Reçu le 14 janvier 1989.

Correlation between the sexually dimorphic aromatase of the preoptic area and sexual behavior in quail : effects of neonatal manipulations of the hormonal milieu

BY

J. BALTHAZART

(Laboratory of General and Comparative Biochemistry, University of Liège, Belgium)

(4 figure)

The aromatase of the preoptic area is significantly more active in males than in females. This sex dimorphism in enzyme activity is still found in birds that have been gonadectomized and treated with a same dose of testosterone. This suggests that the sex difference is not the result of a differential activation by the adult hormonal environment but rather is organized neonatally by steroid hormones. As the central aromatization of testosterone is a limiting step in the activation of copulatory behavior by testosterone, the lower aromatase activity in the preoptic area of females might be responsible, at least in part, for their lower sensitivity to the activating effects of testosterone on behavior. Three experiments were carried out to determine whether early manipulations of the hormonal environment, which are known to differentiate sexual behavior, also affect in a permanent way the aromatase activity in the preoptic area. Injection of estradiol benzoate into male embryos on day 9 of incubation decreased the preoptic aromatase activity in parallel to its demasculinizing effect on behavior. Unexpectedly the same treatment tended to increase enzyme activity in females so that the physiological relevance of the observed enzymatic change remains questionable. In two independent experiments, we confirmed that neonatal ovariectomy of female quail interferes with their behavioral differentiation. Females gonadectomized at 4 days post-hatch showed significantly more male-type sexual behavior as adult in response to testosterone than females gonadectomized at the age of 5 weeks. These experiments also confirmed that the preoptic aromatase activity is higher in males than in females but no evidence for an effect of the age of gonadectomy on the enzyme activity could be obtained. The sex difference and experimental modifications observed in the aromatase activity of the preoptic area were not seen in the posterior hypothalamus demonstrating that these effects are specific. The mechanisms controlling the sex difference in aromatase activity are discussed. The difference might be organized by the action of embryonic steroids as suggested by the changes observed in males injected with estradiol benzoate in egg. Alternatively, activational mechanisms cannot be ruled out at present. In one experiment, the activity of the preoptic aromatase was positively correlated with the sexual activity of the birds. In addition, the enzyme activity was found to be numerically (although not statistically)

higher in males that had been exposed to females than in birds kept in social isolation. The differential interaction with the stimulus females should be studied in detail as this might be the cause rather than the consequence of the enzymatic dimorphism.

Introduction

We demonstrated recently that the aromatase activity of the preoptic area is significantly higher in male than in female Japanese quail (SCHUMACHER & BALTHAZART, 1984*b*). The activity of this enzyme is strongly controlled by testosterone (T). It decreases after castration and returns to the levels typical of adult sexually mature males within a few days after the implantation of silastic capsules filled with T (SCHUMACHER & BALTHAZART, 1986). Interestingly, the sex difference in preoptic aromatase activity was still observed in birds which had been gonadectomized and treated with a same dose of T (SCHUMACHER & BALTHAZART, 1986) suggesting that it may represent a sexually differentiated brain characteristic organized in a permanent way by neonatal steroids.

The aromatase is a key enzyme in the control of male reproductive behavior in quail as in many species of birds and mammals. In castrated quail, copulatory behavior can be activated by aromatizable but not by non aromatizable androgens (ADKINS, 1977; ADKINS & PNIEWSKI, 1978; ADKINS *et al.*, 1980; SCHUMACHER & BALTHAZART, 1983). In addition the behavioral effects of T can be blocked by the stimultaneous administration of antiestrogens (ADKINS & NOCK, 1976; ALEXANDRE & BALTHAZART, 1986) or of aromatase inhibitors (ADKINS *et al.*, 1980; BALTHAZART *et al.*, 1988; BALTHAZART *et al.*, 1989). It is thus clear that the aromatization of T is required in order to activate male behavior even if the exact role played by the estrogens and their interaction with androgenic steroids remains somehow unclear (SÖDERSTEN & GUSTAFSSON, 1980; SÖDERSTEN *et al.*, 1986; BALTHAZART, 1989). The studies of the aromatase itself also support the notion that this enzyme controls the male reproductive behavior. The highest levels of activity are found within the medial preoptic nucleus of the preoptic area (SCHUMACHER & BALTHAZART, 1987) which is a critical nucleus in the activation of copulation as demonstrated recently by stereotaxic implantation of T (BALTHAZART *et al.*, 1988; WATSON & ADKINS-REGAN, 1989). The induction by T of the enzyme activity is dose- and time-dependent and parallels the activation of the behavior (BALTHAZART *et al.*, 1986; BALTHAZART *et al.*, 1989). Finally the sex difference in preoptic aromatase also correlates well with the behavior which is know to be stimulated by T in males but not in females (ADKINS & ADLER, 1972; ADKINS, 1975; BALTHAZART *et al.*, 1983). Thus, the differential induction of the aromatase activity in males and females may be part of the mechanism responsible for the behavioral sex difference. This raises the questions of how the sex difference in enzyme activity becomes established and whether there is a similarity between the hormonal mechanisms which sexually differentiate the preoptic aromatase activity and those which differentiate the copulatory behavior.

Two types of manipulations of the early hormonal milieu have been shown to affect permanently the copulatory behavior in quail. Injections of estradiol into male eggs completely abolish the capacity of the adults to show copulatory behavior in response to T treatment. They are demasculinized (ADKINS, 1979; ADKINS-REGAN *et al.*, 1982; SCHUMACHER *et al.*, 1989). This type of treatment is only effective if applied before day 12 of incubation suggesting that the sexual differentiation of behavior is completed at that age (ADKINS, 1979; ADKINS-REGAN, 1983). According to

this type of experiment, the female quail would be demasculinized before day 12 by their endogenous estrogens which are known to be present in higher concentration in the plasma of females than in that of males at this stage of development (SCHUMACHER *et al.*, 1988). However, it was also demonstrated that if females are ovariectomized just after hatching, they are still capable of showing male-type sexual behavior as adult if treated with T (HUTCHISON, 1978; BALTHAZART & SCHUMACHER, 1984; SCHUMACHER & BALTHAZART, 1984a). This finding suggests that sexual differentiation may not be completed on day 12 of incubation and is still in progress during the first two weeks after hatching.

The possible ways in which this apparent discrepancy could be resolved have been discussed elsewhere (SCHUMACHER & BALTHAZART, 1985; BALTHAZART & SCHUMACHER, 1987; SCHUMACHER *et al.*, 1989). The aim of the present study was to research whether these early manipulations of the steroid environment, which are known to modify irreversibly the behavior, also affect in a permanent way the activity of the aromatase in the preoptic area. During three independent experiments, birds of both sexes were thus submitted to experimental manipulations of the early hormonal milieu. When adult, they were treated with T and their behavior as well as the activity of their aromatase in the preoptic area and in the posterior hypothalamus were quantified.

Material and Methods

a) Animals

The experiments reported here were carried out on male and female Japanese quail *(Coturnix coturnix japonica)* which were bought from a local breeder (Lefèvre, Boneffe-Eghezée) at the age of 3 to 4 weeks or on birds hatched from eggs which were obtained from the same breeder and incubated in the laboratory. All birds were gonadectomized or sham-operated either within 4-5 days after hatching or at about 4 weeks of age under general anesthesia (Hypnodil, *Janssen Pharmaceutica,* Beerse Belgium; 15 mg/kg body weight for older birds, 5 mg/kg for neonates) using procedures which were previously described (SCHUMACHER & BALTHAZART, 1984*a*). Both testes were removed through an unilateral incision on the left side. Only the left ovary of females was taken away. The right one is not developed and does not regenerate even after removal of the left gonad (GIBSON *et al.*, 1975). At the age of about 6 weeks (close to sexual maturity), birds were transferred from the cages in which they were kept in mixed-sex groups to individual cages where they stayed until the end of the experiments. Birds were exposed throughout their life to a photoperiod simulating long days (16L : 8D) and received food and water *ad libitum.*

Immediately before and on several occasions during the experiments, birds were weighed to the nearest gram and the size of their cloacal gland, an androgen-dependent structure (SACHS, 1967) was measured with a caliper (greatest width X greatest length in mm^2 = cloacal gland area). No effect of the experimental treatments on body weight (individual range from 200 to 260 g) was detected in the experiments. The females were however systematically heavier than the males by about 20-30 gr. No further mention of these results will thus be made in text.

b) Hormonal treatments

The activation of sexual behavior in castrated birds was obtained by the implantation under the skin in the neck region of 60 mm (3 X 20 mm) silastic capsules (*Dow*

Corning silastic tubing number 602-252; 1.57 mm i.d.; 2.4 1 mm o.d.; *Dow Corning Co,* Midland, MI, USA) filled with crystalline T (*Sigma* T-1500). Before implantation, capsules were preincubated for about 24 h in isotonic saline solution in order to initiate steroid diffusion through the tube walls.

c) Sexual behavior

On several occasions at the end of the experiments, the copulatory behavior of the quail was observed and quantified during standard presentations to adult egg-laying females. Tests were performed either in an experimental arena (60 × 40 × 50 cm) or in the home cage of the birds. For the tests in the arena, a stimulus female was first introduced into the arena and 30 s later, the experimental male was added and its behavior was observed for 5 min. In the home cage situation, the female was introduced into the residency cage of the bird to be tested and the behavior was immediately recorded for the next 5 min. The frequency of the following behavior patterns were recorded : neck grab (NG), mount attempt (MA), mount (M) and cloacal contact movement (CCM).

d) Killing

At the end of the experiments, birds were killed by decapitation. The completeness of gonadectomy and the presence of the implants were checked. Data relative to birds showing gonadal remnants or birds which had lost some of their implants were discarded before analysis. The number of animals given in the result section is always relative to those which were included in the final analyses. Brains were immediately dissected out of the skull and frozen on powdered dry ice. They were stored at −70°C until assayed for aromatase activity.

e) Aromatase assay

The activity of the aromatase was measured in the preoptic area (POA) and in the posterior hypothalamus (PH) using an *in vitro* radioenzyme assay previously described and validated for quail (SCHUMACHER *et al.,* 1984; SCHUMACHER & BALTHAZART, 1986). The brain were dissected by a free-hand procedure which has been previously described (SCHUMACHER *et al.,* 1983). They were homogenized in 200 (part 3) or 400 (parts 1 and 2) µl ice-old STMM buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 at 20°C, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol) using either glass micro-homogenizer tubes (part 3) or a polytron (part 1 and 2). Parallel experiments demonstrated that higher levels of enzyme activity were measured when brain samples were homogenized with the glass homogenizers and this justifies the change from one technique to the other for the third experiment. The homogenates were immediately snap-frozen in an acetone-dry ice bath. For the assay, an aliquot (100 or 200 µl) of the homogenates was incubated in presence of (1,2-α) ^{3}H-testosterone (*Amersham,* U.K.; specific activity 60 Ci/mmole) at a final concentration of 20 to 25 nM depending on the experiment. At the start of the incubation, 100 µl of STMM containing $NADPH_2$ were added to the tubes to obtain a final concentration of 1.2 mM of this cofactor (= saturating levels). Tubes were then maintained at 41°C for 15 min under constant agitation. The radioactive metabolites produced were then extracted by diethylether, estrogens and androgens were separated by phenolic partition and estradiol was purified by thin layer chromatography on silicagel plates. This assay has been fully validated for the quail brain (SCHUMACHER & BALTHAZART, 1984b;

SCHUMACHER *et al.*, 1984; SCHUMACHER & BALTHAZART, 1986) and the identity of the estradiol after chromatography has been confirmed by recrystallization to constant isotopic ratio (SCHUMACHER *et al.*, 1984). The protein content of the homogenates was determined by the method of Bradford (BRADFORD, 1976) and the amounts of metabolites produced were expressed in femtomoles/mg protein/hour. The mean fresh weight of the samples varied from 4.8 to 5.6 mg according to the experiment and the mean protein content measured was in the range 6.9 to 7.3% of the fresh weight.

f) Statistical analysis.

All data were analyzed by analyses of variance (one-, two- or three way ANOVA) followed when appropriate by the Fisher Protected Least Significant Difference test (PLSD) for multiple comparisons of means. Percentage and cumulative percentages of behaviorally active birds were compared by the Fisher exact probability test. Effects were considered significant for $P<0.05$. All probabilities which are mentioned are two-tailed unless otherwise mentioned. All values in the figures are means ± standard errors.

Part one. Effects of embryonic treatment with estradiol benzoate

Previous experiments had demonstrated that the injection of estradiol benzoate (EB) into quail eggs totally suppresses the adult copulatory behavior of the males (ADKINS, 1975; ADKINS, 1979). This effect could only be obtained if the injection of estrogen was performed before day 12 of egg incubation (ADKINS, 1979). In the present experiment, we investigated whether the treatments, which demasculinize copulatory behavior, also differentiate the aromatase of the preoptic area. Several controls for the specificity of the effects were also performed and included the study of the effects of treatment with EB started too late (day 14 of incubation) to affect behavior. The anatomical specificity of the effects was evaluated by studying in parallel the aromatase of the posterior hypothalamus.

Procedure

Eggs were set in the incubator in the morning of day 0 (38°C and 50-60% of relative humidity). In these conditions, chicks hatch on day 17. On day 9 or on day 14 of incubation, eggs were injected with either 0, 5 or 25 μg of estradiol benzoate (EB; *Sigma* E-9000; 5 to 7 birds of each sex in each group, see fig. 1) as described previously (SCHUMACHER *et al.*, 1989). Control eggs (0 μg group) were injected with 50 μl of the vehicle alone (sesame oil). Twelve groups of birds were available in this way according to their sex, age at injection and dose of EB injected.

All chicks (males and females) were gonadectomized 4 days after hatching under general anaesthesia in order to decrease any possible effects of postnatal steroids. At the age of 4 weeks, birds were isolated in individual cages and were implanted 2 weeks later with 60 mm silastic implants filled with T. Birds were sacrificed 3 weeks after the beginning of the T-treatment. They were tested once in the arena and twice in their home-cages (1 test/day during the last 3 days of the experiment). Their cloacal gland was measured at the end of the experiment. Aromatase activity was measured in the POA and PH as described in the general method section.

Results

The sexual behavior of all these birds has already been described in detail elsewhere (SCHUMACHER *et al.*, 1989). The embryonic treatments with EB had the expected effects on copulatory behavior and T-induced cloacal gland growth. Cloacal movements were seen only in males during the test in the arena. Females never showed this behavior irrespective of their embryonic treatment. However, as a consequence of the early postnatal ovariectomy (SCHUMACHER & BALTHAZART, 1984*a*), they showed a weak sexual activity (almost exclusively mount attempts) when tested in their home cage (see SCHUMACHER *et al.*, 1989). EB injection (5 or 25 μg) on day 9 of incubation completely suppressed the adult copulatory behavior of males but had no effect on day 14. In parallel, EB injection decreased the cloacal gland area of both males and females when injected on day 9 but not on day 14 of incubation.

The mean levels of aromatase activity in the preoptic area (POA) and posterior hypothalamus (PH) measured in the 12 experimental groups are shown in Figure 1.

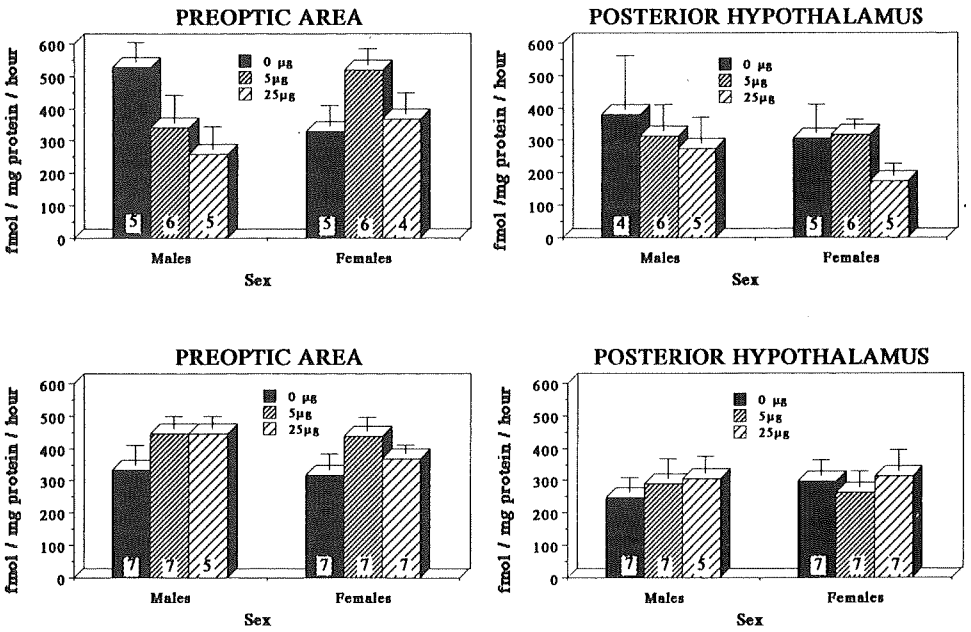

FIG. 1. *Effect of the embryonic injection of different doses (0,5 or 25 μg) of estradiol benzoate on the aromatase activity in the preoptic area and the posterior hypothalamus.* Embryos were injected on day 9 (top) or on day 14 (bottom) of the incubation. All birds were gonadectomized at 4 days post hatch and treated with silastic implants filled with testosterone when adults. Numbers of birds in each group are shown at the bottom of the bars.

The three way ANOVA (sex × age of injection x dose) of the data relative to the POA did not reveal any significant main effect but most of the interactions (second and third order) were very close to significance (*e.g.* sex × dose : $P = 0.054$; sex × age × dose : $P = 0.064$). Data were thus reanalyzed by two way ANOVA's (sex × dose) considering the injections on day 9 and day 14 as independent experiments.

On day 9, there was no overall effect of the sex of the birds or the dose on the level of aromatase activity in the POA but a significant interaction between these factor was detected ($F_{2,25} = 4.18$, $P = 0.027$). One experimental effect only reached significance in the PLSD test : aromatase activity was decreased by the treatment with 25μg EB in males. In the birds that had been injected on day 14, no significant effect of the sex, the dose or their interaction on the aromatase level could be detected although the dose effect came close to significance ($F_{2,34} = 2.83$, $P = 0.073$). The results of these analysis were confirmed by performing two way ANOVA's using now all data from males or females separately (factors were here day of injection and dose). In males there was a significant interaction between the injected dose and the day it was given ($F_{2,29} = 3.62$, $P = 0.039$) but no overall effect of the dose or the day. Two means only were significantly different again : males injected on day 9 with 25 μg EB had a lower aromatase than their controls (males-day 9-0 μg). In females, the two way ANOVA detected a significant effect of the injected dose ($F_{2,30} = 5.45$, $P = 0.009$) but no effect of the day nor of the interaction. The females which had been injected with 5μg EB on day 9 had in fact a higher aromatase that the females injected on day 9 with either 0 or 25 μg. The same numerical trends were visible on day 14 but were not significant. All these analyses (3 and 2 way ANOVAS) performed on the aromatase levels in the posterior hypothalamus yielded results that were very far from significant (all $P > 0.2$).

Contrary to our previously published findings (BALTHAZART *et al.*, 1986; SCHUMACHER & BALTHAZART, 1986), none of the ANOVA had detected differences in aromatase activity that were related to the sex of the birds. The treatments with EB could of course have interfered with the development of this sexual dimorphism in enzyme activity. The data from control birds not treated with EB (0 μg groups) were thus reanalyzed separately. In the pooled data of birds injected with 0μg EB on day 9 or 14, males had an aromatase activity in the POA which was numerically but not significantly higher than that of the females (413.9 ± 175.5 *vs* 320.8 ± 133.5, fmol/mg protein/hour, mean ± SD, $t = 1.46$, $df = 22$, $P = 0.157$). This trend was not visible in the posterior hypothalamus (296.7 ± 214.7 *vs* 302.3 ± 155.9, $t = 0.07$, $df = 21$, ns).

Part two : effects of neonatal castration and of sexual activity on the preoptic aromatase

The first experiment showed that the sexual dimorphism in aromatase activity was of a much smaller magnitude than previously reported (SCHUMACHER & BALTHAZART, 1986) and actually did no reach significance. The explanation of this fact was possibly to be researched in the fact that all birds had been gonadectomized within a few days after hatching to maximize the effects of the embryonic manipulations and avoid the confounding action of postnatal steroids. It was known however that neonatal gonadectomy of quail reduces the behavioral dimorphism in that neonatally ovariectomized females will show weak copulatory behavior (usually only mount attempts) if tested in appropriate conditions (home cage) while normal females ovariectomized after 4 weeks of age will very rarely do so (HUTCHISON, 1978; SCHUMACHER & BALTHAZART, 1984*a*). It was thus conceivable that the neonatal castration was also reducing the sex dimorphism in aromatase activity and this hypothesis was tested in the present experiment.

In addition it was not clear whether the higher aromatase of males compared to females was due directly to a differential response to T or resulted from a differential reaction of the stimulus females : in all previous experiments, birds had been

tested for copulatory behavior before being sacrificed for the measurement of aromatase activity and the behavioral sex difference (males showing, females not showing copulatory behavior) might have been the origin rather than a consequence of the difference in enzyme activity. In the present experiment, a group of males was thus treated with T but not tested for behavior before the aromatase activity was measured.

Procedure

Eggs were incubated in the laboratory as described above. One half of the chicks (males and females) were gonadectomized 4 days after hatching under general anaesthesia while the other half was submitted to the control sham-operation (anesthesia and opening of the abdominal cavity to expose the gonads; see fig. 2 for the numbers of birds in each group) as a control for the possible effect of this procedure. At the age of 5 weeks, all birds which had been sham-operated as neonates were gonadectomized while the neonatally gonadectomized birds now were submitted to the sham-operation. Two weeks later, they were isolated in individual cages and on the next day implanted with 60 mm (3 × 20 mm) silastic implants filled with T. Birds were sacrificed 4 weeks after the beginning of the treatment. They were tested for behavior twice in their home-cages respectively 3 and 2 days before the end of the experiment except for 6 males which had been castrated at 5 weeks of age and who were kept in isolation throughout the experiment in order to test for the effect of performing sexual behavior on aromatase activity. The cloacal glands were measured at the end of the experiment. Aromatase activity was measured in the POA and PH as described above.

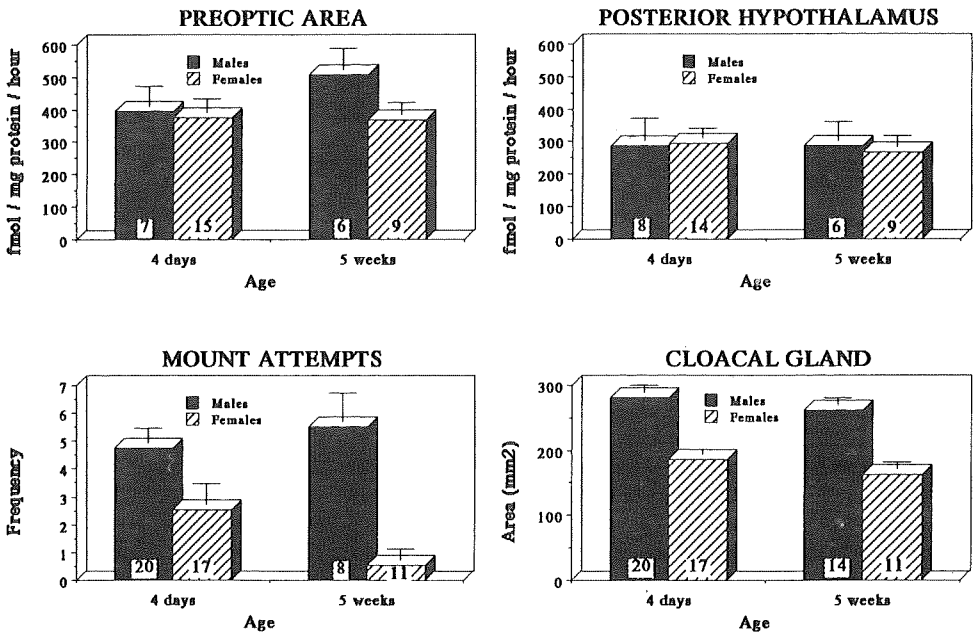

FIG. 2. *Effect of gonadectomy performed at 4 days or at 5 weeks post-hatch on the aromatase activity in the preoptic area and posterior hypothalamus, on the male sexual behavior (frequency of mount attempts during two 5 min tests in the home cage of the birds) and on the cloacal gland area of male and female quail (part 2).* All birds were treated with silastic implants filled with testosterone when adults. Numbers of birds in each group are shown at the bottom of the bars.

Results

As previously demonstrated (SCHUMACHER & BALTHAZART, 1984*a*), the neonatal castration did not change the sexual activity of the males but the early ovariectomy increased the proportion of females which showed weak behavior (mount attempts) when tested in their home cage. These effects are illustrated in Figure 2 by the mean frequency of mount attempt observed during the two tests.

Analysis of these frequencies by two way ANOVA (sex and age castration as factors) confirmed a strong effect of sex ($F_{1,52} = 23.24$, $P<0.0001$) and suggested the existence of an interaction between sex and age of castration ($F_{1,52} = 3.37$, $P=0.071$). The percentage of birds showing mount attempt at least once during the tests was also significantly different from group to group (males-4-day : 18/20; males-5-weeks : 8/8; females-4-day : 10/17; females-5-weeks : 2/11; chi square = 21.36, $P<0.0001$). In particular, the females showed this behavior more frequently if they had been ovariectomized at 4 day rather than at 5 weeks of age ($P=0.035$ by Fisher Exact Probability test one tailed as the direction of the change was predicted on the basis previous experiments).

As demonstrated earlier, the cloacal gland area was larger in males than in females (sex effect in the two-way ANOVA : $F_{1,58} = 79.91$, $P<0.0001$). It was numerically higher in birds castrated at 4 days than in those castrated at 5weeks but the effect did not reach significance ($F_{1,58} = 3.43$, $P = 0.068$). Fisher PLSD tests confirmed that this structure was significantly larger in males than in females irrespective of the age at gonadectomy.

Aromatase activity in the POA was about 20% higher in males that had been tested for sexual behavior than in their controls which had been kept in isolation (507 ± 162 *vs* 424 ± 74.82 fmol/mg protein/hour; mean $\pm$ SD) but this difference was not significant ($t=1.14$, $df = 10$, $P = 0.279$). There was no indication of such a difference in the PH (290 ± 101 *vs* 288 ± 141 fmol/mg protein/hour). However as the difference seen in POA could have been a confounding factor, data from isolated birds were not included in the general analysis of the aromatase activity.

The aromatase was generally more active in the POA than in the PH. In the POA, the enzyme also tended to be more active in males than in females. In the overall ANOVA, this effect was however not significant ($F_{1,33} = 2.20$, $P = 0.147$). The difference in enzyme activity between males and females was numerically larger in birds gonadectomized at 5 weeks than in those which had been gonadectomized at 4 days post-hatch but this difference was not large enough to obtain a significant effect of age ($F_{1,33} = 1.00$, $P = 0.32$) or of the interaction between age and sex ($F_{1,33} = 1.28$, $P = 0.26$) in the ANOVA. The sex difference was marginally significant ($P<0.147$, see above) and actually it could be shown that the distribution of aromatase activities was different between males and females. Within the whole group of birds included in this experiment, two males out of 19 had an aromatase activity lower than 325 fmol/mg protein/hour which was the lowest value observed in "normal" males (castrated at 5 weeks). By contrast, 11 out of 24 females had an aromatase activity below this value. Such a difference is significant by the Fisher Exact Probability test ($2P = 0.023$). There was no suggestion of a sex- or age of castration-related difference in the aromatase activity of the PH (all probabilities > 0.7).

Interestingly, there was also a clear relationship between the aromatase activity in the POA and the sexual activity of the birds during the tests (see fig. 3).

Overall, the birds which showed mount attempt at least once during the tests had an aromatase activity in the POA 40% higher than sexually inactive birds (453 ± 162 *vs* 320 ± 95 fmol/mg protein/hour, mean $\pm$ SD, $t = 2.85$, $df = 35$, $P = 0.007$).

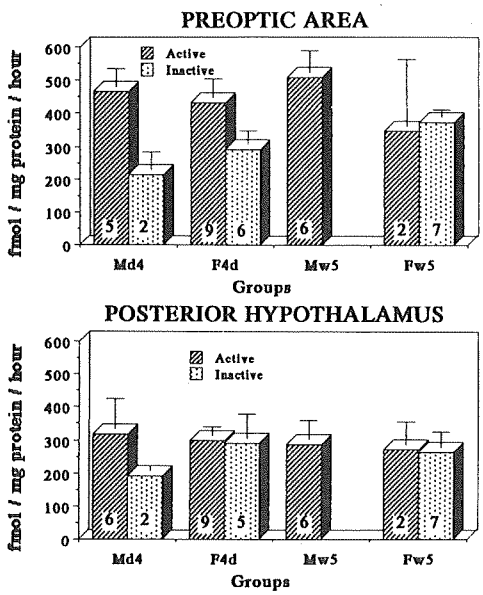

FIG. 3. *Relationships between the aromatase activity in the preoptic area or the posterior hypothalamus and the sexual behavior of male (M) and female (F) quail which were gonadectomized at 4 days (Md4, Fd4) or 5 weeks (Mw5, Fw5) post-hatch.* The figure compares the mean enzyme activity in birds which performed at least once (active) and in those which never performed (inactive) mount attempts during two 5 min tests in the bird's home cage. Numbers of birds falling in each category are shown at the bottom of the bars.

This enzymatic difference was not present in the PH (298 ± 138 *vs* 263 ± 164, $t = 0.76$, $df = 35$, ns). This general analysis confounded the differences between experimental groups with those related to the behavior of the birds. We could confirm however by a two-way ANOVA considering the behavior of the birds (showed or did not show mount attempt) as one factor and the 4 groups of birds as the other factor, that the enzymatic activity was significantly higher in sexually active than in inactive birds ($F_{1,36} = 5.09$, $P = 0.031$). The major contribution to this effect obviously came from the neonatally gonadectomized birds as can be seen on fig. 3 but it must be noted to proper data are actually lacking to evaluate this effect in birds castrated later in life (no inactive males and only two active females were present in the sample). Interactions between the two factors could thus not be tested statistically as data were completely missing in one cell of the general ANOVA table.

Part Three : Effects of neonatal castration on the preoptic aromatase

The previous experiment had confirmed an number of established facts such as the sex difference in copulatory behavior and in T-induced cloacal gland area as well as the effect of neonatal ovariectomy on sexual activity in females. It also suggested that the sex-related difference in preoptic aromatase activity might be larger in birds gonadectomized at 5 weeks of age than in birds gonadectomized right after hatching. However this effect could not be demonstrated clearly and in addition the sex-difference in preoptic aromatase was barely significant. It was thus decided to replicate the experiment with larger numbers of animals in order to test the reliability of the observed differences.

Procedure

Chicks hatched from eggs incubated in the laboratory were treated exactly as in experiment 2 with one half of the birds (males and females) being gonadectomized 4 days after hatching while the others were gonadectomized at the age of 5 weeks (see fig. 4 for the number of subjects in each group). Birds which were not gonadectomized at a given age were always submitted to the sham-operation. Three weeks later, they were isolated in individual cages and on the same day implanted with 60 mm (3 × 20 mm) silastic implants filled with T. Birds were sacrificed 3 weeks after the beginning of the treatment with T. All were tested for behavior twice in their home-cages respectively 4 and 1 days before the end of the experiment. Their cloacal gland was measured one day before the end of the experiment. Aromatase activity was then measured in the POA and PH.

Results

In this experiment again, the neonatal ovariectomy of the females increased their sexual activity (see FIG. 4).

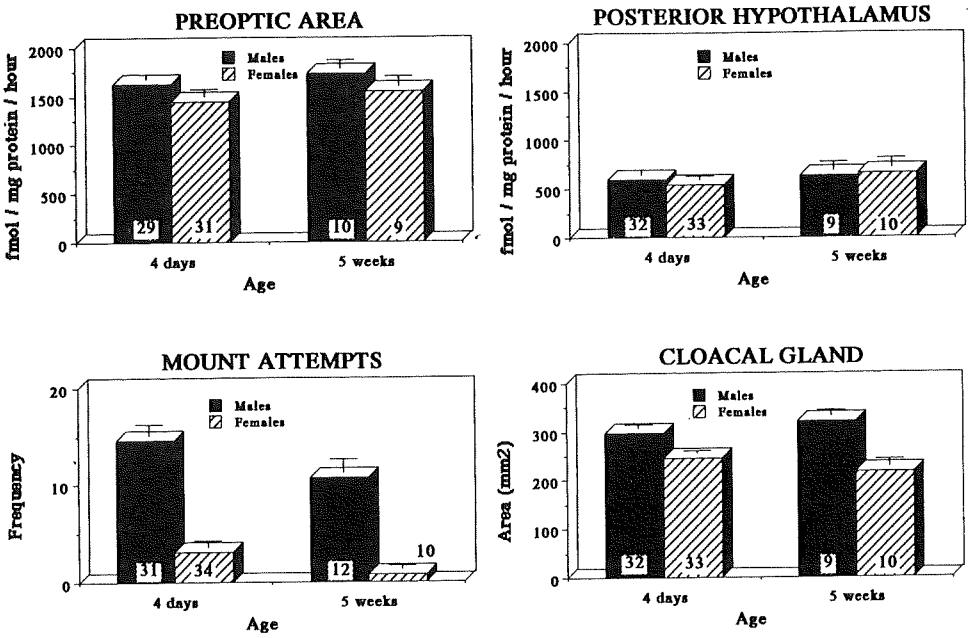

FIG. 4 *Effect of gonadectomy performed at 4 days or at 5 weeks post-hatch on the aromatase activity in the preoptic area and posterior hypothalamus, on the male sexual behavior (frequency of mount attempts during two 5 min tests in the home cage of the birds) and on the cloacal gland area of male and female quail (part 3).* All birds were treated with silastic implants filled with testosterone when adults. Numbers of birds in each group are shown at the bottom of the bars.

The analysis of the mount attempt frequencies by two-way ANOVA (sex and age at castration as factors) demonstrated a significant effect of both factors (sex $F_{1,83} = 76.16$, $P<0.0001$; age : $F_{1,83} = 6.30$, $P=0.014$) but no interaction between these

factors ($F_{1,83}$ = 0.35, ns). Indeed the mount attempt frequency was higher in birds gonadectomized at 4 days than in those gonadectomized at 5 weeks of age. This difference was significant in males and fell short of significance in females by the PLSD test. The percentage of active birds however gave a much clearer picture of the experimental effects. The proportion of birds showing mount attempt varied significantly from one group to the other (males-4-day : 31/31; males-5-weeks : 11/12; females-4-day : 24/34; females-5-weeks : 3/10; chi square = 25,59, $P<0.0001$). The neonatal ovariectomy in particular significantly increased the percentage of females showing the behavior (from 30 to 70%, P = 0.022 by the one tailed Fisher exact probability test) but both groups of females nevertheless remained significantly different from their male control group (2 $P<0.01$ in both cases by the Fisher test).

As reported many times in quail, the cloacal gland area was larger in males than in females and there was no overall effect of the age of castration on this variable (sex effect : $F_{1,80}$ = 48.74, $P<0.0001$; age effect : $F_{1,80}$ = 0.034, ns). There was in addition a significant interaction between these two factors ($F_{1,80}$ = 4.60, P = 0.035). The difference between males and females was larger in birds which had been gonadectomized later in life (significant in both cases by the PLSD test) but the size of the gland was not significantly different the two groups of females.

The preoptic aromatase activity was numerically higher in males than in females irrespective of the age at gonadectomy but this only resulted in a statistical tendency in the general two-way ANOVA ($F_{1,75}$ = 3.12, P = 0.081). There was no significant effect of the age at castration ($F_{1,75}$ = 1.10, P= 0.29) nor of the interaction between these factors ($F_{1,75}$ = 0.003, ns). Considering that the age of castration did not influence the enzyme activity, we decided to pool all the data for both sexes separately and reanalyze them. A student t-test first showed that the preoptic aromatase activity was indeed higher in males than in females (1649 ± 291 *vs* 1664 ± 291 *vs* 1464 ± 449 fmol/mg protein/hour, X ± SD, t = 2.16, df = 77, P = 0.034). The distribution of the individual data was also very different in males and in females. Considering in both sexes the number of data points which fell below or above 1400 fmol/mg protein which is the value just under the mode in the distribution of the male results, it could be shown that a larger proportion of the females had preoptic aromatase activity under this limit compared to males (females : 20 out of 40, males : 8 out of 39, 2 P = 0.009 by the Fisher test). All these statistical analyses were also applied to the aromatase activities in the PH and none of the difference were or even came close to significance.

Like in the previous experiment, the birds which were sexually active (showed mount attempt at least once during the test) had a higher aromatase activity in the POA than the birds which were inactive (1578 ± 390 *vs* 1455 ± 383 fmol/mg protein/hour) but this difference was not significant (t=1.12, df = 76, P = 0.263). This trend was not detectable in the PH (1471 ± 496 *vs* 1455 ± 383).

Discussion

These results confirm and extend a number of previously established facts on the reproductive processes in quail and their sexual dimorphism. We found here in three independent ways that T differentially activates sexual behavior in males and females. A same treatment with T also induced a much larger cloacal gland growth in males than in females. These facts had been observed previously (ADKINS, 1975; ADKINS & ADLER, 1972; BALTHAZART *et al.*, 1983; SCHUMACHER & BALTHAZART, 1984*a*) but they are confirmed here with very large numbers of experimental subjects. It was

known that these sexually dimorphic responses to T are permanently affected by manipulations of the hormonal milieu early in life (ADKINS, 1975; ADKINS, 1979; SCHUMACHER & BALTHAZART, 1984*a*; SCHUMACHER *et al.*, 1989) and this is also fully confirmed here. Treatment of male embryos with estradiol benzoate completely suppressed their capacity for showing copulatory behavior in response to T as adults (see SCHUMACHER *et al.*, 1989 for more detailed discussion of these results). In addition, results of parts 2 and 3 confirmed that if females are ovariectomized during the first week post-hatch, they retain the capacity to display weak male sexual behavior in response to T if tested as adult in an adequate environment. This effect was significant in each of the two experiment ($P = 0.031$ and 0.022 respectively by one tailed Fisher exact probability test) and it is extremely significant when the pooled data for the two experiments are analyzed (number of females showing mount attempt : 4-day-group : 34 out of 51, 5-week group : 5 out of 21, $P = 0.0008$ by the Fisher test). This confirms that the process of sexual differentiation in female quail is not completed before hatching (see SCHUMACHER & BALTHAZART, 1985; SCHUMACHER *et al.*, 1989 for discussion). The neonatal ovariectomy also slightly decreased the sexual dimorphism in the size of the cloacal gland. This effect did not reach full significance in any of the two experiments. This is in agreement with our previous data showing that gonadectomy abolishes the sex dimorphism in cloacal gland growth if performed on day one post-hatch but is no longer effective if performed one week after hatching. Considering that all birds were here ovariectomized on day 4 post-hatch, it is thus normal to observe effects at the edge of significance.

We had previously demonstrated that the preoptic aromatase activity is significantly higher in sexually active males than in egg-laying females (SCHUMACHER & BALTHAZART, 1984*b*) and that this sex difference is maintained in gonadectomized T-treated birds (SCHUMACHER & BALTHAZART, 1986). The enzyme activity was also greatly enhanced by T and was very low in both males and females after gonadectomy. This showed that the aromatase was differentially induced by T in males and females and more recent experiments demonstrated that this differential induction was observed over a wide range of doses (BALTHAZART *et al.*, 1986; BALTHAZART *et al.*, 1989). The magnitude of the sex difference in aromatase induction had been quite variable from experiment to experiment (females values were between 25 and 75% from those of the males). The present set of experiments thus addressed two questions : what is the real magnitude of the sex difference in the induction by T of the preoptic aromatase activity and how does it develop ? The results confirmed that the aromatase activity in the preoptic area is numerically higher in males than in females even when birds are exposed to the same hormonal conditions (gonadectomized and treated with a same dose of T). This sex difference in enzyme activity, even if it was seen in each experiment, was however of a small magnitude and did not always reach statistical significance. We had available three independent sets of data gathered from birds submitted to very similar treatments. The presence of a sex difference in aromatase activity was thus tested in the pooled data. These were first transformed in each case in percentage of the mean value observed in males in order to compensate for the differences in the absolute levels in enzyme activity which had been observed from one experiment to the other. This *post-hoc* analysis confirmed the higher aromatase activity in males compared to females ($100 \pm 27\%$ *vs* $88 \pm 33\%$, $t = 2.305$, $df = 128$, $2P = 0.023$; analysis including all birds from experiments 1 to 3 except those treated with EB part 1). Part of all these birds had been gonadectomized neonatally while the others had been operated at only 5 weeks of age. The analysis of these pooled data by a two-way ANOVA with the sex of the birds and their

age at castration as factors was thus a powerful way to detect any effect of these factors. This in fact confirmed the sex difference in enzyme activity ($F_{1,126} = 5.20$, $P = 0.024$) but gave no indication of an effect of the age at castration ($F_{1,126} = 0.21$, $P = 0.646$) and of the interaction between the two factors ($F_{1,126} = 0.11$, $P = 0.737$). Taken together, these studies thus confirm the presence of a sex dimorphism in the preoptic aromatase activity but shown that its magnitude is very small. In one previously published experiment, the observed magnitude for the difference was very large (activity in females was only about 25% of that in the males) but six independent experiments have now demonstrated a difference in the order of 15 to 25% (SCHUMACHER & BALTHAZART, 1986; BALTHAZART *et al.*, 1986 and present report). The reality of this dimorphism thus appears to be out of question even if its demonstration usually requires large sample sizes due to the small magnitude of the difference. The dimorphism in aromatase activity is probably connected in a specific manner to the control of reproduction because it is not observed in the posterior hypothalamus. In addition when a hormonal manipulation affects aromatase activity it is always specifically in the preoptic area and the enzyme activity is not changed in the posterior hypothalamus.

The way in which this sex difference in enzyme activity differentiates is much less clear. The first experiment suggested that injection of EB into male embryos on day 9 of incubation significantly decreases their adult levels of aromatase activity. The same treatment was ineffective when given on day 14 of incubation which is totally consistent with the way in which copulatory behavior differentiates. However the same treatments performed in females produced unexpected results. The lowest dose of EB (5μg) actually increased aromatase activity while the highest dose (25 μg) had no detectable effect. It is presently difficult to reconcile these results together and with the accepted model of sexual differentiation in quail. Results would be consistent with a model similar to what has been proposed for the differentiation of female sexual behavior in rats (DÖHLER *et al.*, 1984) which suggests that estrogens derived mainly from the central aromatization of T are differentiating stimulus. In this model, exposure to low doses of estrogens would promote the development of a male-type aromatase (and behavior) while higher doses would produce a female type phenotype. It should then be assumed that in physiological conditions, despite the fact that females have higher plasma levels of estradiol than the males (SCHUMACHER *et al.*, 1988*a*), the male POA is exposed to higher levels of estrogens than the females due to the higher local conversion of T to estradiol. No evidence for a such a sex difference in the aromatization in the embryo could be found recently in relatively large brain samples (preoptic area + anterior hypothalamus : SCHUMACHER *et al.*, 1988*b*) but it might well exist in discrete parts of the preoptic area. In males, the addition of exogenous estrogens to the optimal endogenous levels would demasculinize the aromatase and behavior irrespective of the dose. In females normally exposed to suboptimal levels of endogenous estrogens, a low dose of EB would masculinize these characteristics and it is only at the higher dose that the demasculinizing effects would begin to be observed. This interpretation would account for all the trends which were present in the data but it must be stressed that some of these were actually not statistically significant and given the apparent discrepancy between results obtained in males and in females, it is not even assured that embryonic estrogen have permanent effects on the preoptic aromatase activity. Similarly the present experiments did not reveal any permanent effects of the post-hatching steroids on the preoptic aromatase activity. Results of part 2 had suggested that the sex dimorphism in enzyme activity was larger in birds gonadectomized as adults than in those operated

as neonates. However no support for this idea was found in part 3 nor in the analysis of the pooled data.

It is thus unclear whether the early hormonal environment has any permanent influence on the enzyme activity. It is possible that a longer embryonic treatment with estradiol, or a treatment started earlier or an embryonic treatment of birds that would retain their gonads after hatching (all birds in experiment 1 were gonadectomized 4 days post-hatch) would be more efficient and these possibilities should be tested experimentally. Alternatively the notion that the sex dimorphism in preoptic aromatase activity is not organized by early steroids but rather reflects some activational difference should be entertained. It is true that a same treatment with T silastic implants produces identical plasma levels of the steroid so that a differential induction of the aromatase resulting from a different peripheral catabolism of T can probably be discarded (SCHUMACHER & BALTHAZART, 1986). However hormones produced by the adrenals might be slightly different in males and in females and these could modulate the activity of the enzyme. No sex-related difference in the plasma concentrations of five steroids including estradiol and progesterone could be detected in a recent study of gonadectomized quail (BALTHAZART *et al.*, 1987) but subtle differences might be present. Alternatively other hormones such as the gonadotrophins which are known to be secreted differentially in males and females even after gonadectomy (URBANSKI & FOLLETT, 1982; SCHUMACHER & BALTHAZART, 1984*a*) might be responsible for the enzymatic difference (BALTHAZART, 1989). It is also important to point in this context that correlations were found here between the individual levels of activity of the preoptic aromatase and the sexual behavior of the birds (see parts 2 and 3). These are quite specific in nature because they were not observed when the aromatase activity in the posterior hypothalamus was considered. At present, it is unclear whether the differences in enzyme activity are controlled by hormones and cause the differences in behavior (*e. g.* males have a higher aromatase than females and are consequently more active) or the performance of copulatory behavior produces an increase in aromatase activity which generates both the individual correlations and the sex difference in enzyme activity. Previous work has shown that the exposure to a female and the performance of copulatory behavior increase the plasma T levels in a number of bird species including the ring dove (FEDER *et al.*, 1977; O'CONNEL *et al.*, 1981) and the white-crowned sparrow (WINGFIELD & FARNER, 1980). In quail, this could never be demostrated but the exposure to females advances the sexual maturation of the male (DELVILLE *et al.*, 1984). The differential reaction of males and females to a stimulus female could thus be the origin of the dimorphism in aromatase activity. This could be mediated either by an increase in plasma T (unlikely in our conditions because all birds were gonadectomized and treated with exogenous steroid) or by a direct non hormonal action on the brain. No firm support to this hypothesis could be gathered during part 2 in which aromatase activity was not found to be significantly different in male quail which had been tested for sexual behavior and in birds maintained in isolation until their sacrifice. However the activity of the enzyme was about 20% higher in the birds which had been exposed to females. It is thus quite possible that conditions including a more frequent exposure to females, a shorter duration between the last exposure and the sacrifice or more complete social isolation of the non-exposed males (in this study they could not interact but they could see and hear the females) would demonstrate this effect of social stimulation on the enzyme activity.

In conclusion, the present experiments have confirmed that the copulatory behavior and the preoptic aromatase activity are sexually differentiated in Japanese

quail. They have shown that manipulations of the early hormonal milieu irreversibly determine the behavioral phenotype of the birds but effects of these experimental treatments on the preoptic aromatase could not be clearly established. The exact cause of the enzymatic sex dimorphism still eludes us and it is even unsure whether this mechanism is organizational or activational in nature. The very small magnitude of the difference makes additional studies very difficult. We have already shown that the amplitude of the sex difference cannot be increased by manipulating the dose of T given to induce enzyme activity (BALTHAZART *et al.*, 1986) or the duration of exposure to the steroid (in fact inductions shorter than two weeks result in little or no sex dimorphism, Balthazart and Foidart, unpublished data). It is quite possible that the dimorphic aromatase has a very discrete anatomical localization and that the small difference between males and females results from a dilution of dimorphic areas in non differentiated tissue. We recently showed using the Palkovits punch technique (PALKOVITS & BROWNSTEIN, 1983) combined to a sensitive radioenzyme assay that aromatase activity is much higher in the preoptic medial nucleus of the quail than in the rest of the preoptic area (SCHUMACHER & BALTHAZART, 1987). This nucleus is sexually dimorphic (VIGLIETTI-PANZICA *et al.*, 1986), T-sensitive (PANZICA *et al.*, 1987) and is directly implicated in the activation of copulatory behavior (BALTHAZART *et al.*, 1988). If the correlations between preoptic aromatase activity and copulatory behavior observed here reflect causal mechanisms, the aromatase in the preoptic medial nucleus should be more dimorphic that in the whole preoptic area and should be regulated by adult and embryonic treatment with steroids. This hypothesis is now under investigation.

Acknowledgments — We are indebted to Professor E. SCHOFFENIELS for his continued interest in our work. This research was supported by grants from the *Belgian FNRS* (Crédits aux Chercheurs), the University of Liege *(Fonds pour la Recherche),* the Medical School of the University of Liège, and the *NIH* (# HD22064) to JB and by a grant from the *FRFC* (# 2.4518.80) to Professor E. SCHOFFENIELS. We thank C. SURLEMONT for help in preparing this manuscript.

References

ADKINS, E. K. (1975) *J. Comp. Physiol. Psychol.* **89**, 61-71.
ADKINS, E. K. (1977) *Horm. Behav.* **8**, 201-207.
ADKINS, E. K. (1979) *Neuroendocrinol.* **29**, 178-185.
ADKINS, E. K. & ADLER, N. T. (1972) *J. Comp. Physiol. Psychol.* **81**, 27-36.
ADKINS, E. K. & NOCK, B. L. (1976) *Horm. Behav.* **7**, 417-429.
ADKINS, E. K. & PNIEWSKI, E. E. (1978) *J. Comp. Physiol. Psychol.* **92**, 1169-1178.
ADKINS, E. K., BOOP, J. J., KOUTNIK, D. L., MORRIS, J. B. & PNIEWSKI, E. E. (1980) *Physiol. Behav.* **24**, 441-446.
ADKINS-REGAN, E. (1983) in *Hormones and behaviour in higher vertebrates* (BALTHAZART, J., PRÖVE, E. & GILLES, R., eds.) pp. 219-228. Springer-Verlag, Berlin.
ADKINS-REGAN, E., PICKETT, P. & KOUTNIK, D. (1982) *Horm. Behav.* **16**, 259-278.
ALEXANDRE, C. & BALTHAZART, J. (1986) *Physiol. Behav.* **38**, 581-591.
BALTHAZART, J. (1989) in *Advances in Comparative and Environmental Physiology,* vol **3** (BALTHAZART, J., ed.) pp. 105-159. Springer Verlag, Berlin, 1989.
BALTHAZART, J. & SCHUMACHER, M. (1984) *Horm. Behav.* **18**, 287-297.
BALTHAZART, J. & SCHUMACHER, M. (1987) in *Reproduction : a behavioral and neuroendocrine perspective (*KOMISARUK, B. R., SIEGEL, H. I., CHENG, M. F. & FEDER, H. H., eds.) pp. 308-324., Annals of the New York Academy of Sciences, New York.
BALTHAZART, J., SCHUMACHER, M. & OTTINGER, M. A. (1983) *Gen. Comp. Endocrinol.* **51**, 191-207.
BALTHAZART, J., FOIDART, A. & HENDRIKCK, J. C. (1989) *Physiol. Behav.* (in press)
BALTHAZART, J., DELVILLE, Y., SULON, Y. & HENDRICK, J. C. (1987) *General Endocrinol. (Life Sci Adv)* **5**, 31-36.
BALTHAZART, J., SURLEMONT, C., FOIDART, A. & SCHUMACHER, M. (1988) *Soc. Neurosci. Abstr.* **14**, 97.
BALTHAZART, J., DEVOS, F., DOHET, A., FOIDART, A., HUGLA, J. L., RADEMAKER, F. & SCHUMACHER, M. (1986) *IRCS Med. Sci.* **14**, 1188-1189.

BRADFORD, M. M. (1976) *Anal. Biochem.* **72**, 248-254.
DELVILLE, Y., SULON, J., HENDRICK, J. C. & BALTHAZART, J. (1984) *Gen. Comp. Endocrinol.* **55**, 295-305.
DÖHLER, K. D., HANCKE, J. L., SRIVASTAVA, S. S., HOFMAN, C., SHRYNE, J. E. & GORSKI, R. A. (1984) *Prog. Brain Res.* **61**, 99-117.
FEDER, H. H., STOREY, A., GOODWIN, D., REBOULLEAU, C. & SILVER, R. (1977) *Biol. Reprod.* **66**, 666-677.
GIBSON, W. R., FOLLETT, B. K. & GLEDHILL, B. (1975) *J. Endocrinol.* **64**, 87-101.
HUTCHISON, R. E. (1978) *Horm. Behav.* **11**, 363-387.
O'CONNELL, M. E., REBOULLEAU, C., FEDER, H. H. & SILVER, R. (1981) *Gen. Comp. Endocrinol.* **44**, 454-463.
PALKOVITS, M. & BROWNSTEIN, M. J. (1983) in *Brain microdissection techniques (*CUELLO, A. C., ed.) pp. 1-36. Wiley, New York.
PANZICA, G. C., VIGLIETTI-PANZICA, C., CALCAGNI, M., ANSELMETTI, G. C., SCHUMACHER, M. & BALTHAZART, J. (1987) *Brain Res.* **416**, 59-68.
SACHS, B. D. (1967) *Science* **157**, 201-203.
SCHUMACHER, M. & BALTHAZART, J. (1983) *Physiol. Behav.* **30**, 335-339.
SCHUMACHER, M. & BALTHAZART, J. (1984*a*) *Horm. Behav.* **18**, 298-312.
SCHUMACHER, M. & BALTHAZART, J. (1984*b*) *Prog. Brain Res.* **61**, 53-61.
SCHUMACHER, M. & BALTHAZART, J. (1985) in *Neurobiology. Current comparative approaches (*GILLES, R. & BALTHAZART, J., eds.) pp. 203-219. Springer-Verlag, Berlin.
SCHUMACHER, M. & BALTHAZART, J. (1986) *Brain Res.* **370**, 285-293.
SCHUMACHER, M. & BALTHAZART, J. (1987) *Brain Res.* **422**, 137-148.
SCHUMACHER, M., CONTENTI, E. & BALTHAZART, J. (1983) *Brain Res.* **278**, 337-340.
SCHUMACHER, M., CONTENTI, E. & BALTHAZART, J. (1984) *Brain Res.* **305**, 51-59.
SCHUMACHER, M., HUTCHISON, R. E. & HUTCHISON, J. B. (1988*a*) *Brain Res.* **441**, 98-110.
SCHUMACHER, M., SULON, J. & BALTHAZART, J. (1988*b*) *J. Endocrinol.* **118**, 127-134.
SCHUMACHER, M., HENDRICK, J. C. & BALTHAZART, J. (1989) *Horm. Behav.* **23**, 130-149.
SÖDERSTEN, P. & GUSTAFSSON, J. A. (1980) *J. Endocrinol.* **92**, 279-282.
SÖDERSTEN, P., ENEROTH, P., HANSSON, T., MODE, A., JOHANSSON, D., NASLUND, B., LIANG, T. & GUSTAFSSON, J. A. (1986) *J. Endocrinol.* **111**, 455-462.
URBANSKI, H. F. & FOLLETT, B. K. (1982) *J. Endocrinol.* **92**, 279-282.
VIGLIETTI-PANZICA, C., PANZICA, G. C., FIORI, M. G., CALGAGNI, M., ANSELMETTI, G. C. & BALTHAZART, J. (1986) *Neurosci. Ltrs.* **64**, 129-134.
WATSON, J. T. & ADKINS-REGAN, E. (1989) *Horm. Behav.* **23**, 251-268.
WINGFIELD, J. C. & FARNER, D. S. (1980) *Prog. Reprod. Biol.* **5**, 62-101.

J. BALTHAZART
Université de Liège, Laboratoire de Biochimie Générale et Comparée (Bat. L1), 17 place Delcour, B-4020 Liège, Belgium