Unexpected results when assessing underlying aggressiveness in Japanese quail using photocastrated stimulus birds

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ABSTRACT Aggressive behaviors can affect both animal welfare and productivity. Because the expression of aggressive behaviors is dependent on the quality of the opponent, they reflect relative rather than absolute levels of underlying aggressiveness. This study was aimed to characterize the aggressive responsiveness of photostimulated (14:10 h light:dark photoperiod) adult Japanese quail when interacting with a photocastrated (6:18 h light:dark photoperiod) counterpart in a novel test environment and to assesses interindividual variations. This was based on the assumption that photocastrated birds will not actively provoke an aggressive confrontation. Birds were reared in male-female pairs. Frequencies of behaviors (i.e., pecks, threats, chases, grabs, mounts) were determined during 10 min social interactions in a novel environment. A first experiment evaluated 78 encounters between a photostimulated male or female with either a photocastrated male or female (photocastration of sexually mature birds started at 11 wk of age). High interindividual variability was observed and in general, highly aggressive birds (performing 20 or more aggressive interactions) received little or no aggression from their test counterpart. However, unexpectedly, we also found that 37% and 32% of photocastrated males and females, respectively, performed aggressions toward their photostimulated counterparts, and initiated the aggressive interactions in a similar proportion than photostimulated males. Aggressive photocastrated males did not perform reproductive-type behaviors (i.e., grabs, mounts). Aggressiveness in the photocastrated birds was attributed to their social experience prior to photocastration. Therefore, a second experiment evaluated 106 encounters between a photostimulated male or female and a naive photocastrated male (photocastration started at 4 wk of age, prior to sexual development). Photocastrated males performed no aggressions toward their photostimulated counterparts. Consistently with previous studies, our findings show that naive photocastrated males can be used as a non-aggressive stimulus during a social interaction aimed to assess expression of aggressiveness in photostimulated birds. However, caution should be taken when applying the photocastration protocol considering that prior fighting and sexual experience or other physiological changes related with maturation can interfere during subsequent aggressive testing.

Key words: Japanese quail, aggressive behavior, female, male, photocastration

INTRODUCTION

For at least the last 80 years, agonistic behavior and social dominance has been an active area of research. Although aggressions are social interactions present in all animals, in the context of farm animals this type of behavior can affect animal welfare as well as performance and productivity. Aggressiveness has been shown to have a genetic/hereditary component as well as being dependent on interactions between social and physical environmental factors, thus should not be considered as an independent consummatory action (Selinger and Bermant, 1967; Kovach, 1974; Edens et al., 1983; Edens, 1987; Muir, 1996; Rodenburg et al., 2004; Buitenhuis et al., 2009). In poultry, aggressiveness is maximum after social disruption and appearance in the environment of unfamiliar conspecifics (Cloutier and Newberry, 2002). Under these conditions, it is associated with establishment of social hierarchy and/or defense of resources (i.e., territorial behavior). For poultry housed in relatively small groups, dominance appears to be established generally through a pecking order triggered by the invasion of the "personal sphere" of the animal (Guhl, 1958; McBride et al., 1969). On the other hand, in large groups aggressions appear to wine down

and a tolerant social dynamic has been proposed to regulate the social interactions (Estevez et al., 1997, 2003). Behaviors (attitudes) perceived as a threat by the aggressor have been proposed as relevant triggers of aggressive interactions in domestic fowl (Rodriguez-Aurrekoetxea and Estevez, 2014). Thus, the expression of an aggressive behavior is clearly a matter of both the birds´ underlying aggressiveness and the behavior of the potential opponent. The display of aggression towards conspecifics involves threats, followed by pecks which are primarily directed at the head, eyes and neck (Kuo, 1960a,b; Sachs, 1966; Edens et al., 1983). However, behaviors that when directed towards females are associated with reproduction (i.e., grabs, mounts and cloacal contacts), have also been proposed as aggressive behaviors when directed towards other males (Selinger and Bermant, 1967; Tsutsui and Ishii, 1981).

The male aggressiveness during social interactions have been extensively studied and is frequently associated with circulating testosterone level or its metabolites (Silver et al., 1979; Ramenofsky, 1984, 1985), the winner/loser effect (Hirschenhauser et al., 2008), as well as the presence (or not) of an audience (Hirschenhauser et al., 2013). For example, Hirschenhauser et al. (2013) reported that the presence of a familiar mixed-sex audience during the encounter modulated both the testosterone response and the long-term success after a fighting experience. Of those factors, circulating testosterone or its metabolites, have received the most attention. Noteworthy, chirurgical or functional castration (i.e., photocastration) in male quail, not only resulted in a significant decrease in circulating testosterone and the consequent reduction in their cloacal gland size and ceasing of foam production (Balthazart et al., 1979; Busso et al., 2010, 2013) but highly relevant to this study, also led to a cessation of aggressive behaviors (Selinger and Bermant, 1967; Schlinger et al., 1987).

In female domestic hens, many factors including sexual maturity, comb size, color attributes, weight, and success in previous encounters have been reported to relate to both aggressiveness and the outcome of a conflict (Schjelderupp-Ebbe, 1935; McBride, 1958; Rushen, 1982; Zayan, 1987; Martin et al., 1997). In quail, female aggressiveness during social interactions has received far less attention. A few studies have evaluated female agonistic behaviors and dominance when confronted with unfamiliar conspecifics (Rutkowska et al., 2011). Moreover, to the best of our knowledge, the effects of castration on the display of aggressive behavior in female quail have not been evaluated during direct physical contact. In this context it is important to recall that quail exposure to short photoperiods can induce inhibition of the hypothalamic-pituitary-gonadal axis, with the consequent lower levels of plasma steroids (i.e., progesterone, testosterone, estradiol) in comparison to the birds reared under long photoperiods, resulting in the absence of egg laying and null receptivity to mating with males (Brain et al., 1988).

Behavioral tests used to assess aggressiveness and/or dominance of birds usually evaluate attacks during one or a series of interaction with an unfamiliar conspecific of similar characteristics or within a social group (Guhl, 1958; McBride, 1958; Ramenofsky, 1984; Martin et al., 1997). Therefore, the outcome of the interaction is the resultant of a combined behavior of a given pair or set of birds, thus evaluating relative rather than absolute levels of aggressiveness (Selinger and Bermant, 1967). Schlinger et al. (1987) proposed that this situation may explain why actual scores of aggressiveness (e.g., number of events/unit time) do not necessarily correlate with dominance order, nor does the outcome of a fight always predict the result of a subsequent group or pair test. Comparisons would be valid only for birds in the same set, thus limiting the physiological or biochemical experiments which could be carried out to ascertain underlying aggressive mechanisms (Schlinger et al., 1987). Serious problems with the use of dominance hierarchies as a method for quantifying an individual's aggressiveness have also been stated (Schlinger and Callard, 1990), such as the influence of previous encounters of subsequent dominance and group composition (Cloutier et al., 1995; Martin et al., 1997; Hirschenhauser et al., 2013). Ideally, in a behavioral test to assess aggressiveness, the stimulus bird should display a fixed and known level of aggressiveness. Herein, we assumed that castrated birds will not behave aggressively and therefore will not actively initiate an aggressive confrontation. Based on this assumption, we implement the use of photocastrated (6:18 h light:dark cycle) birds as neutral stimulus to assess the underlying level of aggressiveness of a photostimulated counterpart (16: 8 h light:dark cycle), thus allowing the assessment of the test animal's impulse to attack and/or to defend a resource such as its familiar social (male/female) partner.

This study aimed to characterize the aggressive responsiveness of photostimulated quail when directly interacting with a photocastrated counterpart in a novel test environment and to assess interindividual variations in underlying aggressiveness among photostimulated quail during these interactions. We also evaluated whether photostimulated birds respond differentially towards a photocastrated male or a female. Due to the observation in our first experiment of unexpectedly high and intermediate aggressive performances in more than 30% of both photocastrated males and females, a second experiment was performed using stimulus birds that were photocastrated at a younger age to minimize prior aggressive and/or sexual behavior experience, or other physiological changes induced during maturation in long days that would be non-reversible with photocastration.

MATERIAL AND METHODS

The study was performed with Japanese quail (*Coturnix coturnix japonica*) that are considered an
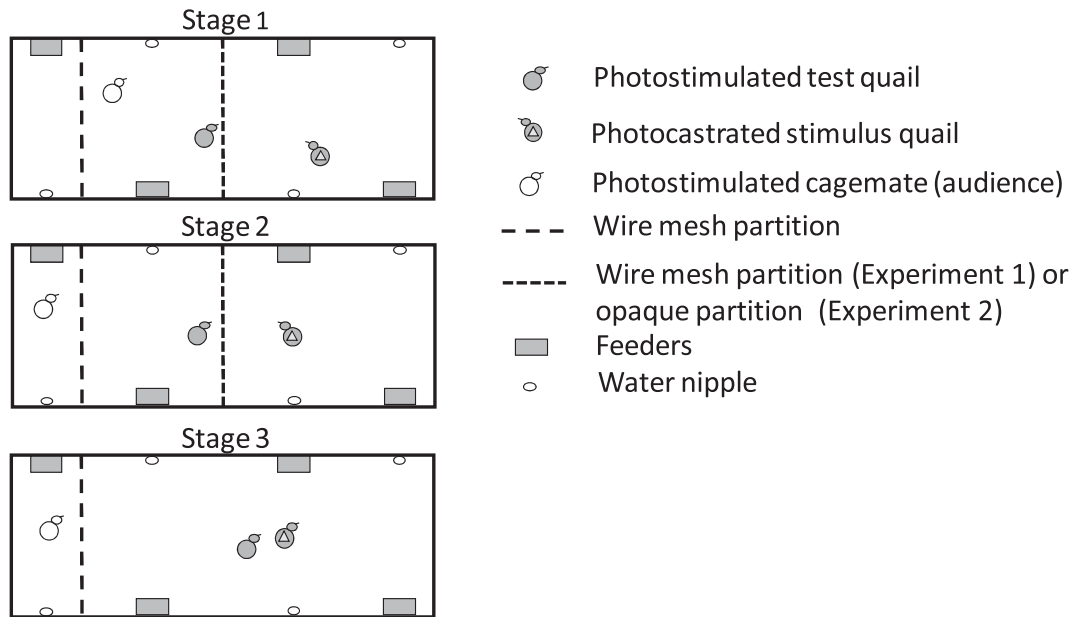
Figure 1. Representative diagram of the experimental setup performed during Experiment 1 and 2 social interactions.

excellent laboratory model for the extrapolation of data to other poultry species with higher commercial relevance because of its high physiological similarity (Jones, 1996). This species has also been widely used for studies covering neuroendocrine control of the reproductive system and social behaviors (Balthazart et al., 2003; Ball and Balthazart, 2010). The animals were bred according to standard laboratory protocols (Shanaway, 1994; Kembro et al., 2012) and according to general rules of care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals, 2011). Mixed-sex Japanese quail hatchlings were randomly housed in groups of 30 to 40 in white wooden boxes measuring $90 \times 80 \times 60$ cm (length × width × height) 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 and a lid prevented the birds from escaping. Brooding temperature was $37.5°C$ 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.

At 28 days of age, animals were sexed by plumage coloration, marked with a wing band, and housed in pairs of 1 male and 1 female in cages of 20 cm × 40 cm × 20 cm (width × length × height respectively). Quail were subjected to a daily cycle of 14 h light (300 to 320 lx): 10 h dark (long photoperiod) during the study with the exception of photocastrated birds that were submitted to a short photoperiod light cycle (see below). Food and water continued ad libitum. Females kept on long photoperiod cycle peaked egg production at 52 days of age and were laying eggs normally throughout the study.

Experiment 1

One hundred and sixty quail were reared under the described long photoperiod conditions until 11 wk of the age. At this time, half of the birds remained on long photoperiod and the other halve were shifted to a short photoperiod (6:18 h light:dark cycle) until the end of the study (16 wk of age). Animals raised under the long photoperiod were considered photostimulated (**PS** birds) showing normal reproductive and behavioral parameters (i.e., males had cloacal glands volumes larger than 1,500 mm³, maximum foam production, exhibited reproductive and also aggressive behaviors towards their cagemate). Taking into consideration that some quail can be non-responsive to photocastration (Dominchin et al., 2017), in this study we only used photocastrated males that after 5 wk of photoinhibition showed a full cloacal gland involution (volume lower than 1,000 mm³ and no foam production (Marin and Satterlee, 2004)) and females that completely stopped egg production. Thus, after 5 wk of photoinhibition all birds used as stimulus were considered fully photocastrated (**PC** birds).

Social Interaction Test. At 16 wks of age, a total of 78 encounters between an unfamiliar PS male or female and a PC male or PC female were performed in the presence (audience) of the PS cagemate. Thus, we tested 4 types of social interactions (PS Male—PC Male; PS Male -PC Female; PS Female—PC Male; PS Female—PC Female). Figure 1 shows a schematic representation of the test apparatus that consisted of a rectangular white wooden box measuring 40 cm × 90 cm × 40 cm (width × length × height respectively), divided into 3 compartments. The social interaction test was

divided into 3 stages: In Stage 1, both PS birds from the same cage (male and female) were placed in a central compartment separated by a wire mesh partition from one PC stimulus male or female. Birds were allowed an acclimation setup of 2 min. In Stage 2, either the PS male or the female and the PC stimulus bird remained in the same compartment, while the other PS cagemate was placed in a nearby compartment at one side of the apparatus and used as a social audience (a wire mesh partition allowed visual contact between birds while preventing the audience bird from getting physically involved during test). Immediately after, the Stage 3 began with the removal of the central wire partition and the PS and PC birds were allowed to interact. Direct interaction lasted a maximum of 10 min. Following the Dominchin et al. (2017) procedure, 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. The testing of each pair combination was evenly distributed throughout 4 testing days. 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.

During interactions, behaviors were recorded using ANY-maze behavioral tracking software (Stoelting Co., 620 Wheat Lane, Wood Dale, IL 60191, USA). We used a program's feature that allows a manual registration of behaviors by using the computer keyboard while video recording runs. The following behaviors were recorded for both birds:

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 (adapted from McGary et al. (2003)).

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 (adapted from McGary et al. (2003)).

Threats: one bird stands with its neck and head raised in front of the other bird that usually has its head at a lower level than the first (adapted from McGary et al. (2003)).

Chase: a bird runs after another that is escaping (adapted from Ramenofsky (1984)).

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

Attack initiation: during testing, the subject that first initiated an aggressive interaction (i.e., peck, threat, chase) towards the other animal was recorded as initiating attack (adapted from Ramenofsky (1984)).

Herein, when grabs, mounts, or cloacal contacts were performed by one male to another, they were considered as aggressive behaviors.

During Stage 1 of the test (i.e., when a wire mesh wall separated PS from PC birds (Figure 1)), pecks and threats towards their opponents through the wire mesh wall were also registered.

Experiment 2

A similar experimental setup was used during this experiment, where 53 PS-Males and 53 PS-Females were evaluated in the presence (audience) of the PS cagemate. Because in Experiment 1 (see Result section below) some attempts of aggression were also displayed by birds during the habituation period (Stage 1), in this experiment an opaque wooden partition was used to replace the wire mesh partition (Figure 1). To assess the expression of aggressive behaviors in PS birds (either male or female) we only focused on the use of PC-Males as stimulus birds. PC-birds were housed under same conditions as in Experiment 1 but were submitted to short photoperiod at 4 wk of age (at least 2 wk prior to puberty) instead of 11 wk of age to minimize aggressive and/or sexual behavior experience during and/or after sexual maturation.

Statistical analysis

Two-way ANOVAs (Di Rienzo et al., 2016) were used to determine differences between PS birds (female and male) and PC birds (male and female) and their potential interaction on aggressive behaviors. Data from chases, pecks, and threats and total interactions were transformed to ranks to better fit ANOVA assumptions (Shirley, 1987). Fisher's LSD tests were used for post hoc between-group comparisons. A proportion test (Statistix, 2008) was performed to assess differences between groups in the number of birds that were highly aggressive or that initiated aggressive interactions. In all cases, a P-value of ≤ 0.05 was considered to represent a significant difference.

RESULTS

Experiment 1

Behaviors performed by PS and PC birds during social interactions are shown in Table 1 and 2, respectively. Reproductive behaviors that can also be associated with aggressions (grabs, mounts, and cloacal contacts) were only performed by PS-Males. On the other hand, exclusively aggressive behaviors (i.e., pecks, threats, and chases) were performed by birds in all groups (Table 1 and 2). In particular, PS-Males showed in comparison to PS-Females more chases and more total social interactions independently of whether they were interacting with a PC-Male or a PC-Female ($P < 0.05$, in both cases). Unexpectedly, PC-Males and also PC-Females initiated and performed aggressions

Table 1. Behaviors performed by the photostimulated (PS) male or female quail towards their photocastrated (PC) male or female counterparts during the Social Interaction test.

Counterpart Variable	PS-Male		PS-Female	
	PC-Male (17)	PC-Female (23)	PC-Male (21)	PC-Female (17)
Pecks	2.4 ± 0.7	1.8 ± 0.7	2.4 ± 1.1	1.2 ± 0.8
Grabs	4.6 ± 1.4	4.8 ± 1.5	0 ± 0	0 ± 0
Mounts	1.1 ± 0.5	0.8 ± 0.4	0 ± 0	0 ± 0
Cloacal Contacts	0.2 ± 0.1	0.3 ± 0.1	0 ± 0	0 ± 0
Threat	0.2 ± 0.2	0.2 ± 0.1	0.7 ± 0.4	0.5 ± 0.5
Chases*	3.6 ± 1.3	2.3 ± 0.8	0.5 ± 0.3	0.1 ± 0.1
Claws Attacks	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.4
Pecks + Threats	2.7 ± 0.8	2.0 ± 0.7	3.1 ± 1.4	1.7 ± 1.2
Total Social Interactions*	12.2 ± 3.2	10.2 ± 2.8	3.8 ± 1.8	2.2 ± 1.6

Mean ± SEM.

Total Social Interactions: sum of all registered behaviors. Number of tested encounters are shown in parenthesis.

*PS-Males differed from PS-Females at $P < 0.05$.

Table 2. Behaviors performed by photocastrated (PC) male or female quail towards their photostimulated (PS) male or female counterparts during the Social Interaction test.

Counterpart Variable	PC-Male		PC-Female	
	PS-Male (17)	PS-Female (21)	PS-Male (23)	PS-Female (17)
Pecks	5.2 ± 2	4.1 ± 1.2	3.8 ± 1.7	5.2 ± 2.0
Grabs	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Mounts	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Cloacal Contacts	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Threat	1.4 ± 0.8	1.3 ± 0.5	1.1 ± 0.4	3.0 ± 1.3
Chases	1.2 ± 1.0	0.4 ± 0.3	0.6 ± 0.3	0.2 ± 0.2
Claws Attacks	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Pecks + Threats	6.5 ± 2.4	5.4 ± 1.6	4.9 ± 1.9	8.2 ± 2.8
Total Interactions	7.9 ± 3.0	5.9 ± 1.9	5.8 ± 2.3	8.6 ± 3.0

Mean ± SEM.

Total Interactions: sum of all registered behaviors. Number of tested encounters are shown in parenthesis.

(predominately pecks and threats) towards PS birds (Table 2).

While the group of PS-Males showed the highest proportion value of aggressive birds when interacting with PC-Males, the group of PS-Females showed the lowest proportion value of aggressive birds when interacting with a PC-Female counterpart (Figure 2a). Approximately 37% (14 out of 38) of the PC-Males and 32% (13 out of 40) of the PC-Females performed more than 5 aggressive behaviors towards their male and female PS-counterparts.

Figure 2b shows the proportion of individuals from each group that initiated an aggressive interaction. During PS-Male and PC-Male interactions, no differences ($P = 0.14$) were observed between groups in the number of birds that initiated aggressive interactions. During PS-Female and PC-Female interactions, PC-Females tended ($P = 0.09$) to initiate the attacks more frequently than PS-Females. During mixed-sex interactions (PS-Males and PC-Females, or PS-Females and PC-Males) males initiated the attacks more frequently ($P = 0.001$) than their respective female test counterpart.

In Figures 3a,b and 4a,b the total interactions performed by each animal are shown in sequential descendent order from the most aggressive to the least aggressive birds. Figures 3c,d and 4c,d show for the same animals the number of interactions received from their test counterpart. It is observed that males that performed 20 or more aggressive interactions (highlighted with gray area in Figure 3) received only a very low number of aggressions (if any) from their counterparts. However, for males that performed between 5 and 19 total interactions a higher diversity in opponents reactivity was observed. These PS-Males received a varied number of aggressions from both PC-Males and PC-Females (Figure 3c), while PC-Males only received a very low number of aggressions (Figure 3d).

Figures 3c and 3d also shows that PC-Males only received high levels of aggressions (>20 aggressions) when they performed less than 5 aggressive interactions. On the other hand, Figure 4 shows that females received aggressive and reproductive interactions from their test counterpart fairly independently of their own level of aggressiveness, this phenomenon appears most noticeable when the PC-Females were interacting with the PS-males.

Noteworthy, 50% (6 of the 12) of the highly aggressive PS-Males (birds that performed ≥20 aggressive interactions) were already directing pecks or threats towards
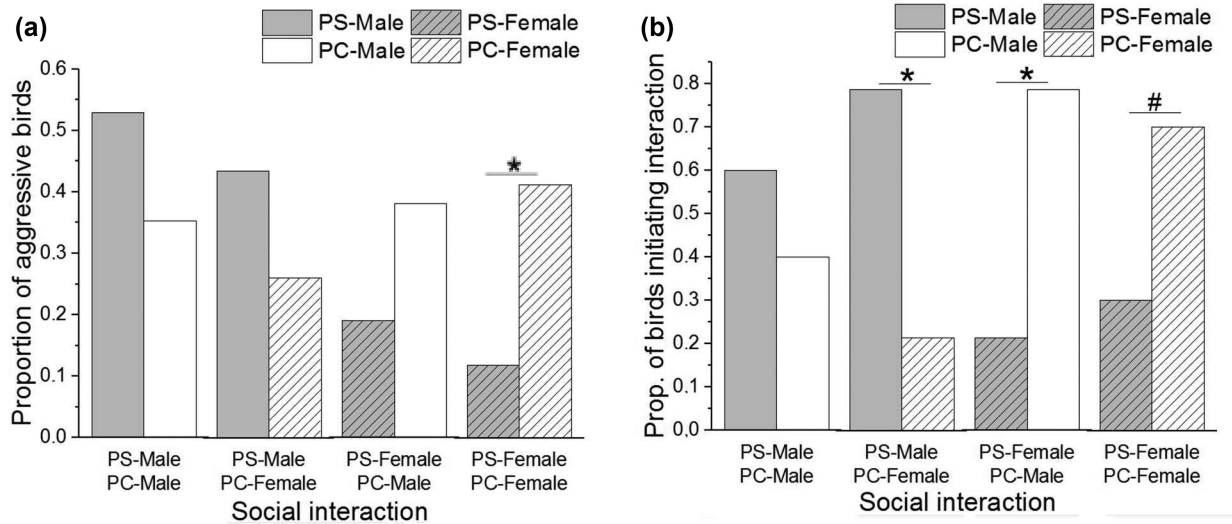
Figure 2. a) Proportion of birds performing 5 or more aggressions, and b) Proportion of birds that initiated an aggressive interaction. PS = Photostimulated. PC = Photocastrated. *Groups differ at $P < 0.05$. #Groups tend to differ at $P \leq 0.09$.
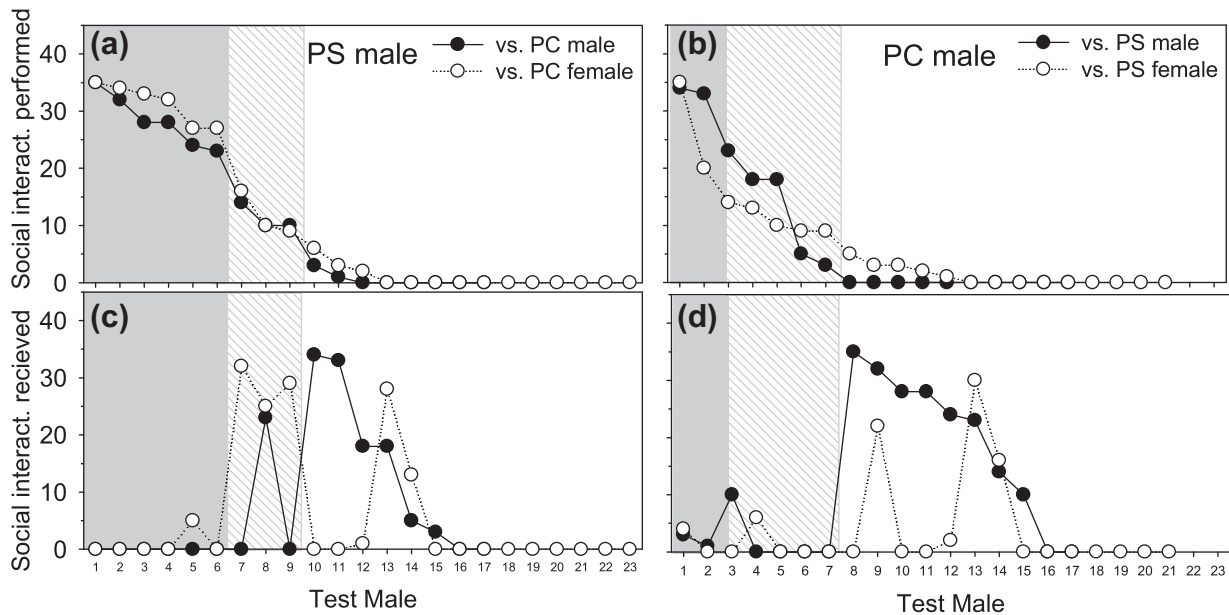

Figure 3. Male aggressive behaviors performed (a,b) and received (c,d). Photostimulated (PS) and Photocastrated (PC) males are ranked in the figure according to a descending order of aggressiveness during the Social Interaction test (i.e., the most aggressive male was assigned the test number 1 and the least aggressive bird the highest test number). The graph section highlighted in gray includes all PS test birds that performed 20 or more total interactions; note that these birds received few if any aggressions from their test counterpart. The region shaded with lines includes all PS birds that performed between 5 and 19 total aggressions.

their PC counterpart before the wire mesh partition was open (during Stage 1 of the test). That phenomenon was found in only 14% (3 of the 22) PS-Males with intermediate or no aggressiveness (≤ 20 aggressive interactions performed) ($P = 0.02$).

Experiment 2

Aiming to minimize the expression of aggressive behaviors by PC birds, the test was performed with naive PC-Males (photocastrated at 4 weeks of age, prior to sexual development, instead of at 11 weeks of age as in Experiment 1). Under this test situation,

none of the PC-Males performed aggressive behaviors toward their PS test counterpart (Figure 5). Furthermore, out of the 53 PS-Females tested, only 3 of them (5.7%) performed aggressions toward their PC-Male counterpart and showed an intermediate level of aggressiveness (between 5 and 20 total aggressive behaviors performed, Figure 5).

In this opportunity, 11.3% of the PS-Males performed 20 or more aggressions, 26.4% of the males performed between 5 and 19 aggressions, and a 62.3% performed no aggressions. Approximately 60% of the behaviors recorded by males were grabs, only <7% were mounts, and no cloacal contacts were registered (Table 3).

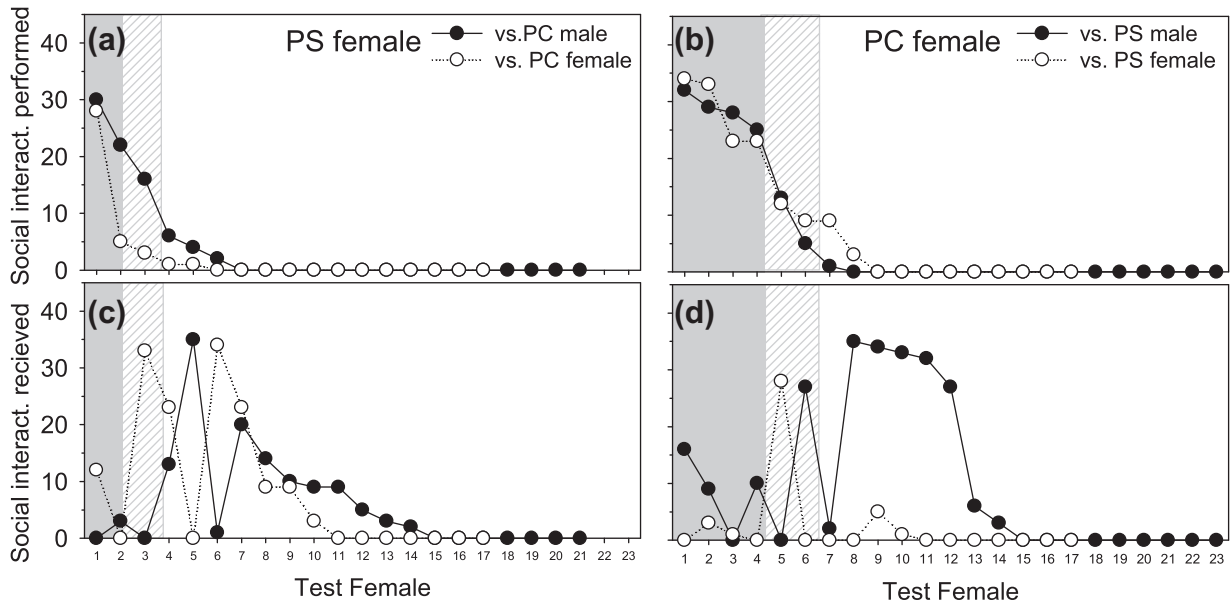**Figure 4.** Female social interactions performed (a,b) and received (c,d). Photostimulated (PS) and photocastrated (PC) females are ranked in the figure according to a descending order of aggressiveness during Social Interaction test (i.e., the most aggressive female was assigned the test number 1 and the least aggressive female the highest test number). The graph section highlighted in gray includes all PS test birds that performed 20 or more total interactions. The region shaded with lines includes all PS birds that performed between 5 and 19 total aggressions.
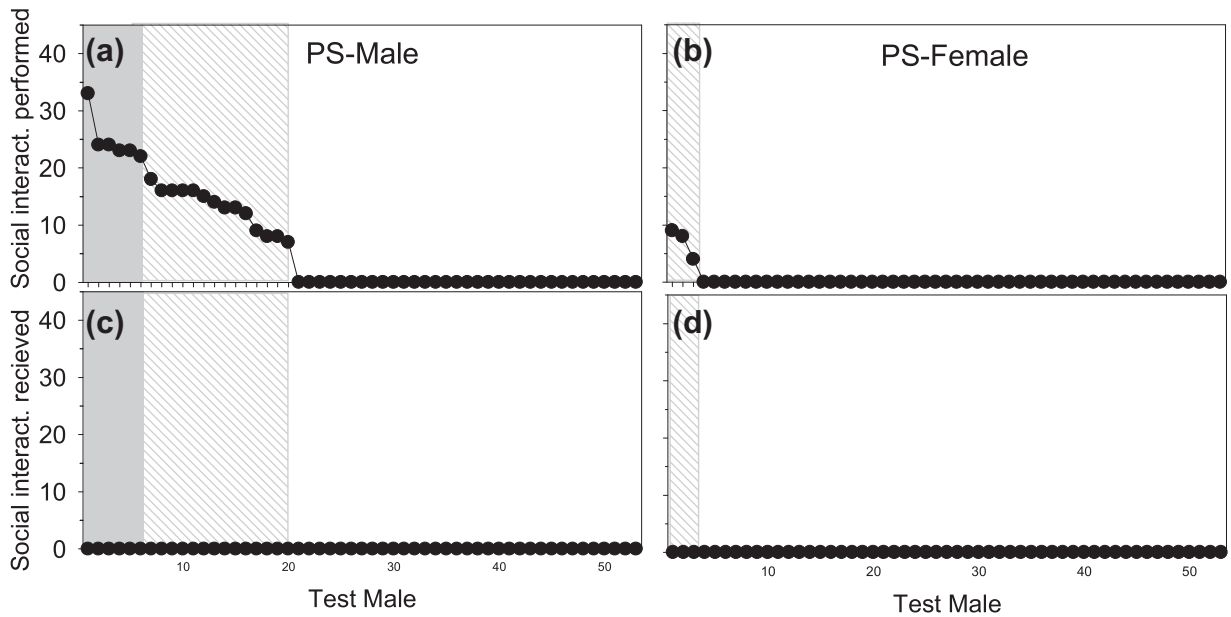

Figure 5. Aggressive behaviors performed (a,b) and received (c,d) by photostimulated test birds vs. photocastrated males. Photostimulated (PS) test males and females are ranked in the figure according to a descending order of aggressiveness during the Social Interaction test (i.e., the most aggressive male was assigned the test number 1 and the least aggressive bird the highest test number). The graph section highlighted in gray includes all PS test birds that performed 20 or more total interactions. The region shaded with lines includes all PS birds that performed between 5 and 19 total aggressions.

DISCUSSION

The main difficulty in the assessment of underlying aggressiveness of an animal lays in discriminating between the impulse to attack and a defensive reaction to attacks from a conspecific. Herein we used a Social Interaction test where the opponent's own potential aggressiveness is minimized through photocastration, therefore allowing assessment of the test animal's impulse to attack and/or to defend a resource such as its social (male/female) partner while it is not being actually threatened. However, during testing there were interesting but unexpected (at least from a frequency point of view) findings. In this regard, more than 30% of both PC-Males and PC-Females presented high or intermediate aggressiveness (performed ≥5 aggressive interactions) toward the experimental PS counterpart. PC-Males even initiated the aggressive

Table 3. Classification of photostimulated (PS) males in Social Interaction test according to high ($\geq$20), Intermediate (between 5 and 19) or No aggressiveness shown towards a photocastrated male.

	PS-Males		
	High aggressiveness (6)	Intermediate aggressiveness (14)	No aggressiveness (33)
Pecks	5.5 $\pm$ 1.4	1.9 $\pm$ 0.4	0 $\pm$ 0
Grabs	14.0 $\pm$ 1.9	8.0 $\pm$ 0.8	0 $\pm$ 0
Mounts	2.5 $\pm$ 1.1	0.5 $\pm$ 0.3	0 $\pm$ 0
Cloacal Contacts	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Threats	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Chases	2.8 $\pm$ 0.6	2.5 $\pm$ 0.5	0 $\pm$ 0
Attacks with claws	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Total Aggressions	24.8 $\pm$ 1.7	12.9 $\pm$ 1.0	0 $\pm$ 0

Mean $\pm$ SEM.
The number of birds within each category is mentioned in parenthesis.

interactions against their PS-counterparts in about half of the encounters (Experiment 1; Figure 2b). Moreover, in most of the cases, PS-Males exhibited a high level of aggressiveness during interactions only when PC-Males performed few or no attacks towards them (Figure 3d) which suggests an inhibition of the expression of PS aggressive behaviors by their aggressive PC counterparts. These results highlight the relevance of using a non-aggressive test counterpart to avoid potential interference of the expression of underlying aggressiveness in the experimental birds.

The aggressive behaviors (pecks, chases, and threats) performed by PC-Males in the first experiment were no longer observed in the PC-Males evaluated during the second experiment. From our point of view, the main difference between PC-Males in Experiments 1 and 2 lies in the age at castration (11 and 4 wk of age, respectively). The observed aggressive performance by PC-Males in the first experiment was certainly not expected, at least not in the observed frequency, if we consider that i) a chirurgical castration at 70 days of age has previously been shown to lead to inhibition of aggressiveness (Tsutsui and Ishii, 1981); ii) the exposure to short days during 5 wk also showed a significant reduction in the number of aggressions during male-male encounters (resident vs. intruder test) (Dominchin et al., 2017), and iii) the study by Schlinger et al. (1987) using immature PC-Males (5 to 6 weeks old) as stimulus birds also showed a low locomotor activity and a lower number of pecks directed towards a glass partition that separate them from an older PS-Male. However, it should also be considered that in all three of those previous studies, males (PS and PC) were housed in isolation from other birds and may have only experienced a few short social interaction situations as adults. That social environmental situation is in stark contrast with our Experiment 1, where birds remained in pairs of 1 male and 1 female since 4 weeks of age until full adulthood. Furthermore, it is known that PS-Males that have been housed in isolation behave more aggressively than birds that have been reared socially (Middlebush et al., 1968). Thus, in the previous studies, it is possible that the aggressive expressions of those PS-Males were also exacerbated and therefore inhibit-

ing or reducing potential expression of aggressive behaviors in their castrated counterparts. Kovach (1974) proposed that once aggressive attacks are conditioned, they take precedence over other strongly motivated behavior patterns such as feeding or sexual behavior. Thus social interactions between cagemates as mature adults (prior to photocastration) in our first experiment could help explain a priori highly unexpected aggressiveness of PC-Males. However, we cannot rule out that other non-reversible physiological changes related with maturation could also interfere during subsequent aggressive testing.

Although aggressive behaviors were observed in PC-Males during Experiment 1, no behaviors associated with reproduction (grabs, mounts, or cloacal contacts) were performed by those males (Table 2), which is consistent with previous studies showing that copulatory behaviors disappear within a few days after exposure to a short photoperiod or after chirurgical castration (Beach and Inman, 1965; Sachs, 1969; Ball and Balthazart, 2010). Since in our study all PC-Males showed a full cloacal gland regression (volume <1,000 mm^3), testosterone was expected to be minimal (Sachs, 1967; Ottinger and Brinkley, 1978; Delville et al., 1984) as well as the associated reproductive behaviors. In contrast, as expected, PS-Male performed reproductive related behaviors towards both PC-Male and PC-Female counterparts. When those behaviors are performed by PS-Males towards other males, they are usually considered as aggressive behaviors aimed to demonstrate dominance (Adkins and Adler, 1972; Tsutsui and Ishii, 1981; Schlinger and Callard, 1990). This assumption is based on observations that when several males are kept together in one cage, dominant individuals display grabs, mounts, and/or cloacal contacts more frequently than their subordinates (Tsutsui and Ishii, 1981). However, more recently, Adkins-Regan (2015) proposed that male-male sexual behavior is an incidental by-product of strong mating motivation, that is, males may have been selected to mate rapidly, vigorously, and fairly indiscriminately, which although costly if the other bird happens to be another male, would be sufficiently beneficial whenever the other bird is a female to have a net adaptive advantage. Our results showed that about

half of the interactions performed by intermediate to highly aggressive PS-Male towards their PC counterpart are behaviors associated with reproduction (Tables 1 and 3), while PC-Males although highly aggressive did not perform reproductive related behaviors, which is in line with Adkins-Regan proposal. Seemingly, behaviors associated with reproduction are highly based on hormone levels, while aggressive behaviors (pecks, chase, and threats) appear also highly related to prior social experience (Kovach, 1974; Kovach and Wilson, 1975). Although further experiments aimed to address specifically these point are still needed, it is well known that the reproductive sequence (grabs, mounts, cloacal contacts) with a female depends mostly on the transformation in the brain of androgens into estrogens (e.g., 17β-estradiol), a process known as aromatization (Adkins. et al., 1980; Schumacher and Balthazart, 1983; Balthazart et al., 2004). There is, however, a synergistic action of androgens and estrogens on the copulatory sequence: low doses of 17β-estradiol or of 5α-dihydrotestosterone that are almost behaviorally inactive by themselves induce a substantial level of behavioral activity if given concomitantly to castrated males (Ball and Balthazart, 2010). Aromatase activity in the hypothalamus/preoptic area is positively correlated with behavioral intensity (locomotor and pecking activity) towards a female separated by a glass partition in PS males (Schlinger and Callard, 1989). Photocastrated males that display little or no aggressive behaviors have been shown to have not only low testosterone levels but also lower levels of brain aromatase as compared to PS males (Schlinger et al., 1987). This hormone control of this reproductive sequence during male-male interactions and the effect of prior experience upon PC-Males to our knowledge have not been explored, and could be useful to shine light on the role of reproductive-like behaviors between males.

In the first experiment PC-Females showed intermediate to high levels of aggressiveness. Furthermore, PC-Females showed almost 5 times more pecks and chases combined toward PS- Females, than from PS-Females toward PC-Females. Moreover, PC-Females initiated the majority of the first interaction towards PS-Females (Figure 2b). To the best of our knowledge, heightened aggressiveness in PC-Females has not been previously reported in poultry. As stated previously, short photoperiods have been shown to induce inhibition of the hypothalamic-pituitary-gonadal axis, with consequent declines in steroid hormones related to the regulation of ovulation, oviposition (Brain et al., 1988), and sexual behaviors. Thus, the heightened aggressiveness in PC-Females apparently should not a priori be attributed to testosterone levels.

In males, independently of photostimulus during rearing, in the first part of our study we show that in general high levels of aggressiveness in one male (≥ 20 total social interactions performed, gray area in Figure 2) consistently coincides with a low or no aggressive performance in their bird counterpart. Obviously,

this could be due to two motives, the first being that an animal with a high underlying aggressiveness is prone to attack a non-aggressive animal, and second the high level of aggressiveness shown inhibits the opponent's response, favoring an escape rather than confrontation. In this regard, it should be noted that 17% of the interactions evaluated in Experiment 1 were ended prematurely by the experimenter when escape behavior was observed, or in anticipation of behaviors that could potentially result in wounds. In contrast, females received social interactions (both aggressive and reproductive) from counterpart fairly independently of their level of aggressiveness, this is most notable for PC-Females interacting with PS-Males (Figure 3), which is probably due to a male sexual motivation during the social interaction. Given that this sexual motivation per se (and not exclusively aggressiveness) could drive interactions of PS-Males towards PC-Females, in Experiment 2 only PC-Males were used as stimuli to assess aggressiveness. Moreover, the heightened aggressiveness of PC-Females especially towards PS-Females (Table 2 and Figure 4A) further supports the use of PC-Males (and not PC-Females) as test stimuli to assess the expression of underlying aggressiveness.

In Experiment 1, during PS-Male and PC-Male interactions, no differences were observed between groups in the initiation of the aggressive interactions (Figure 2b). However, during female interactions, PC-Females tended to initiate the interaction more frequently than PS-Females, while during mixed-sex interactions, males initiated significantly more attacks than their respective female test counterpart. In this regard, Hirschenhauser et al. (2013) showed that fight initiation was a good predictor of winning an encounter between PS-Males in the presence of a mixed-sex audience. As expected, PS-Male group was the most aggressive with a 35% of males classified as high aggressive (≥ 20 interactions) and 18% classified as intermediate aggressive (between 5 and 19 interactions) independently of the opponent's sex (Figure 2a). Although most classification methods for determining aggressiveness are based on one or a series of confrontations, Schlinger et al. (1987) proposed a method based on locomotor and pecking activity directed towards an immature PC conspecific separated by a glass partition. In Experiment 1, during Stage 1 of the test (i.e., when a wire mesh wall separated PS from PC birds), about half of PS-Males classified as highly aggressive also directed pecks or threats towards their opponents through the wire mesh wall regardless of their sex. These results are in line with Schlinger et al. (1987) showing that the expression of PS-Male behavior was not dependent on the sex of the PC counterpart. However, because only half of the aggressive birds directed aggressions through the wire partition, it appears that keeping the birds separated can also lead to underestimation of the number of aggressive birds.

The few aggressive PS-Females observed in both experiments ($\sim$6%) is consistent with Rutkowska et al.

(2011) reporting that behaviors between two sexually mature females during 5 min interactions were either avoidant or neutral. These results could also be due to the short duration of the test, and in the future similarly to other studies in hens (Cloutier et al., 1995; Agnvall et al., 2014) longer test durations should be considered for female quail aggressiveness testing.

The main finding of Experiment 2 was that PC-Males were no longer aggressive toward PS-Males. Because Experiments 1 and 2 were performed independently, it still remains inconclusive whether the presence of a non-aggressive PC-Male actually reduces the overall expression of PS-Male aggressions. Nevertheless, comparing the total number of aggressive interactions shown by PS-Males when interacting with PC-Males between Experiments 1 and 2 (12.2 and 6.21, respectively), it seems that the presence of a non-aggressive PC counterpart helps to reduce aggressive behaviors, most likely due to the lack of need to respond to attacks. Experiment 2 was also part of a larger project assessing whether there are differences between PS-Males with high, intermediate and no aggressiveness in BW and cloacal gland volume. No differences between groups were found in those variables ($P > 0.24$, in all cases; Caliva, Kembro, Guzman, and Marin, unpublished data). Thus, findings are consistent with previous studies showing that aggressive behaviors are not correlated with BW, cloacal gland (Ball and Balthazart, 2008), or with plasma testosterone level (Tsutsui and Ishii, 1981).

In all, our findings suggest that naive photocastrated males can be used as a non-aggressive stimuli during a social interaction aimed to assess expression of aggressiveness in photostimulated birds. However, caution should be taken when applying the photocastration protocol considering that prior fighting and/or sexual experience, or other non-reversible physiological changes related with maturation could interfere during subsequent aggressive testing.

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