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Lucas Court, Laura Talbottier, Julie Lemarchand, Fabien Cornilleau, Emmanuel Pecnard, et al.. Exploring neuronal markers and early social environment influence in divergent quail lines selected for social motivation. *Scientific Reports*, 2024, 14, pp.23554. 10.1038/s41598-024-74906-3. hal-04735026

HAL Id: hal-04735026

<https://hal.science/hal-04735026v1>

Submitted on 14 Oct 2024

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OPEN Exploring neuronal markers and early social environment influence in divergent quail lines selected for social motivation

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Many species, including humans exhibit a wide range of social behaviors that are crucial for the adaptation and survival of most species. Brain organization and function are shaped by genetic and environmental factors, although their precise contributions have been relatively understudied in the context of artificial selection. We used divergent lines of quail selected on their high versus low level of motivation to approach a group of conspecifics (S + and S-, respectively) to investigate the influence of genetic selection and early social environment on sociability. We observed distinct sex- and brain-region-specific expression patterns of three neuronal markers: mesotocin, and vasotocin, the avian homologues of mammalian oxytocin and vasopressin, as well as aromatase, the enzyme that converts androgens into estrogens. These markers displayed pronounced and neuroanatomically specific differences between S + and S- quail. Additionally, in a second experiment, we assessed the influence of early social environment on social skills in juvenile birds. Mixing S + and S- resulted in more S- males approaching the group without affecting the sociability of S + or other behaviors, suggesting that the early social environment may influence the results of genetic selection. In conclusion, the divergent quail lines offer a valuable model for unraveling the neuronal and behavioral mechanisms underlying social behaviors.

Keywords Mesotocin, Vasotocin, Aromatase, Social behavior, Social environment, Japanese quail

In vertebrates, social behaviors – the interactions between at least two conspecifics – encompass multiple forms of behavior, including sexual, aggressive and parental behaviors. They play a crucial role for the adaptation and survival of most social species, including humans^{1–4}. Conversely, some neurological disorders including autism spectrum disorder (ASD) are characterized by social impairments^{5,6}, leading to difficulties in adapting to the social environment. Social experience shapes neural circuits during development and influences social abilities in adults^{7,8}. For example, social deprivation alters sociability while enriched social environment improves social skills in models of ASD^{9–13}. However, few studies have examined the influence of early social environment and genetic factors and their interplay on sociability in the context of artificial selection.

In vertebrates, social behaviors are regulated by an evolutionary conserved set of interconnected brain nuclei forming the social behavior network^{14–18}. Oxytocin and vasopressin neuropeptides, as well as mesotocin (MT) and vasotocin (VT) homologs in birds, are primarily synthesized in neurons located in the paraventricular (PVN) and the supraoptic nuclei of the hypothalamus^{19–21}. These neurons project to key brain structures within this network, including medial preoptic nucleus (MPN), bed nucleus of the stria terminalis (BNST), lateral septum (LS), ventromedial nucleus of the hypothalamus (VMN), as well as the pituitary^{22–25}. Moreover, receptors for these neuropeptides, as well as aromatase (ARO) that converts androgens into estrogens, and estrogen receptors are highly conserved and expressed in most brain nuclei within this network, thereby modulating social and socio-sexual behaviors^{7,26–37}. Thus, MT, VT and ARO are valuable markers to study sociability.

Birds exhibit a wide range of social behaviors that can be modulated through *in ovo* manipulations^{38,39} or genetic selection⁴⁰. They are useful models to study brain and neural mechanisms, as well as environmental and

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genetic factors affecting social behaviors. Divergent lines of Japanese quail (*Coturnix japonica*) have been selected over 45 generations for either low or high motivation to approach a group of conspecifics and designated as S- and S+, respectively⁴⁰. Subsequent studies have confirmed the social divergence in their approach towards the group while mixed effects were also observed in their expression of sexual behavior, aggressiveness and reaction to humans^{41–47}. Notably, S+ quail exhibit increased locomotor activity in response to social isolation^{45,48,49}. These lines serve as valuable genetic models to investigate sociability and the influence of distinct social environments. However, the identity of the neuronal markers implicated in the sociability of Japanese quail, particularly in these divergent lines, remains to be determined.

In this study, we explore the effects of quail genetic selection on three neurochemical markers that have been associated with sociality: MT, VT and ARO. Results reveal a differential expression of these markers between S+ and S- quail lines, particularly in the PVN and MPN. In addition, in a second experiment, we tested the influence of early social environment on social abilities by mixing S+ and S- quail lines. S- male quail exposed to S+ males showed a partial improvement in social motivation when the percentage of birds approaching a social group was examined. These results suggest that divergent quail lines selected for sociability offer a valuable animal model for investigating the interplay between social environment and decades of genetic selection.

Results

Morphological differences between S+ and S- quail

From hatching to adulthood, S+ quail exhibited significantly smaller body mass compared to both their S- counterparts and the control (CTL) group from the non-selected line in 7-month-old quail. As expected, female quail were significantly heavier than males^{50,51} (Fig. 1A and Supplementary Fig. S1A–B. Raw data, statistical tests, and p-values are presented in Supplementary Table S1). Similarly, S+ brain mass was significantly decreased compared to S- adult quail (Fig. 1B). In parallel, all brain measurements collected in the PVN, MPN, BNST, and LS were significantly smaller in S+ than in S- brains (Fig. 1C). Therefore, subsequent quantifications were normalized based on body mass or brain measurements.

In 7-month-old quail, S+ males exhibited significantly smaller testes than S- but no line-related change was found in the cloacal gland area of males or of ovarian follicle mass and cloacal opening of female quail after correction for body mass (Fig. 1D–E; no difference was similarly present without correction, see Supplementary Table S1). At the age of 3 weeks, the cloacal gland area or opening was larger in S- birds but these differences disappeared after correction for body mass (Supplementary Fig. S1C–D and Supplementary Table S1).

Brain region- and sex- specific expression of MT, VT and ARO markers in socially divergent quail lines

S+ and S- quail lines were compared to determine whether MT, VT, and ARO expression patterns had been impacted by the genetic selection.

MT-immunoreactivity (-ir) somas were mainly located in the PVN while the highest density of MT-ir projecting fibers was observed in the MPN, BNST and LS (Fig. 2, Supplementary Fig. S2 and S3). Following the neuroanatomical nomenclature provided by Puelles and colleagues⁵², MT-ir somas and fibers were also identified in regions corresponding to the prethalamus (plates 14–15 of his atlas), the intermediate nucleus of the supraoptic decussation (plate 22), the tectothalamic tract (plate 22), the pregeniculate nucleus (plate 15), the arcuate hypothalamic nucleus (plate 27), the periventricular stratum (plate 27), the intercollicular area (plate 27), the periaqueductal gray (plate 27) and the parvicellular or magnocellular nucleus of the posterior commissure (plate 28) (Supplementary Fig. S3).

Quantification revealed a significantly lower absolute number or normalized number of MT-ir neurons in the PVN of S+ compared to S- quail, as well as a significantly lower density of MT-ir fibers in the MPN of S+ males (Fig. 2A, Supplementary Fig. S4A and raw data, statistical tests, and p-values in Table S2). No difference in MT-ir was found in the BNST and LS. Although sex differences in oxytocin expression have rarely been described in mammals and birds^{53,54}, we observed, for the first time in quail, a significantly higher density of MT-ir fibers in MPN, BNST, and LS in males compared to females, while no sex difference was found in the number of neurons in the PVN (Fig. 2 and Supplementary Table S2).

We observed a significant difference in the absolute number of VT-ir neurons in the PVN but this difference disappeared after normalization (Fig. 3, Supplementary Figure S4B and Table S2). A significantly lower density of VT-ir fibers was also observed in the MPN and BNST of S+ quail compared to S- but not in the LS. In line with previous studies^{55–57}, large sex differences were observed in the density of VT-ir fibers, with significantly higher densities of VT fibers being present in the MPN, BNST and LS of male quail compared to females (Fig. 3 and Supplementary Table S2).

Finally, a significantly lower absolute number or normalized number of ARO-ir cells was detected in the MPN of S+ quail compared to S-. A significant difference between lines was also found in the BNST, but this difference became only a statistical tendency following normalization. No difference between lines was observed in the VMN (Fig. 4, Supplementary Figure S4C and Supplementary Table S2). Contrary to previous studies^{58,59}, we found no sex differences in the MPN nor in the BNST. This discrepancy might be due to the higher sociability displayed by the female quail in our study, unlike previous studies that used different quail strains, where most females remained immobile when given the choice to engage in social interaction⁶⁰. A significantly higher number of ARO-ir cells was however observed in the VMN of males compared to females (Fig. 4 and Supplementary Table S2).

In conclusion, our results provide evidence that the three neuronal markers, MT, VT and ARO, are differentially expressed between the two divergent lines. This suggests that the divergence of these quail lines, based on their social motivation, has also impacted neuronal expression levels.

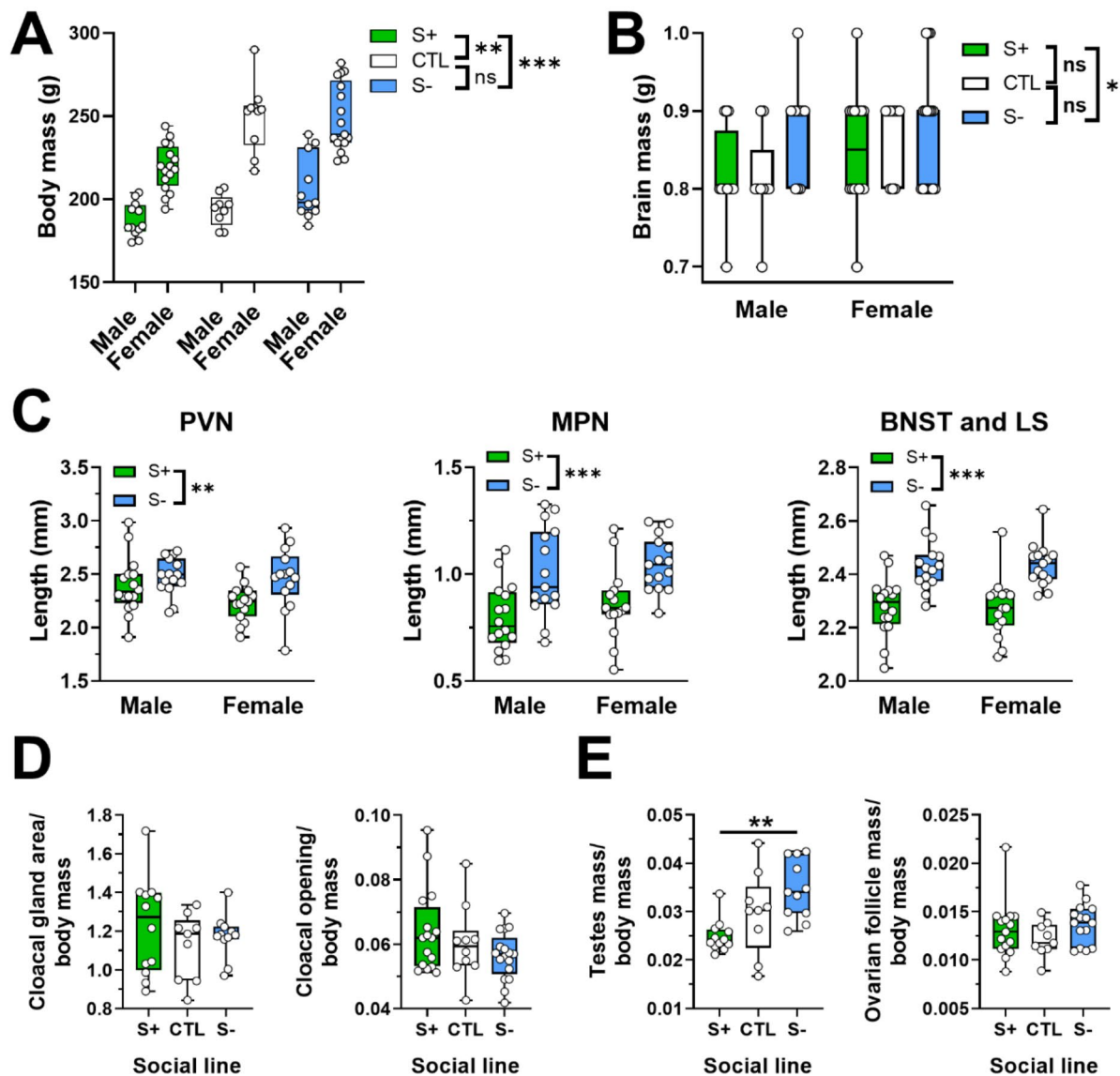


Fig. 1. Morphological data and brain measurements in adult quail lines selected for high versus low levels of social motivation (S+ vs. S-) along with the non-selected control quail line (CTL). **(A)** Body mass. **(B)** Brain mass. **(C)** Brain measurements corresponding to the length of PVN, MPN, BNST and LS brain structures. **(D)** Cloacal gland area and cloacal opening normalized by body mass. **(E)** Testes and ovarian follicle masses normalized by body mass. Sample size includes 12 S+, 9 CTL and 11 S- male quail and 16 S+, 10 CTL, 17 S- female quail (batch 1; A, B, D, E) as well as 16 S+, 15 S- male and 15 S+, 15 S- female quail (batches 1 and 2; C). Morphological data were analyzed using two-way ANOVAs or one-way ANOVAs followed by Tukey's (A-C) or Holm-Sidak's (D-E) post-hoc tests when the interaction was significant. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$. **Abbreviations:** BNST: bed nucleus of the stria terminalis, LS: lateral septum, MPN: medial preoptic nucleus, PVN: paraventricular nucleus of the hypothalamus.

Early social environment improved social ability in some S- males

Following immunohistochemical studies that confirmed differential expression of these neuronal markers between S+ and S- lines, we conducted a second experiment to assess the influence of distinct social environments from hatching to 3–4 weeks of age on social and non-social (not targeted) behaviors in juvenile males and females across 3 social groups (Fig. 5A–B). The first group exclusively consisted of S+ quail [S+], the second group comprised only S- quail [S-] (representing a low social environment for the S- quail), and the third group included quail raised with in a mix of 50% of S+ [S+(-)] and 50% of S- [S-(-)] (representing an enriched social environment for the S- quail).

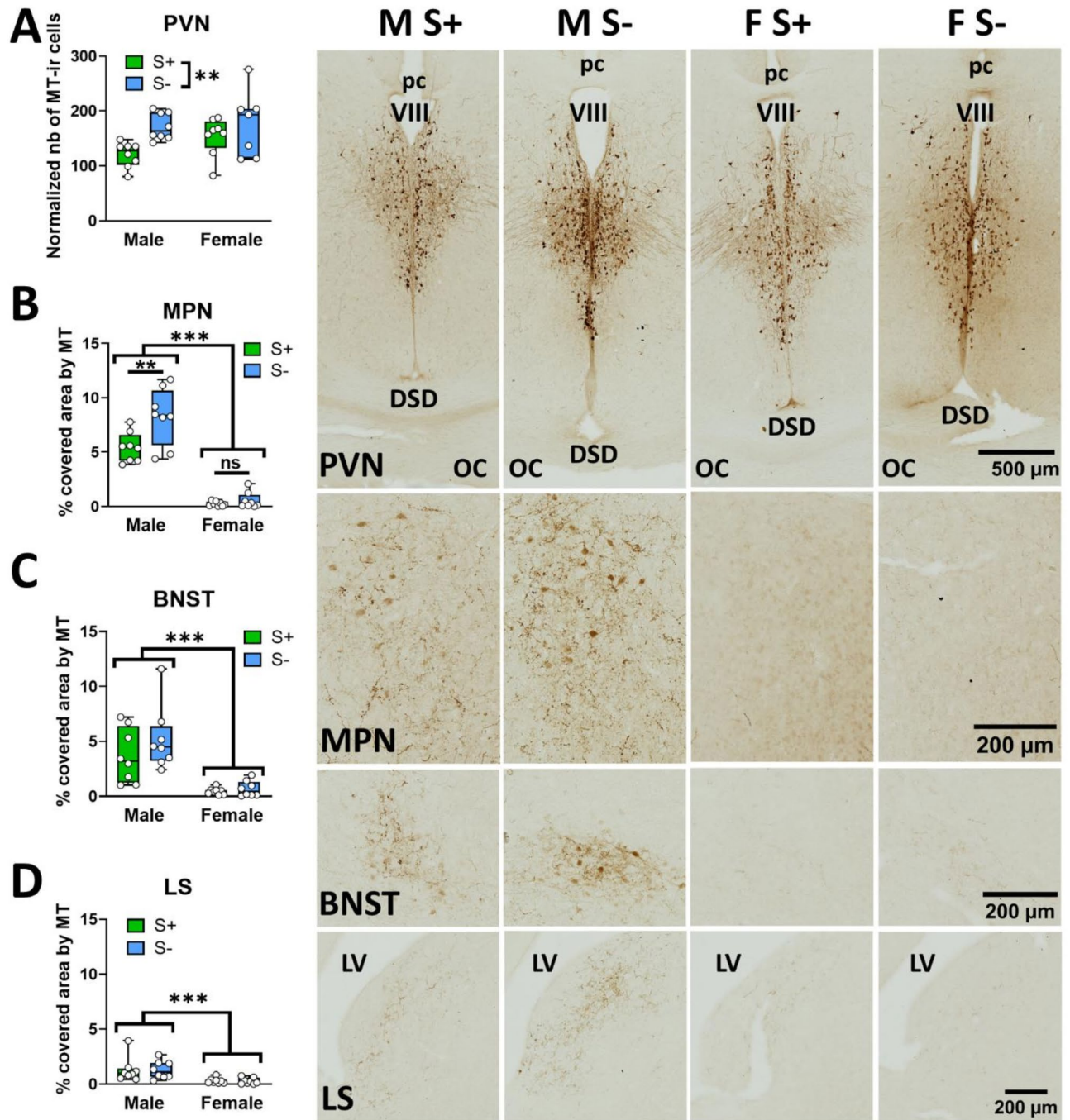


Fig. 2. Sex- and brain region- specific expression of mesotocin (MT) in divergent quail lines selected for sociability. **(A)** Normalized number of MT-immunoreactive (-ir) cells in the paraventricular nucleus (PVN) of adult male and female quail selected for a low level of sociability (S-; $n = 8$ by sex) compared to age- and sex-matched quail selected for a high level of sociability (S+; $n = 8$ by sex; batch 1). **(B-D)** Percentage of area covered by MT-ir fibers in the medial preoptic nucleus (MPN; **B**), bed nucleus of the stria terminalis (BNST; **C**) and the lateral septum (LS; **D**). Photomicrographs illustrate the MT-ir cells and fibers in the PVN, MPN, BNST and LS of male (M) and female (F) quail from S+ or S- phenotypes. Data were analyzed using separate two-way ANOVAs for each brain nucleus followed by Sidak's post-hoc test when the interaction was significant. $**p < 0.01$, $***p < 0.001$, ns = not significant. *Abbreviations:* DSD: dorsal supraoptic decussation, LV: lateral ventricle, OC: optic chiasm, pc: pallial commissure, VIII: third ventricle.

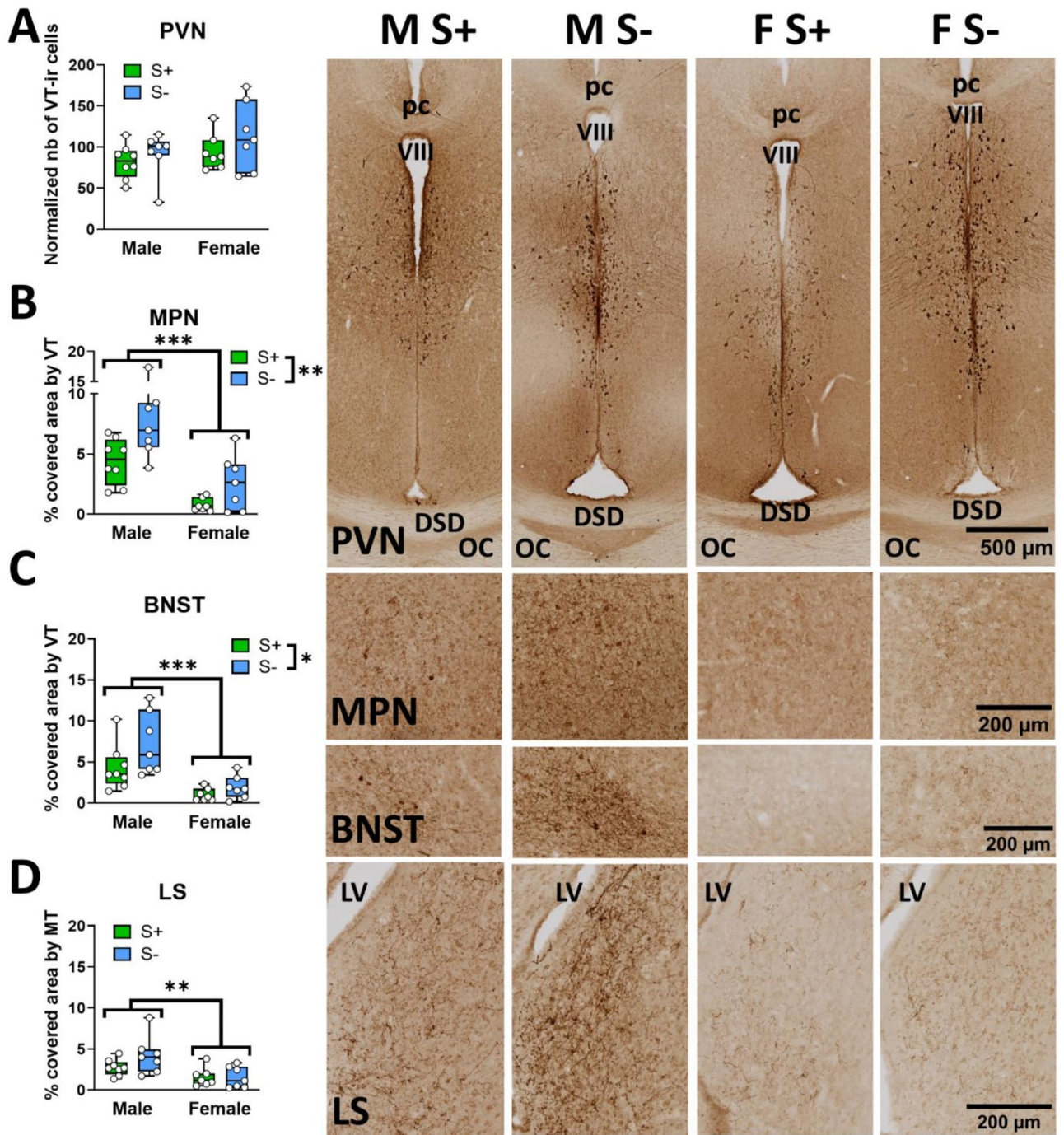


Fig. 3. Sex- and brain region- specific expression of vasotocin (VT) in divergent quail lines selected for sociability. **(A)** Normalized number of VT-immunoreactive (-ir) cells in the paraventricular nucleus (PVN) of adult male and female quail selected for a low level of sociability (S-; $n=7$ by sex) compared to age- and sex-matched quail selected for a high level of sociability (S+; $n=8$ male and 7 female quail, batch 2) **(B-D)** Percentage of area covered by VT-ir fibers in the medial preoptic nucleus (MPN; **B**), bed nucleus of the stria terminalis (BNST; **C**) and the lateral septum (LS; **D**). Photomicrographs illustrate the VT-ir cells and fibers in the PVN, MPN, BNST and LS of male (M) and female (F) quail from S+ or S- phenotypes. Data were analyzed using separate two-way ANOVAs for each brain nucleus. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *Abbreviations:* DSD: dorsal supraoptic decussation, LV: lateral ventricle, OC: optic chiasm, pc: pallial commissure, VIII: third ventricle.

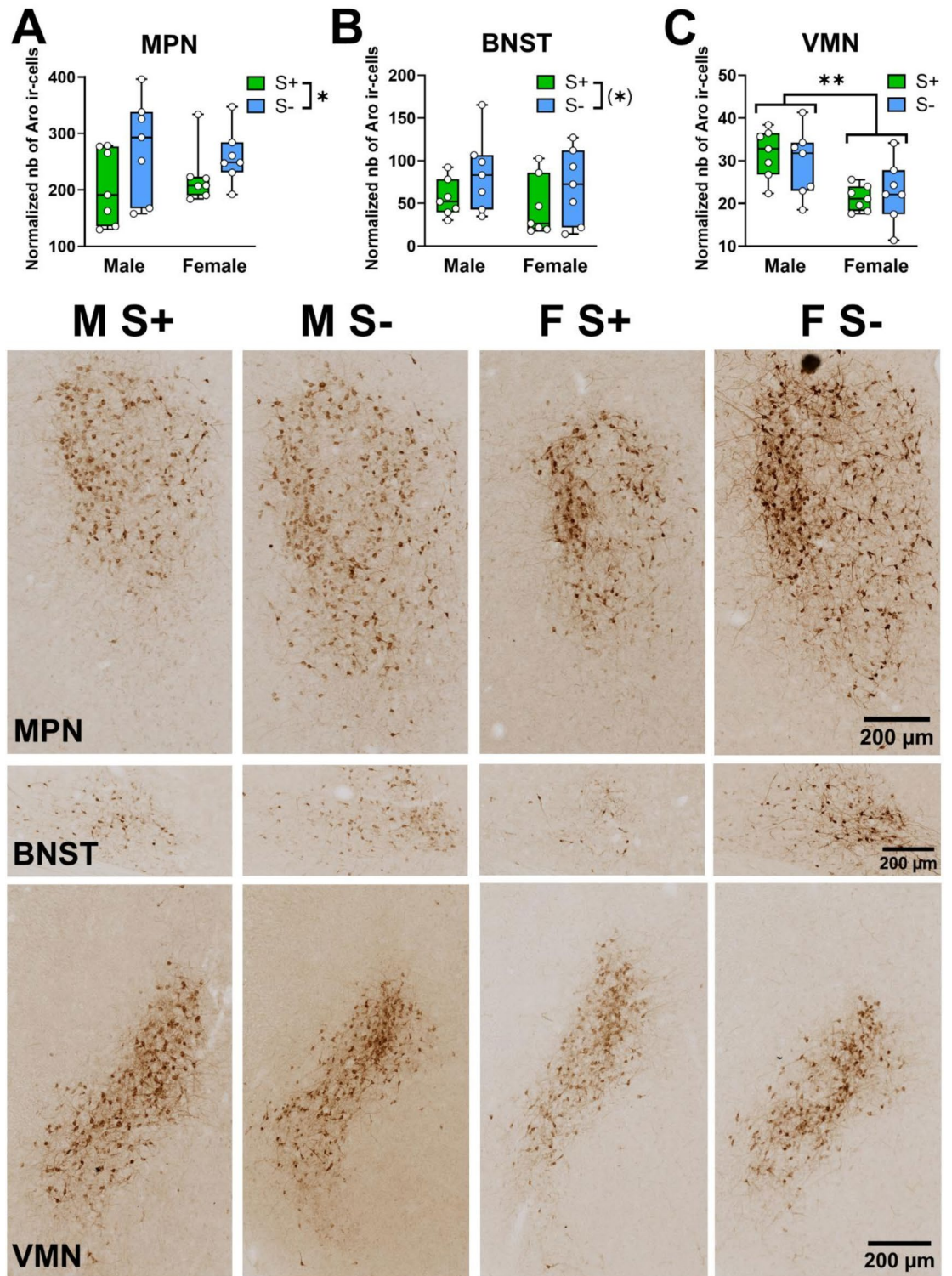


Fig. 4. Sex- and brain region- specific expression of aromatase (ARO) in divergent quail lines selected for sociability. Normalized number of ARO-ir cells in the medial preoptic nucleus (MPN; **A**), bed nucleus of the stria terminalis (BNST; **B**) and the ventromedial nucleus of the hypothalamus (VMN; **C**) of adult male and female quail selected for a low level of sociability (S-; $n = 7$ by sex) compared to age- and sex-matched quail selected for a high level of sociability (S+; $n = 8$ male and 7 female quail, batch 2). Photomicrographs illustrate the ARO-ir cells in the left part of the MPN, BNST and VMN of male (M) and female (F) quail from S+ or S- phenotypes. Data were analyzed using separate two-way ANOVAs for each brain nucleus. (*) $p < 0.10$, * $p < 0.05$, ** $p < 0.01$.

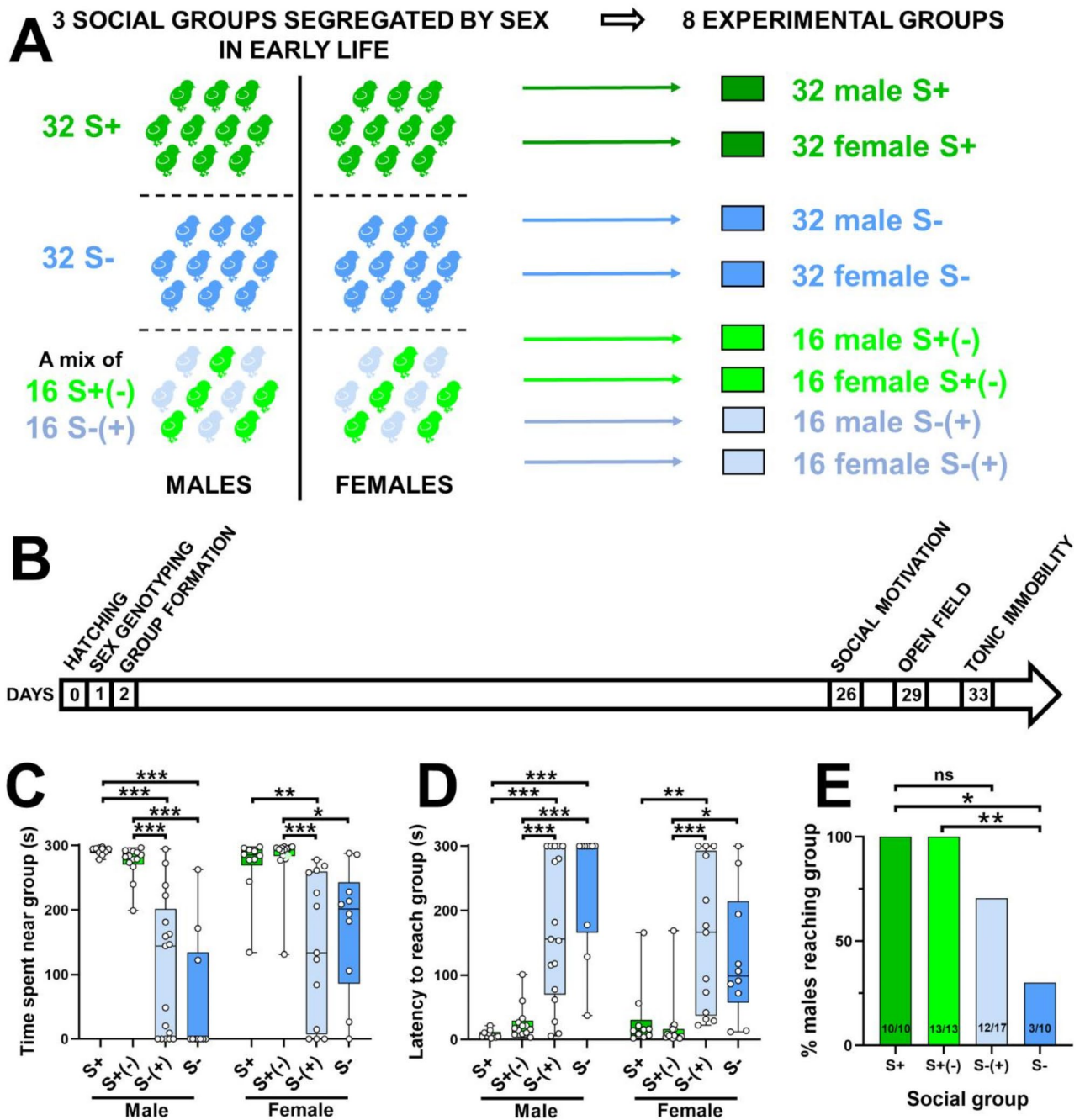


Fig. 5. Influence of early social environment on distinct social responses in juvenile quail selected for sociability. **(A)** Scheme of the experimental design consisting in groups of quail housed separately by sex under three distinct conditions from hatching until six weeks of age: S+ quail selected on a high level of sociability, S- quail selected on a low level of sociability and a mix of 50% of S+ and 50% of S- (32 males + 32 females per condition). For the behavioral tests, we selected a subgroup of S+ ($n = 10$ by sex), a subgroup of S- ($n = 10$ by sex) and all birds in the two mixed subgroups: S+(-) ($n = 13$ males and 15 females) and S-(-) ($n = 17$ males and 14 females; in batch 3, one male had been erroneously sexed as female at hatching). **(B)** Experimental timeline. **(C-D)** Time spent in **(C)** and latency to reach **(D)** the compartment adjacent to a group of 5 age- and sex-matched control quail from the non-selected line by the experimental male or female quail during the social motivation test. **(E)** Percentage of male quail able to reach the compartment adjacent to the group, at least once, during the social motivation test. Data were analyzed using two-way ANOVAs followed when significant by Tukey's post hoc tests **(C-D)** or by Fisher's exact probability test **(E)**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

Given the observed differences in locomotion^{45,48}, which could potentially influence sociability, the social motivation test was performed without activating the treadmill, unlike the methods used for the selection process⁴⁰. In this adapted behavioral test, S- male quail exhibited a significantly reduced time in the compartment adjacent to the group of conspecifics and a significantly longer latency to reach the group compared to S+ males (Fig. 5C-D and raw data, statistical tests, and p-values in Supplementary Table S3). Furthermore, only 30% of the S- males reached the compartment adjacent to the social group, while 100% of the S+ males rapidly joined the group (Fig. 5E). Although no significant difference was observed between S+ and S- female quail, likely due to the high inter-individual variability and the number of statistical comparisons that decrease the statistical power, the social divergence was confirmed in both sexes when considering the social line (S+ vs. S-) in the statistical analysis (Supplementary Table S3). These results, particularly in males, confirmed the divergence in sociability between the two quail lines independently of treadmill use.

In the group composed of a mix of S+ and S- quail, S+(-) male quail were not affected by the presence of S- males, while S- (+) inter-individual variability increased (Fig. 5C-D). Indeed, 70% of S- (+) male quail raised with S+ males approached the social group while only 30% of S- males did (Fig. 5E). Notably, the proportion of S- (+) males reaching the group was no longer different than S+ and S+(-), suggesting that an early enriched social environment with S+ partially restored S- social abilities.

Subsequently, we examined the influence of these distinct early social environments in the open field and in the tonic immobility tests. In the open field, S+ male and female quail traveled a significantly longer distance compared to the S- quail (Fig. 6A), and S- (+) performed similarly to S- quail, despite a high inter-individual variability. Furthermore, irrespective of the social environment they had been exposed, S+ and S- quail lines spent similar time in the center of the open field (Fig. 6B) or immobile (Fig. 6C, Supplementary Table S3), two measures considered as an index of anxious-like behaviors or of an innate behavioral response to predators.

In conclusion, our results confirm the social divergence between the S+ and S- lines in males, but also in females when the number of groups was reduced by excluding the condition with a mix of S+ and S-. Furthermore, a second experiment suggested that an early enriched social environment with S+ partially restored S- social abilities in some male quail without affecting non targeted behaviors.

Discussion

In this study, we found a reduction in body and brain masses in the S+ quail line relative to S-, along with similar differences in the size of the male gonads and brain structures. This difference in body mass was previously reported⁴⁵ and might be partly explained by the genetic selection using a treadmill. Indeed, quail lines exhibiting low and high levels of social motivation were selected based on the speed and traveled distance to rejoin the social group⁴⁰. Thus, the observed morphological differences might result from a selection of multiple phenotypic traits including social motivation, but also locomotor performance in the treadmill. It should also be noted that only the S+ line displayed a difference in body mass compared to the non-selected line (S- were not different), in agreement with the idea that the genetic selection possibly concerned locomotor ability. Co-selection of multiple factors occurs during genetic selection and has been described in multiple species⁶¹. In

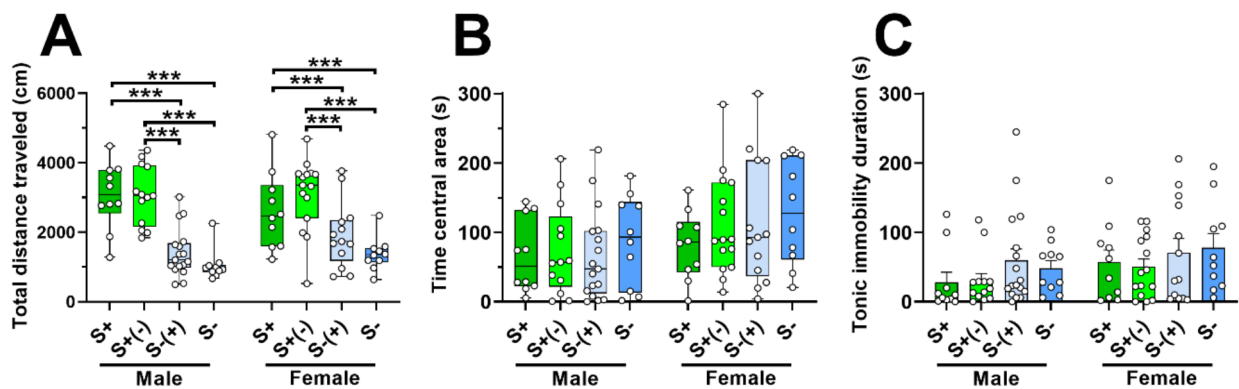


Fig. 6. Influence of early social environment on non-social behaviors in juvenile quail selected for sociability. Tests were performed in 3 groups of experimental quail (32 males + 32 females) segregated by sex from hatching. Among those, we selected for behavioral studies a subgroup of quail selected on a high level of sociability (S+; $n = 10$ by sex), a subgroup of quail selected on a low level of sociability (S-; $n = 10$ by sex) and all birds in the two mixed subgroups: S+(-) ($n = 13$ male and 15 female quail) and S- (+) ($n = 17$ male and 14 female quail; batch 3, one male had been erroneously sexed as female at hatching), each containing 50% S+ and 50% S- quail. (A) Total distance traveled used as a measure of locomotor activity and social response induced by short-term isolation in the open field. (B) Time spent in a central area of the open field as a measure of anxious-like behavior. (C) Duration of immobility quantified by the time spent by an experimental quail on its back in the tonic immobility test, used as a measure of innate behavioral response to predators. Data were analyzed using two-way ANOVAs followed when significant by Tukey's post-hoc tests. *** $p < 0.001$.

addition, as these differences were present from hatching and persisted throughout life, we speculate that the genetic selection targeted genes that might be involved in developmental processes. In an attempt to compensate for these size differences, cell numbers were presented as absolute values and after normalization by the length of each brain structure. Note however that differences in immunoreactive cell numbers or fiber densities were not found in all brain regions of the social behavior network (e.g., LS or VMN), suggesting that their differential expression did not systematically mirror the differences in body mass.

In the present study, we showed that the length of several brain structures, the number of MT neurons in the PVN, as well as the density of MT or VT fibers and the number of ARO cells in the MPN are reduced in the S+ quail line compared to the S- line. These differences clearly indicate that the genetic selection for high or low sociability had a major impact on the expression of several neurochemical markers in the social behavior network. The direction of the observed changes is however counterintuitive: a priori one would expect that the more social line would display higher densities of markers such as VT or MT (avian homolog of the mammalian OT and VP) that have been shown to be associated with social behavior. Two obvious reasons might explain this apparent discrepancy. First, it must be recalled that the S+ line also displayed a marked decrease in testis size and previous research has demonstrated that in mammals and birds, including quail, the expression of MT/OT, VT/VP and ARO is activated by testosterone (e.g.^{27,29,33,55,58,62}). If as suspected the decreased testis mass was associated with a lower testosterone circulating concentration, this could explain the decreased density of markers in males of the S+ line. This mechanism could also potentially apply to females even if the difference in gonadal development is less obvious in this sex. The second reason that could explain the unexpected changes in neurochemical markers is that the density of an immunocytochemical marker reflects both its production (synthesis) turnover and its rate of release and use by the brain. A lower density of the immunocytochemical signal might thus reflect a decreased synthesis and/or a faster release of the corresponding peptide (MT, VT) or protein (ARO). It is indeed well-known that some neuropeptides are very difficult to reveal by immunocytochemistry if one does not previously block their release by a drug such as colchicine. The lower density of fibers observed here in the S+ quail might thus reflect a higher activity of MT, VT and ARO neurons resulting in a more intensive release. Additional work collecting information on the rate of synthesis of these markers, using quantitative *in situ* hybridization, quantitative PCR for the corresponding mRNAs or MT/VT peptide dosage, would be needed to test this interpretation.

Previous studies using optogenetic or neurochemical manipulations have indeed highlighted the functional role of OT, AVP and ARO on social behaviors across various species. In male mice, inhibiting or activating PVN-OT neurons respectively impaired or facilitated social behavior^{63,64}. Similarly, in male rats, activating the magnocellular OT neurons of the PVN, supraoptic and accessory nuclei of the hypothalamus, resulted in a higher social motivation towards an opponent in a social defeat paradigm⁶⁵. Moreover, in zebra finches, knockdown of OT and VT synthesis in the PVN resulted in sex-specific effects on gregariousness and aggressiveness⁶⁶. Similarly in mice, deleting AVP expressing cells in the PVN increased social investigation in females⁶⁷ while knockdown of AVP expression in BNST reduced social investigation, sexual and aggressive behaviors in males⁶⁸. Similar approaches should be used to assess MT and VT activity and peptide release in these divergent quail lines.

Finally, the role of brain aromatase in the control of social behavior has been poorly investigated. In quail, chronic intracerebroventricular administration of an aromatase inhibitor affected social motivation depending on the sex of the stimuli used in the group³³. In addition, site-specific inhibition of ARO in the male quail MPN or BNST reduced the expression of sexual behavior^{69,70}. Optogenetic or chemogenetic manipulations of aromatase-expressing neurons similarly affect aggressiveness and some components of sexual behavior in mice⁷¹. However a study examining sexual or aggressive behaviors in the divergent quail lines found no differences between S+ and S- quail at the age of 7 weeks⁴⁵. The present results demonstrating differences in ARO cell number between S+ and S- quail would then suggest that this neuronal marker could be implicated in social motivation besides its potential role in sexual behavior. Overall, further pharmacological or genetic manipulations to target PVN or MPN would be needed to confirm a differential functional role of MT, VT and ARO in the expression of social motivation or in other forms of social behavior.

Although the treadmill was not used in the present study and the sociability tests were performed in 6-week-old quail rather than quail chicks, the social divergence between S+ and S- males was confirmed without any associated effect on anxious-like behaviors or on a presumed innate behavioral response to predators. These findings confirm previous studies^{40,45,48} and support the idea that the differential expression in MT and VT observed here are specific to social abilities and do not relate to anxious-like behaviors or stress. These neuropeptides are indeed implicated in both processes (review in⁷²). Interestingly, the early enriched social environment mixing S+ and S- male quail led to an intermediary profile of sociability in S-(+) males relative to S+ and S- counterparts, without affecting other behaviors. This suggests that a subset of S-(+) males exhibit behavioral flexibility and can be influenced by their conspecifics.

It is important to acknowledge two limitations of this proof-of-concept experiment. First, each bird within a given large social group was considered as an independent unit, but we cannot rule out the possibility that a few individuals' behavior may have influenced the behavior of the entire group. This issue is challenging to avoid in this context, as studying sociability requires the use of large social groups with substantial sample sizes, which introduces ethical (number of experimental subjects enrolled) and spatial (availability of housing space) constraints. Second, males and females were housed in separate rooms to avoid potential intersex influences on the development of social behaviors. Despite these limitations, the observed social divergence between lines (S+ versus S-) was consistent with previous studies^{40-45,47} (when analyzed in the same way, without considering the mixed groups), supporting the validity of this experiment. Repeating this experiment as well as using different ratio of S+ and S- in mixed social groups in subsequent studies would strengthen the effect of the early social environment observed in this proof-of-concept experiment.

In summary, this study offers novel insights into the influence of early social environments in divergent lines of animals selected for sociability. More generally, few studies have been conducted on the influence of early social environment on genetic models. Consistent with our results, improved social interactions were found in a mouse model of social deficits, the inbred BTBR T1tf/J mouse line or in mice exposed *in utero* to valproic acid, when reared with control cage mates^{11,12}. Furthermore, *Shank3* knock-out mice also display differential social skills when exposed to low or enriched early social environment¹³. Overall, the divergent quail lines selected for social motivation are a valuable model to study the genetic, neural, and behavioral aspects underlying social skills and provide insights into potential behavioral strategies to optimize animal welfare and human conditions associated with social deficits.

Methods

Animals

Japanese quail chicks (*Coturnix japonica*) were selected on their high versus low levels of motivation to approach a group of conspecifics (S+ and S-) while ensuring no difference in the tonic immobility test⁴⁰ over 45 generations. In parallel, a control (CTL) line of non-selected descendants, derived from the first generation, was maintained throughout these generations. Independent batches of 7-month-old male and female quail were used to characterize potential morphological differences between the divergent quail lines (batch 1 only; males: N=12 S+, 11 S- and 9 CTL; females: N=16 S+, 17 S- and 10 CTL, respectively) and to perform brain measurements and immunohistochemistry for neuronal markers (batch 1, N=8 individuals by group and batch 2, N=7–8 individuals by group). Quail were provided by an independent service responsible for breeding within the Poultry Experimental Facility. The animals were raised on the ground floor, then organized in small groups of one male and two females of the same line and transferred to cages containing colored balls, which served as potential enrichment or distractors (batches 1 and 2).

In a second experiment, a third batch of birds was used to investigate the influence of early social environment on the later sociability of the divergent lines in juvenile quail. Eggs were provided by an independent service responsible for breeding within the Poultry Experimental Facility. From hatching to the end of the experiment, quail were housed separately by line and by sex on the ground floor that was covered with wood chips and enrichments were composed of tunnels made with plastic mesh (batch 3; detailed N in the ‘Early social environment’ and ‘Behavior’ sections).

In all batches, juvenile or adult quail were maintained under a neutral 12 h light and 12 h dark photoperiod. Food and water were provided *ad libitum*. All animal procedures were performed in accordance with the Ethics Committee CNREEA Val de Loire N°19 animal care committee’s regulations, with relevant named guidelines, and French and European Directives (Advisory CE 19-2022-2505-1). The experimental procedures were performed following the ARRIVE guidelines.

Incubation

Eggs were selected and gathered by quail lines for incubation at 37.5 °C and 55% of relative humidity over 15 days (Egg Incubator Zundel). On incubation day 15, eggs were candled and if considered as developed, they were gathered in stainless steel hatching baskets (93 × 32 × 8 cm) to prevent mixing of chicks from distinct quail lines. Humidity was increased to 75% to facilitate hatching. On incubation day 19, newly hatched quail chicks were labelled with a ring placed on the right wing. Chicks were then placed in a brooder at a temperature of 38–40 °C that was gradually decreased over two weeks to reach room temperature (about 22 °C).

Sex genotyping

Sex identification of newly hatched quail was adapted from a previous study⁷³. Five down feathers were collected behind the neck of the quail chicks with a pair of forceps washed with 1% liquid detergent RBS followed by distilled water between each bird. Feathers were placed into a 1.5 mL Eppendorf tube and stored at room temperature. On the same day, genomic DNA extraction was performed by incubating feathers in 200 µl of 10% Chelex® 100 Resin (Bio-rad, 142–1253) in distilled water and 2 µl of proteinase K (20 mg/ml; Qiagen, RP103B) for 1 h at 55 °C in a heating-shaking dry bath, followed by centrifugation at 1000 rpm and supernatant collection. PCR was performed using 2 µl of 10X PCR DNA-free MgCl₂ buffer, 1 µl of 50mM DNA free MgCl₂, 0.4 µl of 10 mM dNTP, 0.2 µl of Invitrogen Platinum Taq DNA polymerase (Thermo Fisher Scientific, 10966034), 1 µl of sample and 0.4 µl of the three primers (ZF, WF and P2) mixed in a total reaction volume of 20 µl of nuclease-free water. Primers for sex determination were as followed ZF (5'-CTCTGGGTTTTGACTGTATTG-3') and WF (5'-CATCTGTTTTCCCCCAAAA-3') forward primers for the Z and W allele, respectively, as well as P2 common reverse primer (5'-TCTGCATCGCTAAATCCTTT-3'). The PCR program was set at 95 °C for 5 min followed by 30 cycles of 45 s for each step at 95 °C, 58 °C and 72 °C and a final extension at 72 °C for 10 min. 5 µl of PCR volume were migrated on a 2% agarose gel stained with GelRed (Biotium). Based on PCR results, the 2-day-old quail chicks were separated by sex. At 3 weeks, sex was confirmed through the identification of the breast feather color, with males having brownish-red feathers and females having gray feathers with black speckles. The sex of a few animals was not correctly identified at 3 weeks and they had to be transferred a bit later in the group corresponding to their sex.

Morphology and brain measurements

Total body mass (g) was measured at three distinct time points: hatching, 3 weeks at the end of the social motivation test (batch 3), and at 7 months (batch 1). The cloacal gland area (length x width) and the cloacal opening (width) considered as indirect markers of circulating concentrations of androgens^{74–76} and estrogens^{77–79} respectively were also assessed at 3 weeks (batch 1) and 7 months (batch 3). Additional measures of the brain

mass, the mass of the two testes and the mass of the largest ovarian follicle (g) were performed in the S+, S- and CTL phenotypes (batch 1).

Brain measurements were obtained using neuroanatomical markers specific to the brain sections containing the PVN, MPN, BNST, LS and VMN (batch 1 and 2). The rostral part of the PVN is located at the level of the hippocampal pallial commissure and the beginning of the supraoptic decussation, corresponding to plates 15 and 16 of the chick brain atlas⁵². This atlas provides descriptions that are relatively closer, more detailed and updated than the earlier quail brain atlas version⁸⁰. In the brain section containing the PVN, the length between the top of the pallial commissure to the top of the dorsal supraoptic decussation was measured within the medial part of the brain section, at the level of the third ventricle. The left and right caudal MPN, rostral BNST and medial LS are located in brain sections corresponding to plate 14⁵² where the anterior commissure reaches its largest extension. In the brain section containing MPN, the length was measured between the bottom of the anterior commissure to the top of the periventricular fibers at the level of the third ventricle (for MT- or VT-immunostained brain sections) or between the bottom of the anterior commissure to the top of the optic chiasm (for the ARO-immunostained brain sections) at the level of the third ventricle. In the same brain section containing the BNST and LS, the length was measured between the internal edge of the left and right lateral ventricles. Additional measures of the area of MPN, BNST and VMN containing ARO-ir cells were obtained by drawing a polygon shape around the population of these immunoreactive cells. Finally, the rostral VMN was located at the beginning of the median eminence when the optic tectum is not fully developed corresponding approximately to plates 22 to 23⁵². At the VMN level, the length used for standardization was measured between the two central external parts of each hemisphere.

Immunohistochemistry

Seven-month-old birds were transcardially perfused with 4% formaldehyde (VWR Chemicals BDH, 20910.363) in 0.01 M phosphate-buffered saline (PBS, pH = 7.2) following anesthesia with sodium pentobarbital (Exagon 400 mg/ml; 150 μ l per quail) and a transcardiac injection of 100 μ l heparine (25 000 UI/5 ml; Cheplapharm France). Perfusions were conducted over two separate days, alternating social line and sex throughout the entire day (morning and afternoon) to minimize the potential influence of time of day or of the specific day on the observed effects in the brain. Brains were removed from the skull, post-fixed in formaldehyde 4% overnight and then rinsed three times in PBS (0.01 M, pH 7.4). Brains were then immersed in 30% sucrose with 0.5% sodium azide (Sigma-Aldrich, 8.22335) until they sank (approximately two nights), frozen on dry ice and stored at -70°C until further use. Seven to eight brains of S+ and S- male and female quail (MT, batch 1; VT and ARO, batch 2) were cryosectioned in four series of 30 μ m thick coronal slices from the septopalliomesecephalic tract, which marks the rostral end of the medial preoptic nucleus, to the third nerve indicating the caudal end of the hypothalamus and stored in antifreeze at -20°C until further use. Antifreeze was prepared using 40% v/v PBS 0.01 M, 1% polyvinylpyrrolidone (Sigma-Aldrich, P5288), 30% saccharose (VWR Chemicals BDH, 27478.296), 30% v/v ethylene glycol (VWR Chemicals BDH, 24041.297), which was adjusted with PBS to achieve the final volume. Brain sections were immunostained for each neuronal marker using validated antibodies against MT, VT and ARO according to previous studies^{37,53,57}. Sections were first rinsed three times in 0.01 M tris-buffered saline (TBS, pH 7.6) for 5 minutes each (same duration applied for all following rinses). For ARO and MT, peroxidase activity was blocked with 0.6% of hydrogen peroxide (H₂O₂; Merck Millipore, 1072090250) in TBS at room temperature (RT) for 20 or 30 minutes, respectively. After rinses with TBS (ARO) or TBS containing 0.1% Triton X-100 (TBST; MT), sections were blocked and permeabilized for 1 hour in 5% (ARO) or 20% (MT) normal goat serum (NGS; ARO: Merck Millipore, S26, MT: Cell signaling technology, 5425S) in TBST. For VT, peroxidase activity was blocked with 1% of H₂O₂ at RT for 30 minutes with 10% NGS (Merck Millipore, S26) in TBST. Sections were then incubated with a rabbit anti-oxytocin primary antibody (1/10000, Immunostar, Ref: 20068), rabbit anti-arginine vasopressin primary antibody (1/5000, Merck Millipore, Ref: AB1565) or a rabbit anti-aromatase primary antibody (1/3000, Harada QR 02/05) with 2% (MT and VT) or 5% (ARO) NGS in TBST for two nights at 4°C. Sections were rinsed in TBS (VT and ARO) or TBST (MT) and incubated for 2 hours at RT with a goat anti-rabbit biotinylated secondary antibody (1/250 for MT and 1/400 for VT and ARO respectively, Jackson ImmunoResearch, Ref: 111-065-003) in TBST. Sections were washed in TBS (VT and ARO) or TBST (MT) and incubated for one hour and a half in the vectastain Elite ABC-HRP Kit, Peroxidase in TBST (1/200, 1/1000, 1/800 of each reagent for MT, VT and ARO, respectively; Vector Laboratories, PK-6100) at RT, then rinsed in TBS (VT and ARO) or TBST (MT). The peroxidase was visualized with 0.04% of 3,3'-diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich, D537) used as chromogen along with 0.012% of H₂O₂ in TBS at RT. Sections were finally rinsed, mounted on slides, dried overnight, left 10 min in xylene and cover-slipped using Eukitt (Sigma-Aldrich, 03989).

Image acquisition and analysis

Photographs of brain sections were acquired using a microscope slide scanner (Zeiss, Axio Scan.Z1) with an objective 20x/0.8 and a HV-F202SCL Hitachi camera. High-resolution images were generated by the Zen Blue 3.1 software (Carl Zeiss, Oberkochen, Germany) and visualized with QuPath software, version 0.5.0⁸¹. Specific brain regions were delimited by rectangular quantification fields of identical dimensions across social lines and sexes in the left and right hemispheres for each brain region of interest using QuPath. These rectangular fields were then exported for further analyses in ImageJ 1.54f⁸².

For the MPN, the rectangular quantification field was placed in the corner formed by the ventral edge of the anterior commissure and the lateral edge of the third ventricle. For MT and VT staining, small anatomical inter-individual differences prevented the positioning of a standardized quantification field covering the entire immunoreactive (ir) fibers of the MPN, without including magnocellular MT- or VT-ir cells and fibers from the periventricular region. Fiber density was thus quantified in a rectangular field covering the dorsal portion of the

MPN, which remained identical across all brain sections regardless of social line and sex. For the ARO staining, the quantification field included the entire MPN, as no interfering signal was present in adjacent regions. In the BNST, LS and VMN, the quantification fields were placed to cover the entire immunoreactive part of the brain nucleus located dorsally to the most lateral edge of the anterior commissure, approximately under the lateral ventricle (BNST), adjacent to the lateral ventricle (LS), or in the corner formed by the lateral edge of the third ventricle and the ventral limit of the brain (VMN).

The number of MT- and VT-ir cells within the rostral PVN was determined manually using the cell counter plugin in ImageJ. The number of ARO-ir cells in the left and right part of the caudal MPN, rostral BNST and rostral VMN was determined by an automatic method using the cyto2 model from the Cellpose deep learning-based segmentation method⁸³ in ImageJ.

The percentage of area covered by MT- or VT-ir fibers in the left and right part of the caudal MPN, rostral BNST and medial LS was determined by a semi-automatic method using the threshold method in ImageJ where threshold was manually adjusted for each subject. The values from the left and right quantification field were averaged to obtain one single value for each brain region. The number of cells was normalized based on the length between neuroanatomical markers identified in the brain sections containing each brain nucleus of interest (detailed in the [Morphology and brain measurements](#) section).

For all measurements and analyses, the investigator was blind to the social line or the sex of the birds.

Effect of early social environment

For experiment 2, two-day-old quail chicks were segregated by sex from hatching until six weeks of age and housed under three distinct conditions, also referred as three social groups. These conditions included a group of 32 S+, a group of 32 S-, and a mixed group of 16 S+ with 16 S-, resulting in a total of 192 quail (32 individuals per condition \times 3 conditions \times 2 sexes). This experimental design generated 8 groups to compare statistically: two groups (males and females) comprised solely S+ quail [S+], two groups only S- quail [S-] and two groups that contained a ratio of 50% of S+ [S+(-)] and 50% of S- [S- (+)] quail. The sample size was sufficiently large to ensure that each social group consisted of at least 30 individuals, thereby minimizing the risk of potential attrition.

Nineteen days after hatching, quail were transferred in larger spaces comprising two rooms of 5.6 \times 3.6 m (one for each sex) divided in three equal compartments to receive the animals from the three distinct housing conditions (S+, S- and S+(-) with S- (+) respectively). Compartments were separated by wooden board at the bottom and wire mesh at the top to prevent visual contacts with the other groups. A third room was dedicated to the control non-selected quail line ($N=25$ quail of each sex) whose individuals were used as stimuli in subsequent behavioral tests.

Behaviors

Three- to four-week-old juvenile quail were used to conduct the behavioral tests in order to avoid the surge of testosterone production and the activation of sexual behaviors^{84–86}. We randomly selected a subgroup of S+ ($n=10$ individuals per sex), S- ($n=10$ individuals per sex), and all birds in the two mixed groups, including S+(-) ($n=13$ male and 15 female quail) and S- (+) ($n=17$ male and 14 female quail; batch 3, one male had been erroneously sexed as female at hatching), to perform the series of behavioral tests assessing social motivation, open field activity, and tonic immobility, respectively. Subgroups of S+ and S- individuals were selected rather than the total number of birds in each condition ($n=32$ individuals by condition) to enable the testing under standardized conditions, thereby avoiding a “day” effect within the same test. An arbitrary rest period of three to four days between each test was implemented. Birds were carried individually to the testing room in a transport box (15 [length] \times 15 [width] \times 20 [height] cm) made of opaque polycarbonate, alternating between sex and social group. The testing apparatus was cleaned using water and paper towels between subjects. Behavioral scoring and analyses were carried out by the investigator who was unaware of the social group to which the birds belonged.

Social motivation test

Three-week-old experimental quail placed at one end of a long corridor (245 [length] \times 30 [width] \times 30 [height] cm) was offered the choice to approach and stay close to a group of 5 age- and sex-matched quail from the non-selected line located at the other end. The corridor made with wire mesh was divided into five virtual compartments (C1-5) of equal length. The experimental quail was placed for 10 s for habituation in C1 defined by a transparent Plexiglas. Then, the partition was removed, and the experimental quail could freely move for 5 min within compartments C1 to C4 and get closer visual access to the group confined in C5 at the opposite end of the corridor. The full test was recorded. The time spent in each compartment was measured, as well as the latency to reach the compartment adjacent to the social group (C4).

Open field test

Four-week-old experimental quail were individually placed for 5 min in the center of a square arena (80 [length] \times 80 [width] \times 50 [height] cm) made of wood with the floor covered by a plastic surface and surrounded by an opaque green curtain. A light and a camera positioned above the center of the arena was used to record the behavior. Locomotor activity, also used in previous studies as a proxy for social response induced by short-term isolation^{48,87} and anxious-like behaviors were assessed by quantifying the mean distance traveled and the time spent in the squared central zone, respectively. These measures were automatically measured using the EthoVision XT tracking software.

Tonic immobility test

Tonic immobility is an innate behavioral response to predators in birds^{88–90}, which has been counter-selected during the divergence process in these two lines. A quail was placed and maintained on its back by the experimenter's hand for 10 s (induction) in a plastic U-shaped cradle (20 [length] x 10 [width] x 10 [height] cm) covered with a cloth. Then, the experimenter's hand was gently removed from the bird and the time spent by the bird to stand up was quantified (tonic immobility duration per se). When the induction phase failed, the attempt was repeated. After 5 attempts, the tonic immobility duration was scored as 0 s. Conversely, a score of 300 s was attributed when a quail failed to stand up after the maximal duration of the test set at 5 min. The tonic immobility duration and the number of induction attempts were quantified in this test.

Statistics and writing

Considering social line and sex as independent factors, morphological and behavioral data were analyzed by two-way ANOVAs followed by Sidak's or Tukey's post-hoc tests when significant. The data from the cloacal gland area, the cloacal opening and the testes or ovarian follicle masses were analyzed by ordinary one-way ANOVAs followed by Holm-Sidak's post-hoc tests when significant. The percentage of male quail reaching the compartment adjacent to the social group in the social motivation test were compared using the Fisher's exact probability test followed by a Bonferroni correction for multiple comparisons and thus, all p values were multiplied by the number of comparisons (adjusted p or p_{adj}). All analyses were performed with GraphPad Prism version 8.0.2. Normality and homoscedasticity were assessed for all statistical tests. In a small number of cases where these assumptions were not met, non-parametric tests were performed to verify the validity of the results. Since the non-parametric tests always yielded similar outcomes (significant results were always identical with the two approaches and non significant results remained identical), we only report here the p-values from the parametric tests to facilitate readability. Results were considered significant for p value < 0.05 and were presented by boxplots with individual data points, median, first and third quartiles and whiskers indicating the smallest and largest value. English language editing was partly provided by ChatGPT 3.5 developed by OpenAI.

Data availability

All data generated and analyzed during this study are included in the Supplementary Tables 1 to 3.

Received: 3 June 2024; Accepted: 30 September 2024

Published online: 09 October 2024

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Acknowledgements

This work was supported by INRAE “SOCIALOME” project (PAF_29). Quail breeding and care was conducted in Poultry Experimental Facility (PEAT) from the INRAE experimental unit 1295 (F-37380 Nouzilly, France, DOI: 10.15454/1.5572326250887292E12). Microscopy was performed through the facilities and expertise of the “Plateforme d’Imagerie Cellulaire” (PIC) of the UMR PRC, INRAE. The authors thank Dr. Nobuhiro Harada for providing the aromatase antibodies.

Author contributions

Lucas Court (LC), JL, FC, JB, CAC, MK, Ludovic Calandreau (LCC) and LP conceived the experiments; LC, LT,

JL, EP, FC conducted the experiments; LC, JL, MK, LCC and LP analyzed the results; LC and LP wrote the paper; MCB contributed analytic tools. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-74906-3>.

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