be observed the dorsal part of the stratum griseum periventriculare (SGPd).

2.8 Quantification of the immunohistochemistry

The slides were then taken to an optical microscope (OptiCam Microscopy 0400S B.) coupled with a digital camera (ToupCam SCMOS0300KPA) for photographic documentation (Fig. 2 A, B). The quantification of c-fos-ir cells was performed on photomicrographs of two anatomical levels. Areas of interest were determined utilizing anatomical landmarks, principally the lateral expansion of the aqueduct (or tectal ventricle) to the neuroanatomical areas as ICo, MLd and SGPd, and nucleus nervi oculomotorii and posterior commissure to GCt (Fig. 2 C, E). The photomicrographs were converted into binary format with a 0.35 threshold relative to the black and white signal level to exclude all faintly, subthreshold stained nuclei (Fig. 2 D, F). For each neuroanatomical area of interest, a square central counting zone was determined. Within this central zone all c-fos-positive nuclei were manually counted and total area of the counting zone (μm^2) was estimated utilizing the ImageJ software [57,58]. The total nuclei counted were then divided by the total area of the counting zone (μm^2), to obtain $N/\mu m^2$ (N= total number of positive nuclei). Raw data is publically available in figshare [59].

2.9 Statistical Analysis

To compare weight, cloacal gland volume, foam production, locomotor behavior and c-fos expression between the different experimental groups in each neuroanatomical region, we used generalized linear mixed models (GLMM). Initial analysis showed, in general, no main effects of the type of environmental housing condition of rearing (EE and STD) neither on c-fos expression nor on the birds´ behavior during SI testing. Thus, information from the two housing conditions were merged and data was reanalyzed using a GLMM model that included the categorical groups associated with the SI test (Ag, nonAg, testCON, and naïve) as fixed effect, and the type of environmental condition of rearing (EE and STD) and day of sacrifice as random effects. A normal distribution was used for all variables except distance and speed ambulated where a Gama log distribution was used. In all cases, the GLMM assumptions were verified. In all cases, a two-tailed p-value of ≤ 0.05 was considered to represent significant differences. Fisher´s Least Significant Difference (LSD) post hoc was used for comparisons between categorical groups associated with the SI test. It should be noted that Rostral MLd showed an interaction between categorical groups associated with the SI test and the type of

environmental condition of rearing, although it appeared significant, it could not be properly assessed in this study due to the small sample size.

3. RESULTS

Table 2 shows morphometric characteristics of test males at 113 days of age as well as locomotor and aggressive behavior during the SI test. No differences in weight, cloacal gland size and foam production were observed between groups. During the SI test, no differences were found between groups in percent of time mobile. However significant differences between the categorical groups associated with the SI test (Ag, nonAg, testCON and naïve) were found in total distance ambulated ($F_{2\text{-}23}$=2.33; p=0.04) and in the speed of ambulation (i.e. distance ambulated divided by test duration) ($F_{3,23}$= 5.09; p = 0.02). Ag males showed significantly higher distances ambulated than testCON and higher speeds than their nonAg and testCON counterparts.

Quantification of c-fos-ir cells showed significant differences between the categorical groups associated with the SI test (Ag, nonAg, testCON and Naive) in all brain areas evaluated in the intermediate level (Fig. 3A), GCt ($F_{3,19}$= 10.04; p = 0.0004), ICom ($F_{3,19}$= 7; p = 0.0023), MLd ($F_{3,19}$= 12.72; p = 0.0001), ICol ($F_{3,19}$= 11.77; p = 0.0001), SGPd ($F_{3,19}$= 6.09; p = 0.0044) and rostral level (Fig. 3B), GCt ($F_{3,19}$= 9.94; p = 0.0004), ICom ($F_{3,19}$= 6.16; p = 0.0042), MLd ($F_{3,19}$= 15.73; p = 0.0001), ICol ($F_{3,19}$= 7.94; p = 0.0012), SGPd ($F_{3,19}$= 16.66; p = 0.0001). LSD post hoc analysis showed in all areas a greater expression of c-fos in the males that actively exhibited aggressive behaviors during the SI test, in comparison with the nonAg, testCON and Naive (Fig. 3).

4. DISCUSSION

The SI test has been proposed as a useful tool to assess the propensity to behave aggressively in male quail. Because males are tested against PC (non-aggressive) counterparts, it has the advantage of detecting birds that are inherently aggressive (i.e. birds that actively attack even when the opponent never shows aggressiveness towards them) as well as birds that do not show any sign of aggressiveness [26]. The five mesencephalic areas studied (GCt, ICol, ICom, MLd and SGPd) showed heightened c-fos expressions in the males showing aggressive performances in comparison to the males showing no signs of aggressiveness. Moreover, no differences in the percent of time spent mobile was seen between groups during SI testing (Table 2). Interestingly, aggressive males moved further (larger distance ambulated) than testCON and ambulated faster (larger speed) than both nonAg and testCON counterparts, possibly associated with the observed chasing behavior of these animals. In all, these results suggest that activation of mesencephalic areas are dependent of whether males are actively expressing aggressive behaviors, and concomitantly show higher locomotor speeds. In previous studies in zebra finches [24], after a resident intruder test, dominant males showed a greater expression of c-fos in the ICom area while a lower expression was observed in the GCt area in comparison to subordinate males (i.e. those who received a greater number of aggressive behaviors). The differences between our study and that with zebra finches in c-fos expression in the GCt area may be associated with fundamental differences between the tests used. The resident intruder test used in the zebra fiches study, unlike the SI test, does not have the potential to control the opponent's reaction. The subordinate bird when attacked by the dominant, can behave defensively responding with aggressions or escaping [60] and therefore, affecting the dominant performance.

As stated previously, the GCt/ICo complex of the avian midbrain is thought to be comparable to the mammalian PAG [25]. In rodents, the PAG integrates hypothalamic/limbic inputs with motor and autonomic brain stem and spinal cord outputs coordinating defensive and aggressive behavioral responses, as well as hormonal levels (e.g. Testosterone, Corticosterone. etc.) [61]. Specifically, the dorsolateral portion of the PAG receives projections (via the dorsal premammilary nucleus) from the ventromedial hypothalamic nucleus [22] a region that was shown to be active during male aggressive responses as well as in mating behavior [62,63]. This aggression regulating hypothalamic/PAG circuit is relatively understood in rodents (for a review see [22]).

Nonetheless, in avian species, although de GCt/ICo receives input from the hypothalamus [64], to our knowledge, there are no functional studies on the neural circuitry regulating avian aggression as a whole. Furthermore, there is little hodological evidence about the ICo and GCt connections with hypothalamic sub-regions. Here we provide functional evidence that avian midbrain regions are involved with the display of aggression, however, further experiments are necessary in order to elucidate the circuitry (its hodology as well as its neurochemical characteristics) and its role in regulating behavioral and hormonal responses is birds.

For over the last 25 years it has been shown in rats that locomotion coincides with c-fos expression in caudal brainstem and spinal cord [65] areas of inferior olive and cerebellar nuclei [66], as well as the mesencephalic locomotor region [67]. Recent studies have shown that locomotor speed and interlimb coordination (gait) are controlled by several brainstem structures that transform signals from higher brain centers into meaningful commands to initiate, stop or modulate locomotor frequency and gait [67–75]. Specifically, in regard to the contribution of mesencephalic locomotor region in freely behaving mouse, glutamatergic neurons of the cuneiform nucleus have been proposed to initiate locomotion and induce running gaits, whereas glutamatergic and cholinergic neurons of the pedunculopontine nucleus modulate locomotor pattern and rhythm, contributing to slow-walking gaits [76]. Moreover, in mice, speed and gait selection were proposed to be controlled by glutamatergic excitatory neurons (GlutNs) segregated in two distinct midbrain nuclei: the cuneiform nucleus and the pedunculopontine nucleus. GlutNs in both of these regions contribute to the control of slower, alternating-gait locomotion, whereas only GlutNs in the cuneiform nucleus are able to elicit high-speed, synchronous-gait locomotion. Additionally, both the activation dynamics and the input and output connectivity matrices of GlutNs in the pedunculopontine nucleus and the cuneiform nucleus support explorative and escape locomotion, respectively[77]. In mammals, projections to the cuneiform nucleus arise from de dorsolateral column of the PAG (dlPAG). Interestingly, chemical stimulation (with Glutamatergic agonist N-Methyl-D-aspartate) of both the dlPAG and the cuneiform nucleus elicit escape behavior in rats. Although in avian species the mescencephalic locomotor region has received less attention, mesencephalic activation shown herein in Ag males could also be associated with the higher locomotor speed shown due to chasing activities during SI testing in comparison to nonAg and testCON males.

In quail, the c-fos expression in the GCt has also been associated with consummatory and appetitive sexual behavior [78]. Moreover, previous tract-tracing studies demonstrated the existence of projections from the medial preoptic nucleus (POM) to the GCt in quail [79]. It has been proposed, the aromatase-immunoreactive (ARO-ir) fibers located in the lateral part of the POM may control the premotor aspects of male copulatory behavior through their projection to GCt and suggest that GCt activity could be affected by estrogens released from the terminals of these ARO-ir neurons [79] and the activation of that circuit is also known to be testosterone dependent [37]. Herein, 51% of behaviors performed were grabs and mounts considered aggressive due to the fact that they were directed from a male towards another male [5,80]. However, these same behaviors when directed towards females are associated with reproduction, and thus, they could also be considered as male-male sexual behaviors. Indeed, Adkins-Regan (2015) proposed that male-male sexual behavior in quail is an incidental by-product of strong mating motivation (i.e., males may have been selected to mate rapidly, vigorously, and fairly indiscriminately) [50]. In this context, GCt activation during male-male interactions could be associated not only to their underling aggressiveness but also with consummatory and appetitive sexual behavior.

In our study, handling and exposure to the novel test environment in themselves did not induce modulation of mesencephalic c-fos expression. Melleu et al. [25] also showed in pigeons, in same neuroanatomical areas no effect of handling on c-fos expression. In our study, as in Melleu et al [25], birds were handled previous to testing, thus birds could present some degree of habituation to handling. These authors also observed a significant increase in the rostral level of the SGPd and in the intermediate ICom, ICol, MLd and SGPd in the expression of c-fos cell inmunoreactive after a tonic immobility, indicating that a highly stressful situation could induce mesencephalic c-fos expression [25]. Tonic immobility is considered a defense mechanism [63, 75], suggesting that in novel/threatening situations both the display of defensive as well as aggressive behaviors could be modulated by the same neurological areas.

Herein, our initial analysis showed no influence of the environmental rearing condition on body weight, cloacal gland volume, and foam production, in the proportion of aggressive male, nor in the number of aggressive behaviors displayed during SI testing (Fig. S1). In previous studies, exposure to complex or enriched environments was seen to attenuate aggressive and defensive behaviors in both avian and mammalian species [31,32,82–86]. However, tonic immobility duration, but not other defensive responses,

was higher in pigeons reared in an enriched environment when compared to birds housed in isolation [87]. In poultry, the effect of enrichment on the expression of aggression has shown to be dependent on the study. Enrichments have been shown both to reduce [88,89] or to not affect [90] aggression and feather pecking. We did not detect changes in c-fos expression in relation with the rearing environments. However, it cannot be discarded that this lack of effect is due to the EE protocol implemented in itself or the low number of birds tested in those group combinations. Therefore, a larger study focused on testing different housing conditions should be carried out to find conclusive evidence.

5. CONCLUSION

In conclusion, this study shows that the mesencephalic activation is involved when males are actively expressing aggressive behaviors, and concomitantly show higher locomotor speeds. However, because during aggressions, male-male interactions also included sexually related behaviors (grabs and mount), it cannot be rule out that the observed activation could also be related to consummatory and/or appetitive male- male sexual behaviors. The overall phenomena are shown regardless of both the environmental stimuli provided during the birds´ rearing and the potentially stressful stimuli during the SI trial.

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Figure legends

Fig. 1. Schematic representation of experimental design. Half the birds were reared under standard and half under enriched environmental conditions from 7 days of age until sacrificed as indicated by the black and green boxes, respectively. Between 118 and 130 days of age male quail were tested in the Social Interaction (SI) test (photograph of apparatus shown in central bottom panel), behavioral data was recorded, and according to their performance were classified as aggressive (Ag) or non-aggressive (nonAg). Two controls were used, males exposed to the SI test protocol but without a photocastrated opponent (testCON) or birds that remained in home cages a were never exposed to the SI test (Naïve). Four quail from each group were perfused, and brains were fixed, cut and c-fos immunohistochemistry was performed on mesencephalic sections. For statistical analysis, data from birds from standard and enriched environmental conditions within each SI test condition (Ag, nonAg, testCON, and naïve) were pooled, thus providing a total of 8 birds per group. Right panels show Giemsa stain mesencephalic frontal sections at intermediate (top) and rostral (bottom) levels delimiting the areas of interest: Griseum centralis (GCt), medial intercollicular nucleus (ICom) and lateral intercollicular nucleus (ICol), nucleus mesencephalicus lateralis pars dorsalis (MLd) and dorsal part of the stratum griseum periventriculare (SGPd). Escale bar 500 µm. In the bottom is included a photograph of the Social Interaction setup. Photostimulated (PS) test male, photocastrated (PC) stimulus opponent and audience (A) female cagemate are observed. The grey arrow shows the position where the opaque partition is placed in Stage 1 (see further description in Social Interaction Section). The dashed grey line indicates wire wall separating the audience compartment from test compartment.

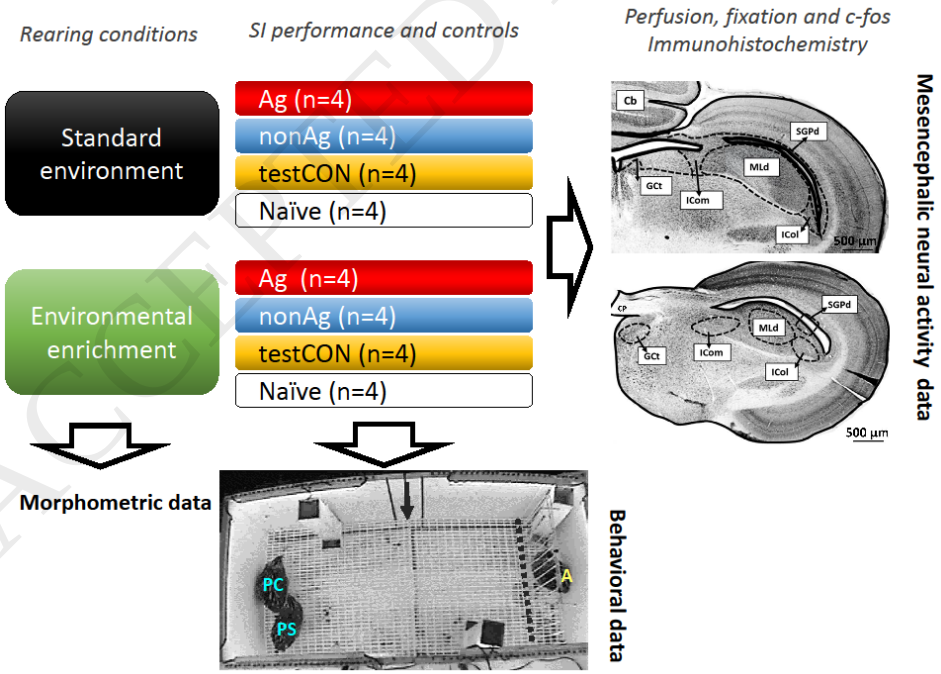

Fig. 2. Representative microphotographes of distributions of c- Fos labeling in intermediate mesencephalic areas in Japanese quails classified as Aggressive (A,C,D) or non-Aggressive (B, E,F) in the social interaction test. A-B) Intermittent lines are lateral intercollicular nucleus (ICol), nucleus mesencephalicus lateralis pars dorsalis (MLd), and dorsal part of the stratum griseum periventriculare (SGPd), and a continuous line are delimiting cerebral aqueduct (Aq). Scale bar 250 µm. C, E) Higher magnification of the microphotographes shown in panels A and B, respectively, in the GCt area. scale bar 15 µm. D,F) Image with threshold processing of images (ImageJ software) shown in C and E, respectively.

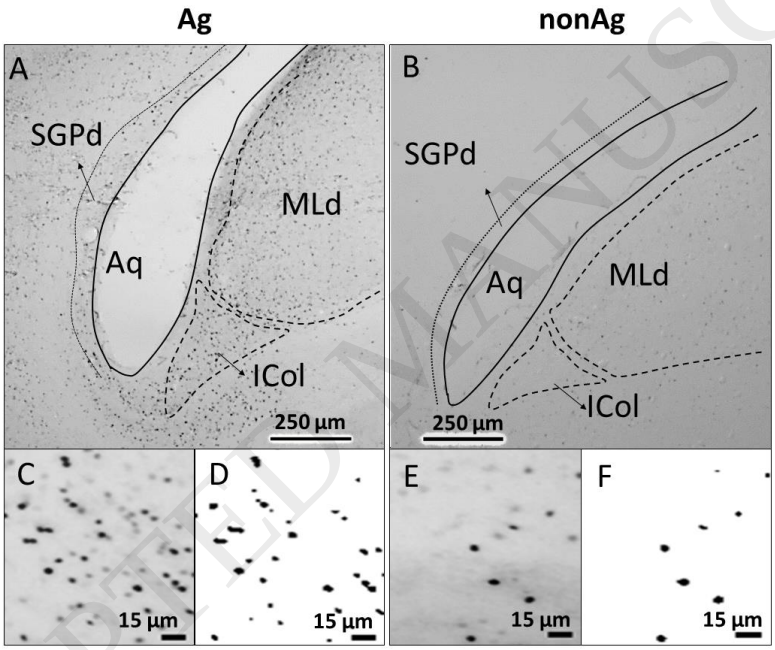

Fig. 3. C-fos-immunoreactive nuclei (N/mm^2) in neuroanatomical areas in two level, A) intermediate and B) Rostral of the Griseum centralis (GCt), medial intercollicular nucleus (ICom) and lateral intercollicular nucleus (ICol), nucleus mesencephalicus lateralis pars dorsalis (MLd) and dorsal part of the stratum griseum periventriculare (SGPd), in males

classified as Aggressive (Ag) or non-Aggressive (nonAg) during the Social Interaction test. Figure included groups of males exposed to the test setup without a decoy opponent (testCON) and control males that remained in their cages (naïve). A total of 32 males were evaluated (n=8). Data was analyzed using a GLMM model that included the categorical groups associated with the SI test (Ag, nonAg, testCON, and naïve) as fixed effect, and the type of environmental condition of rearing (EE and STD) and day of sacrifice as random effects. A normal distribution was used for all variables. *Aggressive males show significantly higher values in comparison to all other categorical groups. + Naïve group is significantly lower than testCON in the intermediate GCt area.

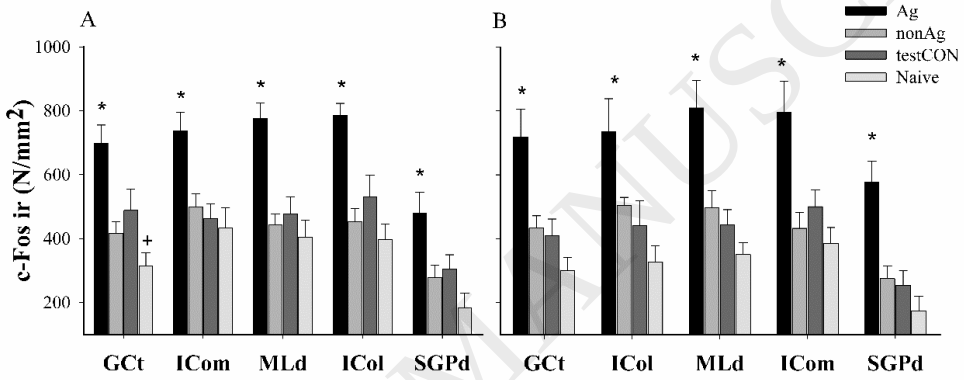

Table 1. Description of enrichment sets applied weekly to quail during each time period.

HOUSING	PERIOD	ENRICHMENT SET
Brood boxes	8 to 29 days of the age (stage chick / juvenile)	Three wooden platforms
		Hanging bottle caps, hanging colored wool, and three Velcro cylinders
		Hanging bottle caps, two Velcro cylinders and two wooden platforms
Home cages	29 to 50 days of the age (stage end of juvenile until started puberty)	Two hanging plastic balls of 2.5 cm in diameter of different colors
		A wooden platform of size 10 x 7 x 1 cm (width x length x height)
		A mirror (15 x 15cm) on the lateral wall
	50 to 71 days of the age (beginning of puberty until maximum male gonadal development).	Synthetic grass (alternate substrate) size 15 x 10 cm
		Three hanging colored strips
		A sandbox of 15 x 10 x 5 cm (width x length x height)
	71 to 92 days of age (animals are sexually active)	Three hanging bottle caps
		A branch of plastic green leaves (alternate substrate)
		A 20 cm long perch
	92 to 113 days of age (animals are sexually active)	A wooden platform of size 10 x 7 x 1 cm (width x length x height)
		Synthetic grass (alternate substrate) size 15 x 10 cm
		Two hanging plastic balls of 2.5 cm in diameter of different colors
	113 and 130 days of age (SI testing)	A sandbox of 15 x 10 x 5 cm (width x length x height)

Table 2. Adult morphometric analyses and locomotor and number of aggressive behaviors during the third stage of the Social Interaction (SI) test in males classified as Aggressive (Ag) or non-Aggressive (nonAg) during the Social Interaction test. Groups included males exposed to the test setup without a decoy opponent (testCON) and control males that remained in their cages (naïve).

	Ag (8)	nonAg (8)	testCON (8)	naïve (8)	P-value
Morphometrics					
Weight (g)	229.38 ± 12.06	217.48 ± 4.22	223.65 ± 13.06	216.75 ± 9.05	0,80
CGV (mm^3)	1741.38 ± 134.56	1822.77 ± 97.63	1887.12 ± 238.08	1942.27 ± 147.60	0.84
Foam prod.	2.38 ± 0.18	3.50 ± 0.50	3.25 ± 0.41	3.13 ± 0.35	0.20
SI test					
Test Durat. (s)*	462.25 ± 60,08	600.00 ± 0.00	600.00 ± 0.00		
Dist. Amb. (m)*	46.49 ± 13,92^{A}	25.66 ± 6.96^{AB}	18.08 ± 6.06^{B}		0.04
Mobile (%)	67.42 ± 4,64	52.95 ± 8.02	63.50 ± 4.27		0.41
Speed (cm/s)	0.10 ± 0,02^{A}	0.03 ± 0.01^{B}	0.04 ± 0,.1^{B}		0.01
Lat. 1st ag (s)*	106.78 ± 40,51				
Lat. 5 ag (s)*	139.69 ± 45,23				
Pecks	11.00 ± 1,81				
Grabs	13.88 ± 4,49				
Mounts	1.50 ± 0,85				
Cloac.Cont.	0.00 ± 0,00				
Chases	4.05 ± 2,25				

Morphometric measurements were performed at 99 days of age. *CGV*: cloacal gland volume was estimated as (4/3 x 3.5414 x a x b2), where a = 0.5 x length, and b =0.5 x width (Marin et al 2004). *Foam prod*.: Foam production. *Test durat*: Social interaction test duration. *Dist. Amb*. : Distance ambulated. *Lat. 1st ag* : Time from the begining of the third stage of the SI test to the first agression towards opponent. *Lat. 5 ag* : Time from the begining of the third stage of the SI test to the completion of 5 aggressions towards opponent. *Pecks*: Number of pecks. Grabs : number of grabs. Mounts : number of mounts. *Cloac. Cont*.: number of cloacal contacts. P-Values estimated with a GLMM that included the categorical groups associated with the SI test (Ag, nonAg, testCON, and naïve) as fixed effect, and the type of environmental condition of rearing (EE and STD) and day of sacrifice as random effects. A normal distribution was used for all variables except distance and speed ambulated where a Gama log distribution was used (marked with *). $^{A-B}$Groups that present differnet letters significantly differ using a LSD post-hoc test. Blank spaces in table indicate measures that given the experimental setup were not taken.