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Sexual Differentiation of Brain and Behavior in Quail and Zebra Finches: Studies with a New Aromatase Inhibitor, R76713

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In many species of vertebrates, major sex differences affect reproductive behavior and endocrinology. Most of these differences do not result from a direct genomic action but develop following early exposure to a sexually differentiated endocrine milieu. In rodents, the female reproductive phenotype mostly develops in the absence of early steroid influence and male differentiation is imposed by the early action of testosterone, acting at least in part through its central conversion into estrogens or aromatization. This pattern of differentiation does not seem to be applicable to avian species. In Japanese quail (*Coturnix japonica*), injection of estrogens into male embryos causes a permanent loss of the capacity to display male-type copulatory behavior when exposed to testosterone in adulthood. Based on this experimental result, it was proposed that the male reproductive phenotype is "neutral" in birds (i.e. develops in the absence of endocrine influence) and that endogenous estradiol secreted by the ovary of the female embryo is responsible for the physiological demasculinization of females. This model could be recently confirmed. Females indeed display a higher level of circulating estrogens that males during the second part of their embryonic life. In addition, treatment of female embryos with the potent aromatase inhibitor, R76713 or racemic vorozole™ which suppresses the endogenous secretion of estrogens maintains in females the capacity to display the full range of male copulatory behaviors. The brain mechanisms that control this sexually differentiated behavior have not been identified so far but recent data suggest that they should primarily concern a sub-population of aromatase-immunoreactive neurons located in the lateral parts of the sexually dimorphic preoptic nucleus. The zebra finch (*Taeniopygia guttata*) exhibits a more complex, still partly unexplained, differentiation pattern. In this species, early treatment with exogenous estrogens produces a masculinization of singing behavior in females and a demasculinization of copulatory behavior in males. Since normal untreated males sing and copulate, while females never show these behaviors even when treated with testosterone, it is difficult to understand under which endocrine conditions these behaviors differentiate. In an attempt to resolve this paradox, we recently treated young zebra finches with R76713 in order to inhibit their endogenous estrogens secretion during ontogeny and we subsequently tested their behavior in adulthood. As expected, the aromatase inhibitor decreased the singing frequency in treated males but it did not affect the male-type copulatory behavior in females nor in males. In addition, the sexuality differentiated brain song control nuclei which are also masculinized in females by early treatment with estrogens, were not affected in either sex by the aromatase inhibitor. In conclusion, available data clearly show that sexual differentiation of reproductive behaviors in birds follows a pattern that is almost opposite to that of mammals. This difference may be related to the different mechanisms of sex determination in the two taxa. In quail, the ontogeny of behavioral differentiation is now well understood but we only have a very crude notion of the brain structures that are concerned. By contrast, in zebra finches, the brain mechanisms controlling the sexually differentiated singing behavior in adulthood have been well identified but we do not understand how these structures become sexually dimorphic during ontogeny.

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INTRODUCTION

The existence of behavioral differences between males and females has long been recognized in many animal species and even in humans but it is only recently that this question became a subject of scientific investigation. The demonstration of sex differences in behavior raises two main groups of questions: (a) what are the proximal causes of these differences in the adult subjects? and (b) how do these differences develop during ontogeny?

An active research effort initiated in the sixties largely as a consequence of the seminal paper of Phoenix *et al.* [1] and almost exclusively focussed on mammalian species has led to the formulation of several responses to these questions that are widely, although not universally, applicable. First, sex differences in behavior are thought to be controlled, to a large extent, by morphological, physiological and/or neurochemical sex differences in the brain. Second, these brain differences between males and females appear to develop mostly under the epigenetic action of gonadal steroids produced during the perinatal period [1, 2]. In a small number of cases, a direct genetic differentiation of specific brain characteristics (i.e. morphology of dopamine and prolactin cells in the rodent brain [3–5]) has, however, been identified so that it should not necessarily be assumed that every neural sex difference is the direct consequence of an early action of steroid hormones [6]. Third, the perinatal effects of steroids (organizing action) are irreversible and they only take place during a limited period of the development, usually called the critical period [2]. By contrast, the action of steroids in adulthood (activating action) is transient. It is often required in order to activate the sex-specific brain circuitry that promotes the expression of the adequate sexually differentiated behavior. It must be noted that, even if the dichotomy between organizational and activational actions of steroids has a great pragmatic value, the more recent discovery of a significant morphological and functional plasticity in the adult brain tends to blur the distinction and suggests that these two types of action probably do not reflect fundamentally different processes [7].

A GENERIC MODEL OF SEXUAL DIFFERENTIATION IN RODENTS

In mammals, major sex differences in the ability to respond to gonadal steroids are at the origin of a significant sexual dimorphism affecting reproductive behavior [2]. In gonadectomized rats, for example, a sequential treatment with estradiol plus progesterone activates sexual receptivity (lordosis) in females but has little or no effect in males. The male-type copulatory behavior is, by contrast, less differentiated and can be activated to some extent in both sexes by a treatment with testosterone. It has been clearly established that these behavioral differences are by and large the result of neonatal exposure of males to testosterone and its endogenous metabolite, estradiol. Male pups gonadectomized early after birth will present as adults a female phenotype of reaction to steroids while female pups treated neonatally with testosterone will react like males to endocrine manipulations performed in adulthood [2, 8].

These data illustrate two general principles that seem to hold true for all mammalian species studied so far. First, a young animal growing in the (relative) absence of gonadal steroids will develop a female phenotype (see, however, [9] for a more refined discussion of this concept) and second, the male behavioral phenotype is normally imposed during ontogeny by the action of gonadal steroids secreted by the heterogametic sex (the male in this case). This summary obviously represents an oversimplified version of the current knowledge and is presented here only to permit a comparison with the avian data to be described below. More circumstantial reviews of this literature are available [8, 10–12].

There is also a huge amount of work describing morphological and neurochemical sex differences in the adult mammalian brain. It is beyond the scope of the present paper to consider these data in detail but these have been reviewed by several authors [13–20]. Many of these sex differences in the brain disappear when adult males and females are placed in identical endocrine conditions which strongly suggests that they are activational in nature, i.e. induced by the different endocrine milieu in adult subjects. A number of these differences are, however, still found in gonadectomized subjects treated or not with testosterone and subsequent experiments have confirmed that they are organized by the early exposure to steroids. This is namely the case of the sexually dimorphic nucleus of the preoptic area in rats which is larger in adult males compared to females even when sex differences in circulating sex steroid levels are controlled for [20, 21]. Unfortunately, it must be stressed that, in most cases, the precise relationship between these organized brain differences and the sexually differentiated behaviors are still unclear (e.g. [22, 23]).

SEXUAL DIFFERENTIATION OF BEHAVIOR IN QUAIL

Adult male and female Japanese quail (*Coturnix japonica*) differ markedly in the way they respond to a systemic treatment with testosterone. This sex steroid activates the entire sequence of copulatory behaviors including grabbing the neck of the female, mounting and achieving cloacal contact in males, while these responses are (almost) never observed in females even after the injection of similar (or even much higher) doses of testosterone [24–26]. By contrast, the receptive sexual behavior (squatting), that is characteristic of the female, can be elicited in both sexes by an appropriate

treatment with estrogen [24, 25]. This points to a first difference between birds and rodents: in quail, the behavior typical of the male is sexually differentiated while the female-typical behavior is not. The reverse is observed in rodents: the lordosis (typical of the female) is sexually differentiated but male copulatory behavior is not. This may be related to the fact that females are homogametic in mammals (XX) while males are homogametic in birds (ZZ). Taking this taxonomic difference in the mechanism of sex control into account leads to a unifying concept: the behavior typical of the homogametic sex is sexually differentiated in both birds and mammals while the behavior typical of the heterogametic sex is not [27–29]. This issue will be reconsidered later.

It is clear that, in quail, the sexual dimorphism in copulatory behavior primarily results from early exposure to a different hormonal milieu. During the seventies, experiments carried out mostly in the laboratory of Dr E. Adkins-Regan demonstrated that the injection of estrogens to male embryos before hatching (injections into the egg during incubation) produces adult males with a female phenotype: they fail to mount and perform cloacal contact movements after injection of behaviorally effective doses of testosterone, i.e. they are demasculinized [30–32]. These experiments also demonstrated that demasculinization of male quail by exogenous estrogens was restricted to a critical period of embryonic life. Estradiol benzoate (EB) injections into male embryos demasculinized copulatory behavior only if they were performed before the 12th day of incubation: delayed injections were without effect [32–34] (see [35] for a more detailed discussion of the critical period in quail).

These very robust experimental findings suggested a model of sexual differentiation for the quail in which the hormone (estradiol) secreted by the heterogametic sex (females) would be responsible for the process of differentiation: the absence of male-type sexual behavior in adult females would result from their early exposure to endogenous estrogens, a process that would be experimentally reproduced in males by the injection of exogenous estradiol [30, 32]. It must be noted, however, that until recently this model of differentiation was based on indirect evidence only. It was clearly established that injections of estrogens in male embryos demasculinize their copulatory behavior (see above) but this effect could have been of a pharmacological nature. There was almost no direct evidence demonstrating that the same mechanism was responsible for the physiological demasculinization of females presumably induced by endogenous estrogens (see, however, [36]).

As a first step toward the physiological validation of this model, we measured by a very sensitive radioimmunoassay the concentration of estradiol and of three other steroids in the plasma of developing quail embryos of both sexes [37]. This showed that the estradiol levels are indeed much higher in female than in male quail embryos during the entire period during which estrogens are supposed to exert their organizing action based on experiments administering exogenous steroids to males, i.e. between the 9th day of incubation and hatching. These data demonstrating that the endocrine stimulus, which is supposed to differentiate the behavior, is in fact present in the right sex at the right time brought additional support for the differentiation model originally proposed by Adkins [30, 32].

It was also supported by more direct causal experiments that were recently carried out with the help of a new very potent aromatase inhibitor [38, 39] with great specificity. Aromatase inhibitors are very useful and specific tools for the physiological study of estrogen-dependent events: they suppress the synthesis of the steroid in the gonads and in any other potential sources so that it becomes possible to study the behavior of subjects in the absence of estrogen. The triazole derivative, R76713 (6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1-methyl-1H-benzotriazole) had previously been used to block testosterone-induced copulatory behavior and preoptic aromatase activity in castrated male quail [40]. The same compound was used here to suppress estrogen production in quail embryos and study the behavioral long-term effects of this treatment [41].

In a series of 4 experiments, embryos were injected with doses of R76713 in the range 10–50 μg at various ages between day 6 and 15 of incubation. Controls were injected in parallel with the vehicle solution. All birds were then gonadectomized at the age of 3–4 weeks post-hatch. When adult, the subjects received subcutaneous Silastic implants filled with testosterone, a hormonal treatment sufficient to restore sexual behavior in castrated males, and the effects of this steroid on male copulatory behavior were quantified during standard presentations to sexually mature females [41].

In one of these experiments, illustrated in Fig. 1, embryos were injected on day 9 of incubation with either 10 μg R76713 (15 males and 15 females), or with 25 μg EB (13 males and 5 females), or with both compounds simultaneously (R76713 + EB; 4 males and 4 females) or with the solvents as control (C; 8 males and 18 females). The numbers of birds indicated here correspond to subjects that completed the entire experiment; a larger number of eggs ($n = 245$ in total) was actually treated. The behavior of these subjects confirmed a number of established facts concerning the differentiation of sexual behavior in quail. Copulatory behavior was present in control males but not in control females and it was almost completely abolished in males by an embryonic treatment with EB. Interestingly this behavior was present in females exposed in egg to the aromatase inhibitor that had presumably suppressed the endogenous secretion of estrogens. This interpretation received additional support from the fact that

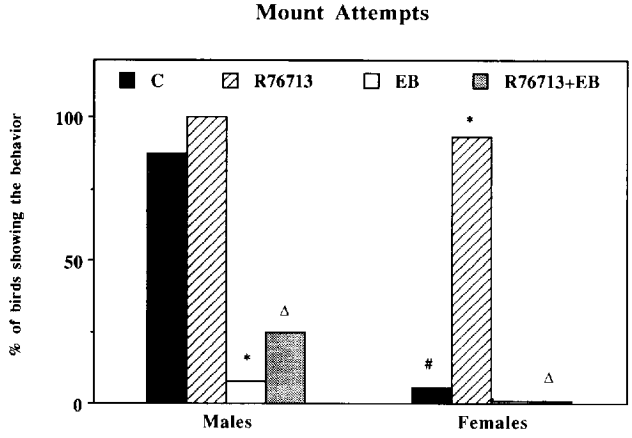

Fig. 1. Effects of the aromatase inhibitor R76713, of estradiol benzoate (EB), of the combined treatment with R76713 and EB and of the control (C) solvent injections on sexual differentiation of masculine sexual behavior in quail. The figure shows the percentage of birds (males and females) that performed at least one mount attempt during the behavioral tests performed in standard conditions in a test arena. All birds were gonadectomized and treated with exogenous testosterone at the time of behavioral testing. The percentages of active birds were analyzed by the Fisher exact probability test and are reported at the top of the columns as follows: $^{*}P < 0.05$ compared to the control (C) birds of the same sex, $^{\#}P < 0.05$ compared to males submitted to the same treatment, $^{\Delta}P < 0.05$ compared to birds treated with R76713 alone.

when R76713-treated females were, in addition, injected with exogenous estrogens (R76713 + EB group), they behaved like control females and they did not show any masculine copulatory behavior. The fact that the embryonic treatment with R76713 promoted copulatory behavior in females by blocking the secretion of their endogenous estrogens was also in agreement with the results observed in males: this compound had no effect on the development of their behavior.

Additional experiments revealed that injections of R76713 during the early phases of incubation (day 6 or 9) blocked female demasculinization to the same extent but that injections performed in the late phase of the incubation (day 12 or 15) only maintained a weak or no copulatory behavior in females. Males were unaffected by all treatments. This confirmed that behavioral demasculinization in quail takes place mainly, though not exclusively, during the early stages of ontogeny.

In a last experiment, we combined an early R76713 treatment (10 μg on day 9) with an injection of EB (25 μg) either on day 9 or on day 14 of incubation. Control birds were injected with the oil vehicle. This experiment showed that the sensitivity to the differentiating effects of estrogens varies with age in a sexually differentiated manner. As shown previously, EB injection on day 9 demasculinized both male and female embryos but if this injection was delayed until day 14, it was no longer effective in males but still caused a partial demasculinization of females (see [41] for more detail).

Taken together, these studies fully confirm the model originally proposed by Adkins which proposes that masculine sexual behavior is lost in female quail during embryonic life under the influence of estrogens. This was suggested by the effect of exogenous estrogens in male embryos but this model received here a direct confirmation by the demonstration that copulatory behavior is present in females in which estrogen synthesis has been blocked during embryonic life. These experiments also show that estrogens are presumably the only hormonal stimulus responsible for behavioral demasculinization of females since blockade of their synthesis results in adult females which have a behavioral phenotype indistinguishable from that of normal males. These data therefore show conclusively that the differentiation of sexual behavior takes place in quail under the influence of steroids secreted by the female. This is, at one level, a pattern of differentiation that could appear opposite to the pattern observed in mammals in which steroids secreted by the male induced differentiation. It must be reminded, however, that the heterogametic sex is the male in mammals but the female in birds. When this is taken into account, a single rule that is applicable to both birds (quail at least) and mammals can then be formulated to describe the mechanism of sexual differentiation as well as the type of behavior that is sexually differentiated (see above).

BRAIN DIFFERENCES IN QUAIL

The available data demonstrate that the sex difference in the copulatory behavior of quail is caused by a sex difference in the sensitivity of the brain to the activating effects of testosterone. Female quail show a form of androgen-insensitivity as illustrated by two facts. First, circulating levels of androgens are only slightly lower in female than in male quail [26, 42, 43] so that plasma testosterone levels should be adequate to activate male behavior in some females at least. Second, treatment of ovariectomized females with doses of testosterone ten times higher than those required to activate sexual behavior in males still does not induce male-type sexual behavior [24, 25, 43]. It has also been demonstrated that Silastic implants filled with testosterone produce similar levels of circulating steroid in gonadectomized males and females so that the differential response to the hormone treatment cannot be attributed to a differentiated peripheral catabolism of the hormone [43, 44]. These data therefore indicate that the behavioral dimorphism in quail is not only a consequence of the different adult endocrine milieu but rather depends on differential properties of the brain areas involved in the control of behavior.

A large number of studies have been carried out in an attempt to identify the sexually dimorphic brain

characteristics that could be mediating (causing) the behavioral dimorphism in copulatory behavior. A large number of morphological and neurochemical differences between the brain of adult males and females have been identified. They, namely, concern the overall volume of the medial preoptic nucleus, POM [45, 46]; the size of the neurons located in the dorso-lateral part of this nucleus [47]; the enzymatic activity of the preoptic aromatase, an enzyme that converts testosterone into its behaviorally active metabolite, estradiol [44, 48]; the number of aromatase-immunoreactive cells located in the preoptic area [49]; the number of cells containing the peptides neurotensin or LHRH in the preoptic area-anterior hypothalamus [50, 51]; and the noradrenergic and vasotocinergic innervation of the preoptic area [52, 53]. Unfortunately, in most cases, these sex differences disappear when the adult birds are placed in similar endocrine conditions (i.e. are gonadectomized and eventually treated with a same amount of sex steroids) so that the differences only reflect a differential activation by steroids in adulthood. They have presumably not been organized by the embryonic estrogens and therefore cannot explain alone the organized sex difference in copulatory behavior. In many other cases, the endocrine control of the sex differences seen in sexually mature birds has not been investigated.

A small number of brain differences have nevertheless been discovered that remain present even when the adult levels of steroids are controlled for (e.g. the neuronal size in the dorso-lateral part of the POM [47] or the preoptic aromatase activity [44]). There is, however, at this time no direct proof that these differences are the result of a differential exposure to estrogens of males and females during the embryonic life. Their precise implication in the control of the behavioral sex dimorphism is also unknown but it is important to notice that these two brain differences that are presumably of an organizational nature are closely related to the mechanisms that control copulatory behavior. The preoptic aromatase that produces estradiol, the active metabolite of testosterone, is known to be a limiting step in the activation of copulatory behavior [54, 55] and the POM that contains larger neurons in males than in females is a sufficient and necessary site of testosterone action in the brain for the activation of male copulation [56, 57]. Current research is devoted to the analysis of the specific relationships of these brain dimorphisms to the control of the sexually differentiated male sexual behavior.

SEX DIFFERENCES IN THE BRAIN AND BEHAVIOR OF ZEBRA FINCHES

By contrast, in the zebra finch (*Taeniopyga guttata*) brain, the only other avian species for which a large amount of experimental data on sexual differentiation is available, major anatomical sex differences have been identified in the brain and their relationship to sexually dimorphic singing behavior have been clearly established. This scientific material has been reviewed many times in recent years and the interested reader can easily find more information on this topic [12, 15, 17, 29, 58–60]. Suffice to say here that in this species, singing behavior is observed in males but never in females even if these are exposed to high levels of testosterone in adulthood. In parallel, a set of interconnected nuclei has been identified in the brainstem (nXIIts and DM) and in the telencephalon (HVC, RA and MAN) of these birds and this nervous circuit (the "song system") controls the learning during ontogeny and production during adulthood of songs. Several nuclei of the song system (e.g. HVC and RA) show an extreme sexual dimorphism and are 4–6 times more voluminous in males than in females [61]. This dimorphism which by and large cannot be manipulated by adult hormone treatments and therefore is presumably of organizational origin correlates nicely with the complete absence of song in adult females. Copulatory behavior is also dimorphic in zebra finches (activated by testosterone in males but not in females [29, 62, 63]) but little or no work has been devoted so far to the identification of the brain mechanisms underlying this behavioral dimorphic trait.

Active research has been going on for the past 15 years in an attempt to identify the endocrine stimuli that induce the sexual differentiation of singing behavior and of the song control system during ontogeny. If a number of reproducible facts have been identified, they fail, however, to produce a consistent pattern that could be integrated in a coherent physiologically based model.

Based on studies carried out mainly on Japanese quail, it has been argued for many years that in birds, males are the "neutral" sex, i.e. the sex whose sex-typical behavior develops in the absence of early steroid hormone influences (see above). This view has been complicated by studies on zebra finch. The sexual differentiation of the song control circuit does not seem to follow the model derived from the work on gallinaceous species: the administration of 17β-estradiol or testosterone to female nestling zebra finches masculinizes many aspects of the song circuit and the capacity for song [64–70]. However, like in quail, a similar treatment demasculinizes reproductive behaviors such as mounting and cloacal contact movements in males [62]. Early estrogen action seems therefore to have two potentially incompatible effects in zebra finches: demasculinization of mounting behavior in males and masculinization of song in females. Since a male zebra finch normally sings and copulates while the female does not do either of these behaviors, it is difficult to understand how the same endocrine environment can lead to these opposite effects on two groups of male-typical behaviors [29, 71].

All attempts to resolve this paradox have been unsuccessful so far. For example, manipulations of the circulating levels of estrogens in nestling zebra finches by gonadectomy and/or injection of exogenous hormones do not identify different sensitive periods for the sexual differentiation of song and mounting so that it is impossible to argue that estrogens masculinize song (and the song system) at one age and demasculinize copulation at another age during ontogeny [62, 63, 72]. In addition, the early gonadectomy of zebra finch nestlings did not alter sexual differentiation [63] perhaps because gonadectomy in nestling songbirds does not fully remove circulating levels of gonadal sex steroid hormones or the gonadectomy was not performed early enough in development to fully remove all steroid hormones [71, 73]. On another hand, the administration of different types of anti-estrogens to nesting zebra finches, paradoxically results in a hypermasculinization of the song system in males [74, 75] presumably because tamoxifen does not block the action of estrogen in the zebra finch brain but rather acts as an estrogen [76, 77]. Finally, analysis of the circulating levels of steroids during the zebra finch ontogeny has failed to identify sex differences in plasma steroids that could potentially explain the process of sexual differentiation: higher levels of estradiol had originally been detected in males from day 3 to day 10 after hatching [78] but subsequent studies by two different laboratories failed to confirm this finding [79, 80].

One experimental approach that would help resolve this problem is to study neural and behavioral development in an hormonal zebra finches (specifically estrogen-free zebra finches). Earlier attempts to reach this goal were largely unsuccessful (see above). We had demonstrated that the new non-steroidal aromatase inhibitor, R76713, was able to completely block estrogen-induced female demasculinization in female quail embryos suggesting that injection of this compound had indeed produced an estrogen-free embryo [41]. Therefore, we decide to use this compound to analyze the sexual differentiation of nestling zebra finches growing in the (relative) absence of endogenous estrogens [81,82].

Zebra finches received at 2–3 days post-hatch one Silastic implant filled with R76713 (racemic vorozole™) or left empty as control. Implants were left in place until the age of about 45 days and birds were then gonadectomized. At the age of 105 days, all these birds received a Silastic implant filled with testosterone. A final group of intact untreated birds was also used as additional control in this experiment. Starting 2 weeks after the implantation of testosterone, all birds were repeatedly tested for singing and copulatory behavior. Behavioral tests were carried out over a 3 week period. All subjects were then perfused and their brain was analyzed by histological methods to establish the volume of the song control nuclei, HVC, RA, area X and MAN. Treatment with R76713 significantly decreased ($\pm 50\%$) the number of song bouts produced by male birds but did not affect their copulatory behavior (see Fig. 2). However, the restoration by testosterone of copulatory behavior in castrated males was only partial and the frequency of this behavior did not reach the level seen in gonadally intact sexually mature birds. Additional studies should therefore be performed in which a better replacement therapy would be provided. The song quality of the males was similar in the 3 groups of subjects. The behavior of females was also not changed by the aromatase inhibitor and, in particular, mounting behavior and singing were never seen in the R76713 treated and in the control females (empty or intact groups). The presence of a strong sex dimorphism in the size of all 4 song control nuclei was confirmed but no significant effect of R76713 on these measures could be detected.

These data are therefore consistent with the idea that estrogens are implicated in the differentiation of singing behavior in the zebra finch but the small amplitude of the behavioral effect observed and the absence of morphological alteration of the song control nuclei suggest that either other factors also play a major role or that higher doses or longer treatments with the aromatase blocker should be tested. We have independent evidence that R76713 is acting in the avian brain with the mode of administration and at the doses that were used here (see [82] for further discussion). It must also be stressed that recent studies from another laboratory using a very similar non-steroidal aromatase inhibitor, fadrozole™, have also failed to affect sexual differentiation of the song control nuclei in zebra finches [83]. Because this failure was observed in conditions where aromatase activity is decreased by at least 80% [84], these studies taken together seriously question the role played by estrogens in the differentiation of the brain and behavior of zebra finches.

CONCLUSION

Research carried out during the last decade has led to a detailed understanding of how reproductive behavior becomes sexually differentiated in quail. It has also been established that the behavioral dimorphism observed in adulthood reflects brain differences in the sensitivity to testosterone but the specific mechanisms responsible for this differential responsiveness have not been identified yet. Major progress has, however, been made in the understanding of the central mechanisms that mediate copulatory behavior in this species. By contrast, the central control of the sex difference in singing behavior is fairly well understood in zebra finches but the mechanisms that control the ontogeny of this sex difference are poorly understood. Although it is clearly established that injection of exogenous estrogen at an early age masculinizes the song control nuclei and the vocal

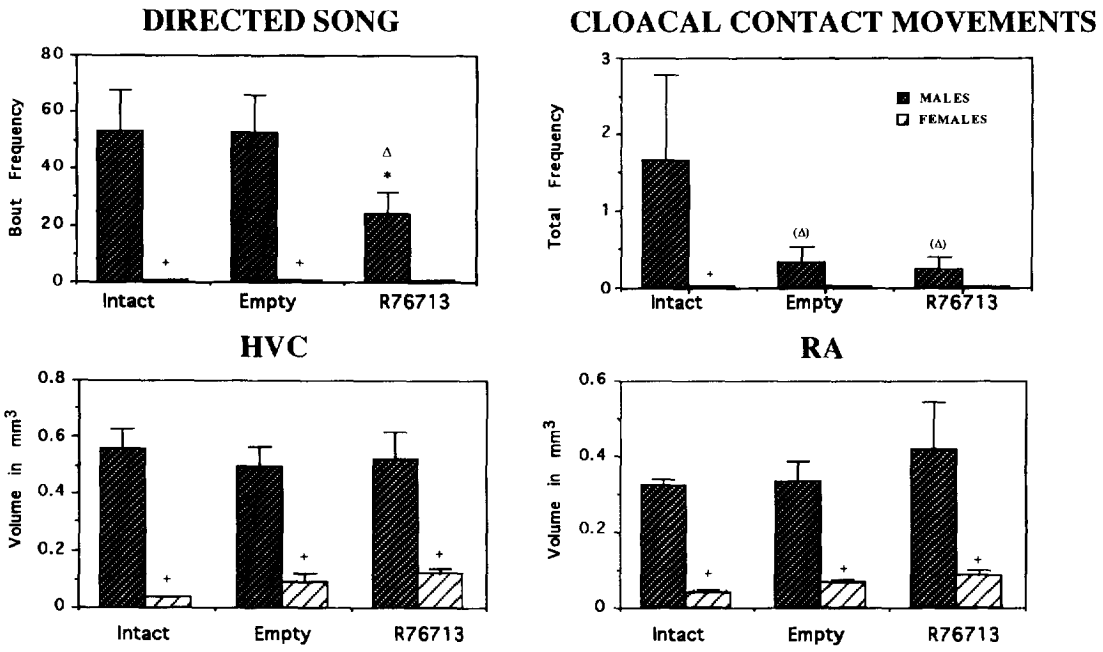

Fig. 2. Frequency of song bouts and of copulatory acts and volume of HVC and RA in male and female zebra finches treated as nestlings with Silastic implants filled with the aromatase inhibitor R76713 or left empty as control. A third group of subjects was submitted to no hormonal manipulation (intact group). All birds in the empty and R76713 groups were gonadectomized and submitted to a standard replacement therapy with testosterone in order to avoid any complication due to a differential activation by steroids in adulthood. All data plotted in the figure are means ± standard error. Data were analyzed by one way analyses of variance (ANOVA) followed by *post hoc* comparisons with the Fisher protected least significant difference (PLSD) tests. These results are reported at the top of the columns as follows: $^{*}P < 0.05$ compared to empty birds of the same sex; $^{\Delta}P < 0.05$ compared to intact birds of the same sex; $^{+}P < 0.05$ compared to males submitted to the same treatment. Parentheses around these symbols indicate that the significant PLSD test follows a non-significant ANOVA and is therefore only indicative.

behavior of females, the same treatment demasculinizes the copulatory behavior of males. It is therefore impossible to understand how a behaviorally competent male that sings and copulates can develop given these constraints. There is today no evidence to support the notion that estradiol acts in physiological conditions during the normal ontogeny of males. The present data suggest that this hormone may play a role in the differentiation of singing behavior but the modest amplitude of the effect observed in our study of birds treated with the aromatase inhibitor [82] and the lack of effect observed by Wade and Arnold [83] suggest that this role may be a minor one.

Alternative mechanisms may have to be considered. These include the action of other hormones such as androgens or of other non-steroid hormones (e.g. thyroxin) or even a direct influence of genetic information. A number of studies have shown that some features of the vertebrate brain can become sexually differentiated in the absence of gonadal steroids. For example, morphological characteristics of dopaminergic or prolactin neurons become sexually dimorphic even if these neurons are placed in culture before the onset of gonadal steroids secretion which argues for a genetically programmed differentiation [3–5]. Given all the difficulties that have so far been encountered in trying to explain the differentiation of the song system based on the idea that gonadal steroids irreversibly organize the brain, such non-conventional processes should perhaps be considered.

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