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Published in:
Physiology & Behavior

DOI:
10.1016/j.physbeh.2009.01.017

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Experience modulates both aromatase activity and the sensitivity of agonistic behaviour to testosterone in black-headed gulls

Albert F.H. Rosa a,b,⁎, Aldina M.A. Franco a, Ton G.G. Groothuis a

a Department of Behavioural Biology, Biological Centre, University of Groningen, Kerklaan 30, 9750 AA Haren, The Netherlands
b Currently at the Department of Biology, University of Neuchâtel, Emile-Argand 11, 2009 Neuchâtel, Switzerland

1. Introduction

It is well known that social experience plays an important role in the development of social behaviour [1–3], while many aggressive and courtship behaviours are facilitated by sex steroids like testosterone and estradiol [4]. The aim of this paper is to study how social experience might interact with these facilitating effects of sex steroids. An interaction between both factors was first demonstrated for the decrease in sexual behaviour after castration. Rosenblatt and Aronson [5] showed that male cats which acquired sexually experience before castration retained their sexual behaviour when exposed to oestrous females in contrast to naïve animals. Subsequent studies on other species have corroborated this finding [6–9]. One pathway by which social experience might exert its effect on testosterone dependent social behaviour is via modulation of the release of sex steroids [10–12]. Alternatively, social experience might modulate the sensitivity of social behaviour to testosterone [7,13].

Testosterone may interact with the neural substrate concerning social behaviour either directly via androgen receptors or indirectly via metabolic pathways of the hormone [14]. One of these pathways is the conversion of testosterone to estradiol in limbic areas such as the hypothalamic preoptic areas [15–19]. These areas play a major role in aggressive and sexual behaviour and estradiol has been found to facilitate some of these behaviours [18,20–23]. Testosterone itself has been shown to increase aromatase activity in the brain of birds [20,24,25]. Because social interactions stimulate testosterone production this might result in a positive feedback loop between increasing aromatase activity and the expression of social behaviour. Interestingly, a direct effect of social stimuli on hypothalamic aromatase activity has been shown for the ring dove [26,27]. Together this suggests that hypothalamic aromatase activity together with estradiol facilitated social behaviours increase in socially experienced animals: (1) via effects of social stimuli on testosterone production; or (2) via direct modulation of aromatase activity by these social stimuli.

Young black-headed gulls (Larus ridibundus) provide a unique model to study the relation between experience, testosterone, estradiol, and agonistic behaviour in a wild species within a functional context. Chicks of this species grow up in dense colonies in which they defend a territory by responding aggressively to young and adult intruders in their territory. During such agonistic interactions testosterone levels were found to be elevated. Although this facilitated the expression of the agonistic behaviours, prolonged experimental
exposure to testosterone was found to be costly in terms of reduced growth rate. Interestingly, both chicks and juveniles of this species exposed to such experimentally elevated levels of testosterone for some time, were found to respond after cessation of treatment with much more agonistic behaviour to a standardized intruder test (challenge) than birds not exposed to such elevated levels of testosterone previously [28]. The authors hypothesized that the group difference in behaviour was due to increased sensitivity of aggressive behaviour to endogenous production of testosterone in the group that had experienced elevated testosterone levels [29]. Such a priming effect of testosterone on agonistic behaviour was demonstrated in an experiment in which gulls treated temporarily with testosterone showed a more rapid increase in agonistic behaviour in a subsequent testosterone treatment than gulls that were treated for the first time with the hormone [30]. Functionally priming might enable young birds to respond to agonistic challenges while having low testosterone production and thus avoiding the costs associated with elevated levels of the hormone. Furthermore, birds primed with testosterone early in life were found to be reproductively active earlier in the season. The first objective of this study was to test whether this priming is mediated by social experience gained during social interactions stimulated by testosterone during the first treatment period. To this end we tested whether animals that were refrained from social interactions during a first treatment period with testosterone show less behavioural responsiveness during a second treatment, sometime later, in a normal social context, than controls that received both testosterone treatments in a social context (experiment 1).

The second objective of our study was to test whether aromatase may play a role in the possible effect of social experience on agonistic behaviour. A role of aromatase in the expression of agonistic behaviour was expected because treatment with estradiol facilitated agonistic behaviour in black-headed gull chicks and in adults of a closely related gull species [31,32]. To this end we conducted two other experiments. In experiment 2 we treated birds with estradiol to test whether aromatization of testosterone may facilitate the agonistic behaviour in the age group under consideration. In the third experiment we replicated experiment 1, but birds were sacrificed early in the second treatment period to analyze brain aromatase activity levels in the hypothalamus. In several avian species, this area, including the preoptic area, contains high levels of aromatase and is a target area for testosterone [33,34].

2. Experiment 1: social experience and t-sensitivity

2.1. Methods

2.1.1. Rearing conditions

Chicks of two weeks of age were collected in June from large gull colonies in the north of the Netherlands and housed in groups of three or four peers in cages of 1×1 m each. At one month of age these groups were re-housed in outdoor cages measuring 1.5×3×2 m. Each aviary contained a water basin. Dry food pellets used in trout farms (Trouvít, Trouw, Gent, Belgium) were ad libitum available in the cages. Juveniles were individually marked on head or back with rhodamine or picrine (ICN Biochemicals, Cleveland, Ohio, USA; chemicals were dissolved in acetone) and colour rings on the legs.

The experiments were carried out with juvenile black-headed gulls of 9–10 months and all experiments were finished two months before the age at which normally testosterone levels start to increase, i.e. in spring for reproduction. Still, young black-headed gulls might before this time show temporary increases in levels of testosterone that play a role in aggressive interactions [29]. Earlier studies showed that this increase in sex steroids and aggressive behaviour can be suppressed by preventing the gulls to be exposed to aggressive challenges from other gulls [1,29]. Therefore birds of different cages were isolated from visual communication with each other.

2.1.2. Design

Fifteen juveniles of 10 months of age distributed over five groups (three juveniles per cage measuring 3×4.3×2 m) received a silastic implant containing testosterone (day 0; start of the T1 period: day 1). Birds of two of these groups were re-housed individually in cages measuring 1.5×4.3×2 m and were visually (but not auditory) isolated from other experimental birds: Iso-group (n=6). Birds of the other three groups stayed in their original cages and social condition: Soc-group (n=9). After 10 days the implants were removed (end of T1 period: day 10; removal implant: day 11). Two days after termination of treatment (day 13), when T1 levels were expected to be low again [28,29], partitions were taken away between the 6 cages of the Iso-group and between the 3 cages of the birds of the Soc-group, resulting in two large aviaries (9×4.3×2 m). This resulted in birds to be socially housed mostly with unfamiliar birds. After 10 days in groups all birds received a testosterone implant for a second time (day 23, start of the T2 period: day 24).

One of the birds of the Iso-group and one of the birds of the Soc-group died during the experiment. Further, one of the birds of the Soc-group lost its implant during the T1 period. These three birds were left out of the analyses.

2.1.3. Hormone treatment

Silicon tubes (Medica BV’s, Hertogenbosch, The Netherlands; internal diameter 1.0 mm, external diameter 3.0 mm) were packed with crystalline testosterone (Diosynth, Oss, The Netherlands; length of the column 10 mm, closed on both ends with 1 mm silicon glue). Such implants have been used by us repeatedly in earlier experiments with juvenile black-headed gulls and are known to induce blood levels of testosterone of approximately 2.4 ng/ml [29,35]. These levels are within the range of adult males during the breeding season [28,36]. Implantation was conducted by placing a small incision in the neck region under local anaesthesia with lidocaine (Xylocaine, Astra, Rijswijk, The Netherlands) and pushing the hormone pellet under the skin. The wound was closed with stitches. Removal of the implants took place by making a small incision in the neck under anaesthesia with lidocaine, and gently pushing the implant out.

2.1.4. Sex determination

Sex of the birds was determined by means of the length of head and bill. In black-headed gulls this parameter is greater for males than females (breakpoint at 8.1 cm) and this method has a reliability of 95% [37,38]. The black-headed gull shows only minor sex differences in testosterone levels in comparison to other species [28,39]. This might be related to the fact that the black-headed gull is a monomorphic species in both nuptial plumage and behavioural repertoire [40]. Moreover, in our earlier studies we did not find differences in behavioural frequencies between the sexes as a consequence of testosterone or estradiol treatment [28–31].

2.1.5. Observations and analyses of behaviour

From a hide at a distance of 6 m from the cages we recorded the behaviour of birds from one to two cages at a time (focal sampling of maximal six birds). Behavioural observations were recorded on audiocassette. Observations were carried out in the morning during sessions of 45 min. As a measurement for agonistic behaviour the Oblique display was chosen. This behaviour is strongly facilitated by testosterone [28,29]. Furthermore, Oblique display is one of the most conspicuous and frequently performed displays in adult black-headed gulls during aggressive and sexual interactions and consists of an erect posture accompanied by a loud call [41,42].
Behavioural observations were carried out on the last day of the T1 period (day 10), half way during the T-out period (day 17), and each day of the T2 period (days 24 to 30).

Behavioural frequencies were Poisson transformed for application of parametric statistical tests [43]. Further details are mentioned throughout the text.

2.2. Results

At the end of the first implantation period, the birds of the Iso-group performed less Oblique display than the birds of the Soc-group (Fig. 1; data day 10, two sample T-test: \( T(10) = 2.23, p = 0.05 \)), which confirmed that the treatment (visual isolation) successfully decreased social stimulation. After removal of the testosterone implant, the frequency of Oblique display decreased to basal levels in both groups (Fig. 1, day 17). In the course of the second implantation, the T2 period, the frequency of Oblique behaviour increased much steeper in the Soc-group than in the Iso-group (Fig. 1, days 24–30). To test this increase statistically an ANOVA was carried out on the behavioural data with the day after implantation in the T2 period as a repeated measurement factor (7 levels), and as independent factors the treatment during the T1 period (2 levels: Iso and Soc) and sex (2 levels). This ANOVA showed a significant effect of treatment (\( F(1,8) = 13.69, p = 0.044 \)), day after implantation (\( F(6,48) = 27.13, p = 0.0001 \)) and a significant interaction effect between treatment and day after implantation (\( F(6,48) = 3.94, p = 0.0028 \)). No significant effect of sex was found (effect sex: \( F(1,8) = 0.96, p = 0.36 \), all interaction effects \( p > 0.05 \)). Thus, experimental deprivation of social stimulation decreased the sensitivity to testosterone in comparison to the control group despite the fact that both groups received the same hormone treatment.

3. Experiment 2: effect of estradiol on behaviour

3.1. Methods

3.1.1. Design

At 10 months of age, 19 birds were randomly chosen from 29 birds housed in eight cages (\( 3 \times 4.3 \times 2 \) m). Nine were implanted with a crystalline pellet of 25 mg 17\( \beta \)-estradiol (Diosynth, Oss, The Netherlands; E-group, day 0). Ten birds received sham surgery (C-group) at 10 months of age. At the end of the experiment, the frequency of Oblique display (mean and sem per hour per bird) during different hormone treatments of juvenile black-headed gulls. Day 10: end of the T1 period (days 1–10); Day 17: during the T-out period (days 11–23); Day 24 to day 34: T2 period. Filled symbols: socially housed (Soc) group; Open symbols: individuals visually isolated (Iso) group.

Fig. 1. Changes in the frequency of Oblique display (mean and sem per hour per bird) during different hormone treatments of juvenile black-headed gulls. Day 10: end of the T1 period (days 1–10); Day 17: during the T-out period (days 11–23); Day 24 to day 34: T2 period. Filled symbols: socially housed (Soc) group; Open symbols: individuals visually isolated (Iso) group.

3.1.2. Blood sampling and radioimmunoassay

Within 5 min of capturing the bird, we collected 0.5 ml blood from the brachial wing vein with a heparin-rinsed needle and syringe. After centrifugation, plasma was stored at \(-20°C\). Radioimmunoassays for 17\( \beta \)-estradiol and testosterone were carried out at the Department of Herd Health and Reproduction at the University of Utrecht. Steroids were extracted from the plasma samples with diethyl ether [44,45].

Previous studies at the Utrecht laboratory showed that in the assay for estimating testosterone levels, the main levels of cross-reactivity with the antisera were 49.7%, 7.54%, and 3.35% for 5α-dihydrotestosterone, 4-androstene-3\( \beta \),17\( \beta \)-diol, and androstenedione, respectively. The interassay coefficient of variation of this assay was 14% [44]. In our assays, the lower detection level was 0.05 ng/ml T and the upper detection level was 4 ng/ml T. The main cross-reactivities of the antisera used in the assay for 17\( \beta \)-estradiol were 1.1%, 0.32%, and 0.16% for estrone, estradiol, and 17\( \alpha \)-estradiol, respectively, and <0.01% for other steroids tested (according to the manufacturer of the antisera, Coat-A-Count TKE; Diagnostic Products Corporation). Previous studies at the Utrecht laboratory showed that the interassay coefficient of variation of this assay was 8.9% [45]. In our assays, lower detection levels of 5 pg/ml were used. Analyses were done within a single assay to avoid interassay variability.

3.2. Results

3.2.1. Steroid levels

E treatment significantly elevated plasma levels of this hormone (mean ± se: C-group: 0.25 ± 0.06 ng/ml \( n = 9 \)), E-group: 0.87 ± 0.20 ng/ml \( n = 6 \); two sample T-test: \( T(13) = 3.6, p = 0.0034 \)). E treatment did not affect average T levels (mean ± se: C-group: 0.32 ± 0.12 ng/ml \( n = 3 \)), E-group: 0.35 ± 0.09 ng/ml \( n = 4 \)).

3.2.2. Behaviour

Four days after the start of the experiment, the frequency of Oblique display started to increase in E-treated birds, and reached maximum levels at seven days (Fig. 2). A three-way ANOVA was
carried out over the transformed data with factors sex (2 levels) and treatment (2 levels: E vs. C) and as repeated factor day of treatment (9 levels). This revealed a significant effect of treatment, \( F(1,15) = 4.55, P < 0.05 \), day of treatment \( F(8,120) = 4.10, P < 0.001 \), and of the interaction of treatment and day of treatment \( F(8,120) = 3.23, P < 0.01 \). No effect of sex was found \( F(1,15) = 1.48, P = 0.24 \), all interaction effects \( P > 0.10 \). Thus estradiol may affect brain areas that facilitate Oblique display.

### 4. Experiment 3: social stimulation and aromatase activity

#### 4.1. Methods

The design of this experiment was kept comparable to that of experiment 1 with some minor differences in the timing of the treatments. At nine months of age one group of four birds and one of five birds were housed in outdoor cages of 3 × 4.5 × 2 m (Soc-group) and two groups of four birds were re-housed individually in social isolation in outdoor cages of 3 × 1.5 × 2 m (Iso-group). The T1 period lasted from day 1 to day 16. At day 17 implants were removed. The T-out period lasted until day 28 at which birds received a new implant. The T2 period lasted from day 29 to day 34.

Behavioural observations were carried out at the end of the first treatment (data averaged over days 15, 16, and 17), 5 days after termination of the first treatment (day 22), and on days 1, 5, and 6 after the start of the second treatment (days 29, 33 and 34). After the observation period on day 34, the birds were sacrificed for brain analyses of aromatase activity.

All other details concerning subjects, rearing condition, observations, and sex determination were the same as in experiment 1.

#### 4.1.2. Brain sampling and enzyme assay

Methods for brain sampling and measuring aromatase activity have been described in [46]. Birds were overdosed with 4 to 10 ml of 6% pentobarbital. The brains were quickly removed and frozen at −196 °C by means of liquid nitrogen. After this, the brains were stored at −70 °C until they were dissected. Brains were transferred to a freezing plate (−20 °C) and cut into 1 mm coronal slices. Samples of similar size and weight (6–9 mg) were dissected with a micro punch under a binocular operating microscope. The following brain areas were selected: the medial preoptic area (POA), the area covering the post-preoptic area and the anterior hypothalamus (POA-AH), the posterior hypothalamus (PH). Also part of the cortex (CTX) was dissected as a control for background activity in the analysis. Dissected samples were re-frozen on dry ice and stored at −70 °C until assayed. Aromatase activity was estimated using the tritiated water releasing method: homogenised tissue was incubated for 15 min. at 40 °C with a standard amount of tritiated testosterone ([1a, 2a,3H] testosterone, approximately 2 × 10^5 d.p.m., sp. act. 53 Ci/mmol: Amersham International Plc. Bucks), and the concentration of tritiated water formed as a result of the aromatisation of the tritiated testosterone to estradiol was measured by radiometry (for further details of the method see [47]).

Aromatase activity was normalized to the total amount of protein in the samples. Protein content in homogenized samples was analysed according to the method described by Simpson and Sonne [48] using bovine serum albumin as standard (the detectable range of protein levels was 0.1–0.2 µg protein). Aromatase activity is expressed as fmol tritiated H2O formed/h/mg protein.

#### 4.1.3. Statistics

Behavioural data were treated as in the previous experiments. Because of difficulties with the sampling method some data on aromatase activity were missing for some brain areas. This concerned different areas in different birds, decreasing the statistical power of an overall ANOVA test. Therefore we tested differences in aromatase activity between the Iso- and Soc groups with Students’ T-tests for each brain area separately, except for the control area CTX. Aromatase levels did not deviate significantly from the normal distribution (Kolmogorov–Smirnov test, all P values > 0.8). We used weighed P values by means of the Holm–Bonferroni method to control for multiple testing.

#### 4.2. Results

#### 4.2.1. Behaviour

During the first implantation (T1 period), the birds of the Iso-group performed almost no Oblique display, whereas birds of the Soc-group showed high frequencies of these behaviours (Fig. 3, day 16). This difference was highly significant (two sample T-tests: \( T(11) = 5.70, p < 0.001 \)). After termination of treatment, the frequency of Oblique display in the T-out period was very low and not significantly different between groups (two sample T-tests: \( T(11) = 1.94, p = 0.1 \); day 29: \( T(11) = 1.21, NS, \) Fig. 3).

4.3. Experiment 3: social stimulation and aromatase activity

Fig. 4. Aromatase activity (mean and sem of fmol/mg protein) in different brain areas after 6 days of testosterone treatment in the T2 period for Soc birds (grey bars) and Iso birds (blank bars). APOA = anterior–preoptic area; POA-AH = area of the posterior preoptic area and the anterior hypothalamus; PH = posterior hypothalamus.
During the subsequent treatment in the T2 period, birds of the Iso-group were almost inactive with respect to Oblique display, in contrast to those of the Soc-group. This difference was significant on the day the birds were sacrificed for measurements of aromatase activity (Fig. 3; two sample T-tests: T(13) = 2.88, p < 0.05).

4.2.2. Aromatase

As expected, levels of aromatase activity in the control area (CTX) were near the lower detection limit of the assay. All other brain areas showed significantly higher aromatase levels than the control area (Fig. 4; matched pairs t-tests: CTX versus APOA, POA-AH, or PH: T (13) > 7.9, p < 0.001, all other comparisons: T(13) < 1.5, p > 0.5). There was no effect of sex on the data of aromatase activity (independent samples t-tests: APOA, POA-AH, or PH: T(12) < 1.0, p > 0.5). This is in agreement with the overall lack of sexual dimorphism in behaviour in this species. Aromatase activity in the POA-AH was significantly lower in the Iso-group during the second period (independent samples t-test: T(12) = 2.9, p < 0.05). This was not the case for the two other areas (APOA: T(14) = 0.58, NS; PH: T(12) = 0.74, NS).

5. Discussion

In previous studies with young gulls it was found that temporary exposure to testosterone increases the sensitivity of agonistic behaviour to a subsequent exposure with this hormone [30]. Such organizing effects of testosterone on behaviour are well known [49,50]. We explored this mechanism in more detail: First, by testing whether social experience gained during the first exposure to testosterone modulates this priming effect; Second, by testing whether this process is mediated by an enhancement of aromatase activity.

5.1. Experience and increased behavioural sensitivity to testosterone

This study shows that birds that were deprived of social interactions during a first period in which testosterone was experimentally elevated, were relatively insensitive to a subsequent testosterone treatment in their social behaviour. The effect was expressed as a steeper increase in the frequency of a sexual and aggressive display, the Oblique, in birds of the Soc-group than in birds of the Iso-group. The results show that experience gained during the first exposure to testosterone modulates this increase in sensitivity. During first treatment isolated birds showed very low levels of social behaviour. It is possible that differences in motor practice with the Oblique might have influenced the differences in behavioural sensitivity between the experimental groups. However, Groothuis and Meeuwissen [28] showed that chicks of the black-headed gulls treated with testosterone were showing full versions of social displays long before the age they are shown in normal ontogeny. This indicates that motor experience is not required for display development in the black-headed gull. We therefore conclude that social experience with display behaviour plays an important role in changes in behavioural sensitivity.

Although plasma levels of testosterone were not measured, it is almost inevitable that these have been the same for both the Soc- and Iso groups. First, both groups received similar implants at the same time in the same way while their rearing conditions have been exactly the same too. Second, endogenous production of testosterone will have been very low in all birds because of the age of the birds and time of the year when the studies were conducted. We tested juveniles at the age of 9–10 months in March. Black-headed gulls normally start to reproduce at 1.5 years of age and will start to show elevated levels of agonistic or courtship behaviour from the end of April. However, free living chicks experience a period of elevated testosterone levels and territorial like behaviours which is induced by aggressive challenges at their nesting territory [29]. In order to reduce such early exposure to testosterone, in the current study social stimulation was minimized by housing the experimental birds in small groups. Treatment with testosterone of chicks and juveniles has been shown to prime agonistic behaviour during a subsequent testosterone treatment [30], which indicates that the priming effect we found in the current study is relevant for the natural situation; Third, autopsies always revealed strongly regressed gonads in birds treated with sex-steroids. Indeed, the control birds of experiment 2, of which plasma levels of testosterone were available, showed low levels of endogenous steroid production as well as low levels of Oblique display. The testosterone implants will have only lowered this production even further because of the negative feedback of the experimentally elevated steroid levels on the gonads. Thus, social stimulation must have exerted its effect directly on the brain, without influencing plasma testosterone levels.

5.2. Experience and aromatase activity

The treatment with estradiol caused a moderate increase in estradiol levels to two-fold of those of untreated birds. This is well within the physiological range of this species [36]. In agreement with results obtained in juvenile laughing gull [32], and in chicks of the black-headed gull [31], the oblique display clearly increased in estradiol treated birds compared to control birds. Thus, both testosterone and estradiol facilitate oblique display. Because testosterone can be metabolised to estradiol by aromatase in the brain, this indicates that aromatase plays an important role in mediating the effects of testosterone on behaviour in the black-headed gull.

Birds that had gained social experience during exposure to testosterone were found to have higher levels of aromatase activity compared with birds that were socially isolated during exposure to testosterone. This likely explains the difference in the behavioural response to a second hormone treatment between the groups in both experiments 1 and 3. The difference in absolute frequency of display between both experiments is likely to be caused by the difference in season in which these were conducted [52]. Nevertheless, since we are only concerned with within experiment comparisons, (treatment and controls matched within experiments), this is of no relevance for our conclusions at all. It is known that testosterone production is increased in birds exposed to social stimuli [12,53]. Such differences in exposure to testosterone may cause differences in aromatase activity [15,20,25]. But as discussed above the differences in aromatase levels could not be due to the effects of the hormone itself.

The effects of social experience on aromatase activity were most significant for the area covering the post-precptical area and the anterior hypothalamus (POA-AH). We were interested in this area because it is known to be a central part of the “social behaviour network” [14]. This group of structures contains high levels of steroid receptors and metabolizing enzymes like aromatase, and coordinates steroid-dependent behaviours including those used in agonistic communication and sexual behaviour. Furthermore, hypothalamic areas are involved in transducing environmental cues into reproductive signals. Therefore the hypothalamus plays a key role when integrating social cues and the hormonal control of social behaviour.

We are aware of only one other experiment showing a relationship between aromatase activity in the POA-AH area and social experience in birds. In this experiment, Hutchison and Steimer [54] treated castrated doves with testosterone and measured aromatase activity in several hypothalamic brain areas. They showed higher aromatase activity in the POA but not in other hypothalamic areas in males that were given the opportunity to interact with females than in males that were not given this opportunity [27]. Although their effect in the POA was of borderline significance it is consistent with our findings. Therefore, we suggest that social stimulation affects the conversion of testosterone in particular brain areas to estradiol independently of actual testosterone levels.

From earlier work on the black-headed gull, we expected that a short exposure to elevated testosterone levels would prime the
behaviour of the birds to later exposure to testosterone [30]. Here we suggest that experience gained during social interactions may affect this behavioural sensitivity to testosterone. It is difficult to answer why juvenile gulls would have such a mechanism. It might be that contingency between variation in androgen levels and social interactions is an important factor in fine-tuning behavioural responses. The age at which we subjected the gulls to our experimental treatment was just before the start of their first breeding season. At this age in gulls priming of behavioural responsiveness is likely to play an important role in their ability to compete with conspecifics for mates and for a good breeding place in the colony. Furthermore, the nature of the experience that has an effect on aromatase needs further study. It may concern the establishment of social relationships (rank order, pair bonds) that enhances aromatase directly, independently of actual plasma levels of testosterone.

Acknowledgements

We thank Dr. John Hutchinson and Dr. Andrew Wozniak of the University of Cambridge for help with analyzing aromatase levels, and Sjoerd Veenstra, Roelie Wiegman, Tosca Boere and Guido Meeuwissen for help with animal care taking. Furthermore, we would like to thank David Gonçalves and several anonymous referees for their help improving the manuscript. The study was supported by grant SLW-805.30.203 of the Dutch Foundation for Research.

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