



The non-aromatizable androgen dihydrotestosterone (DHT) facilitates sexual behavior in ovariectomized female rats primed with estradiol

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ARTICLE INFO

Keywords:

Dihydrotestosterone
Estradiol
Solicitation
Rat
Sexual desire
Preclinical

ABSTRACT

It is still unclear whether Testosterone (T) increases sexual desire through a stimulation of the androgen receptor in relevant brain regions or through its conversion to estrogens. The aim of this study was to clarify the mechanisms of T facilitation of female sexual desire by assessing the effect of a non-aromatizable androgen (Dihydrotestosterone, DHT) in a validated animal model.

Ovariectomized (OVX) Long-Evans rats were treated with oil (O) + O, 10 mcg Estradiol Benzoate (EB) + O, 10 mcg EB + 500 mcg Progesterone (P), O + 500 mcg DHT or 10 mcg EB + 500 mcg DHT (n = 12 per group). EB was administered 48 h, while P and DHT 4 h, prior to 4 sexual behavioral testing sessions in bisected unilevel pacing chambers. Appetitive behaviors (the frequencies of hops/darts and solicitations) were considered as the main outcome measure. Sexual receptivity indexes [lordosis magnitude, expressed as lordosis rating (LR), and lordosis quotient (LQ)], rejection responses, as well as mounts, intromissions and ejaculations received from the male were also coded. The probability of transition among sexual behaviors was evaluated by Transition Matrices; T-Pattern analysis was performed to detect hidden repeated temporal behavioral sequences.

Preliminary analyses found no statistically significant differences between the O + O and EB + O groups, therefore we excluded the EB + O group from further analyses. Rats treated with EB + DHT displayed significantly more appetitive behaviors compared to negative controls (O + O and O + DHT), whereas no difference was observed between EB + DHT rats and positive controls (EB + P); noteworthy, a higher number of appetitive behaviors was observed in the O + DHT group compared to the O + O group. Furthermore, rats treated with EB + DHT showed significantly higher receptivity measures (LR and LQ) and received more mounts, intromissions and ejaculations compared to negative controls (O + O and O + DHT), to levels equivalent to EB + P. No differences were detected in female-male mounts or rejection responses among the 4 groups. Under a qualitative perspective, full solicitation was found exclusively in T-patterns of the EB + DHT group, which was also the only one to display T-patterns of higher order encompassing appetitive behaviors-only events.

In conclusion, the administration of DHT in EB-primed OVX Long-Evans rats enhances sexual behavior measures. Specifically, DHT seems to stimulate sequences of appetitive behaviors separated from copulative/reproductive measures. Our data support an independent role of androgens in the facilitation of female sexual desire.

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<https://doi.org/10.1016/j.psyneuen.2020.104606>

Received 15 July 2019; Received in revised form 22 October 2019; Accepted 3 February 2020

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1. Introduction

Menopause is an ineluctable event in a woman's life, featuring a consistent drop in sex hormonal levels. However, while hormonal levels gradually decline in natural menopause, a sudden and even more pronounced shift of all ovarian hormones (estrogen, progesterone and androgens) is observed in surgical menopause (Davison et al., 2005). These menopause-related hormonal changes result in symptoms that are generally more severe and frequent in women with surgical relative to natural menopause (Bachmann, 2001). Among these is a persistent and distressing decline in sexual desire (Hypoactive Sexual Desire Disorder, HSDD), which affects approximately 9 % of naturally menopausal women, but up to 16–22 % of surgically postmenopausal women (Leiblum et al., 2006; West et al., 2008).

In both naturally or surgically menopausal women, administration of testosterone (T), either alone or in combination with estradiol (E2) as a hormonal replacement therapy (HRT), has been consistently effective in improving many domains of sexual response, especially sexual desire and satisfaction (Davis et al., 2006; Panay et al., 2010; Simon et al., 2005). However, the neuroendocrine mechanisms underlying the influence of T on female sexual behavior are still not completely understood. One complicating factor stems from the ability of T to be converted to both androgenic and estrogenic metabolites. For example, T serves as a precursor hormone that can be converted in both brain and periphery by the enzyme aromatase into 17 β -estradiol, or by the enzyme 5 α -reductase into dihydrotestosterone (DHT). DHT is more potent than T itself, due to its higher affinity for the androgen receptor (AR) and its inability to be aromatized to E2 (Askew et al., 2007). Moreover, either AR and estrogen receptors (ER α and ER β) are expressed abundantly in relevant brain regions associated with both appetitive and consummatory sexual behavior in females (Pfaff, 1980), making it unclear whether T positively modulates sexual desire and satisfaction through a stimulation of the AR, through its conversion to estrogen and the stimulation of ERs, or some combination of the two (Handa and McGivern, 2002).

Female rat models offer an effective tool for untangling this issue, not only because rat appetitive sexual behaviors, like hops and darts or solicitations, are homologous to sexual interest/initiation in women (Gelez et al., 2013; Pfau et al., 2003, 2015), but also because of the ease in manipulating their hormones (Giuliano et al., 2010). Bilateral ovariectomy (OVX) in female rats does indeed approximate sex steroid alterations in natural and surgical menopause (Pfau et al., 2015). Although acute administration of E2 or estradiol benzoate (EB) alone to OVX rats facilitates the display of lordosis when females are mounted by males, the combination of EB and progesterone classically restores not only full lordosis behavior, but also appetitive sexual behaviors in OVX rats (Boling and Blandau, 1939; Pfaff, 1980; Pfau et al., 1999, 2015). Indeed, it has recently been shown that T propionate also facilitates lordosis and appetitive sexual behaviors in OVX rats treated with EB (Jones et al., 2017), suggesting that OVX female rats may constitute a predictive model of women with surgical menopause.

The aim of the present study was to examine whether the administration of the non-aromatizable androgen receptor agonist DHT would facilitate appetitive and consummatory aspects of sexual behavior in OVX rats treated with EB, to levels equivalent to rats primed with EB and progesterone (P), used as positive controls. DHT alone was also compared with a neutral vehicle in non EB-primed rats. 3-month-old OVX rats were used as a model of surgical menopause, which often takes place in women younger than those naturally experiencing menopause. Given that there are multiple aspects of sexual behavior that cannot be captured by the direct measure of a discrete action itself, this study also examined sexual behavior dynamics. The effects of different hormonal treatments on the various stages of sexual behavior as it unfolds over time were therefore assessed using sequential analyses related to the elaboration of transition matrices and T-pattern analysis (Vanderschuren et al., 1995; Magnusson et al., 2016).

2. Material and methods

2.1. Animals

The Long-Evans strain was selected given its proposed superiority in the study of androgen-induced facilitation of desire, due to a high level of baseline sexual inhibition (Jones et al., 2017). Animals were purchased from Charles River Canada (St-Constant, QC) and housed in colony rooms maintained at 21 °C on a 12-h reverse day-night cycle (lights off at 8AM). Upon arrival, females (weight 150–250 g) were housed in pairs in Plexiglas® shoebox cages lined with Beta Chip®, whereas males (200–250 g) were group-housed (4/cage) in large Plexiglas® cages. Standard laboratory chow and tap water were freely available. Environmental enrichment was provided.

The experiments were conducted in accordance with the ethical standards established by the Canadian Council on Animal Care (CCAC) and approved by Concordia University's Animal Research Ethics Committee.

2.2. Ovariectomy

One week after arrival, females were anesthetized with a 2:1 mixture of ketamine hydrochloride (50 mg/ml; Ketaset®; Wyeth Canada, Guelph, Ontario, Canada) and xylazine hydrochloride (4 mg/ml; Rompum®; Bayer Health-Care, Toronto, Ontario, Canada) injected intraperitoneally (IP; mL/kg body weight), and OVX bilaterally through a single or double lumbar incision, and numbered by tail marking. Post-operative care was given with subcutaneous (SC) injections of Penicillin G (0.1 ml), ketoprofen (0.03 ml of a 100 mg/ml solution per rat per day for 5 days), 1 ml of a 0.9 % saline solution, and polysporin ointment applied to cover the incision sites.

2.3. Hormone administration

Estradiol benzoate (EB), progesterone (P) and DHT were dissolved in 0.1 ml reagent grade sesame oil vehicle (Sigma-Aldrich) and administered by SC injection. EB was administered 48 h, while P and DHT administered 4 h, prior to sexual behavior training and test sessions. An equal volume of sesame oil was administered in the control conditions. For tests involving only DHT treatment, the sesame oil vehicle was administered 48 h before testing. All hormone regimens were administered and scored by two trained observers blind to the hormone treatment groups.

2.4. Sexual training

Starting at least one week after surgery, females were made fully sexually receptive by administration of 10 μ g EB 48 h, and 500 μ g P 4 h prior to each of the 5 sexual training sessions in unilevel 4-hole pacing chambers. Training sessions occurred at 4-day intervals to approximate the normal estrous cycle duration, which has been shown previously to induce stable baseline rates of sexual behavior and to reduce variability in sexual responding (Pfau et al., 1999). Females were then given a 2-week hormone washout period before testing, to reduce residual effects of prior hormonal treatments (Jones et al., 2017; Kow and Pfaff, 1975).

Behavioral training and testing were performed in unilevel pacing chambers (38 × 60 × 38 cm) bisected by a clear Plexiglas divider with 4 square holes (4 × 4 cm) in the bottom that rest on bedding. The holes were adjusted to the size of the female in order to allow her to cross to the male's compartment but to prevent the male from doing the same. This allowed the female to initiate and pace the rate of copulatory contact with the male. Prior to sexual contact, randomly selected sexually vigorous males were placed into one compartment for a 5-min acclimation period and only ever used once during a session. Females were then placed into the opposite side for a 30-min copulatory session.

All training and testing occurred during the middle-third of the dark

circadian cycle. The 30-min test sessions were recorded digitally on a GoPro HERO® camera for subsequent scoring with the Behavioral Observation Program customized for rat sexual behavior (Cabilio, 1996).

2.5. Testing procedures

Following sexual training and washout, OVX Long-Evans rats ($n = 60$) were assigned randomly to one of following experimental groups: oil (O) + O ($n = 12$), 10 μ g estradiol benzoate (EB) + O ($n = 12$), 10 μ g EB + 500 μ g progesterone (P) ($n = 12$); O + 500 μ g DHT ($n = 12$); or 10 μ g EB + 500 μ g DHT ($n = 12$). According to previously established protocols (Jones et al., 2017), O + O and EB + O groups were used as negative controls, whereas the group of OVX rats receiving EB + P was used as a positive control. The doses of EB and P were chosen according to previously established protocols (Jones et al., 2013). The dose of DHT was chosen according to previous studies (Beyer et al., 1972). Subsequently, all rats from the different experimental groups were tested at 8-day intervals for 4 tests, to minimize sensitization to estradiol (Jones et al., 2013).

2.6. Behavioral outcome measures

The frequency of partial (hops/darts) and full solicitations were considered as the main outcome measure and analyzed after being combined into a general measure of appetitive sexual behaviors (as in Jones et al., 2015). In unilevel chambers, hops and darts are defined as quick short runs in front of the male (Afonso and Pfau, 2006), whereas full solicitations are defined as a headwise orientation of the female toward the male followed by an abrupt runaway, regardless of whether the female stays on the male's side or exits through the divider (Pfau et al., 1999). Female-male mounting, previously reported as a "super-solicitation" estrogen-dependent behavior displayed when the males do not mount following a normal bout of solicitation, was also coded (Afonso and Pfau, 2006).

As a secondary outcome measure, indices of sexual receptivity were examined, specifically lordosis reflex magnitudes and the lordosis quotient. Lordosis reflex magnitudes were scored on a 3-point scale (Hardy and Debold, 1971) and presented as a mean lordosis rating ($LR = \text{sum of points} / [\text{mounts} + \text{intromissions} + \text{ejaculations}]$). Lordosis quotients (LQ) were calculated as total number of lordosis reflexes/[mounts + intromissions + ejaculations]. If a female did not display lordosis when mounted, she received a score of 0, which was not entered into the LR (as it would thus be redundant with the LQ), but was included in the analyses of sexual behavior dynamics (see below). In the event of a male not mounting a female, a division by zero occurred, and the subject was removed from the analysis on lordosis, but was included in all other analyses. Mounts (front paws on female's flanks), intromissions (pelvic thrust with a dismount) and ejaculations received from the male were also coded, as they are known to provide further insight into the females' sexual receptivity (Pfau et al., 1999). Finally, the frequency of visits to the male's side, time spent in the male's side and rejection responses [(kicks, side-ways takedowns, boxing postures, prone positions, as in Barnett (1967)] were measured.

Outcome measures were analyzed for distribution and found not-normally distributed; therefore, the appropriate non-parametric tests were used for statistical analyses.

2.7. Analysis of sexual-behavior dynamics: transition matrices and T-pattern analyses

Two different approaches to test the effect of hormonal treatments on sexual-behavior dynamics were carried out. Elaboration and analysis of Transition Matrices (TM) provided a full picture describing the probability of transition among the behaviors included in the study (Casarrubea et al., 2009; Spruijt and Gispén, 1984). On the other hand,

T-pattern analysis was employed to detect and describe different real-time sequences characterizing the sexual behavior of the study groups (Magnusson et al., 2016).

2.7.1. Transition matrices

TMs are numerical tables expressing the number of sequential transitions that occurred among behaviors during the observation. TMs were collected per rat and summed in a total TM per group. Results from TMs were provided transforming original TMs in Probability Matrices (PMs) and Adjusted Residuals Matrices (ARMs).

PMs were obtained by transforming transitions into probabilities of transition. To this aim any transition from a given behavior "A" to another behavior "B" was expressed as a ratio of the total number of transitions included in the original TMs. Probabilities of transition were displayed per group by the mean of Path Diagrams. When the number of transitions was $>$ the total sample size, a non-parametric Kruskal-Wallis test followed by Dunn's post-hoc was employed to test differences among groups. Bonferroni correction was applied to reduce the risk of type-I error.

To refine the results of TMs from a statistical point of view, ARMs were obtained for the EB + P and EB + DHT groups. ARMs compared for each behavioral transition the observed values versus the expected values of a given equal distribution of transitions per each row of the matrix (Vanderschuren et al., 1995; van den Berg et al., 1999; van Lier et al., 2003). Positive or negative residual values indicated transitions occurring respectively more often or less often than expected. ARMs can be expressed according to a Z-distribution and therefore p values can be found in a Z-table. Considering an $\alpha = 0.05$, a residual value $\geq +1.96$ indicates transitions occurring significantly more often and residual values, whereas a residual value ≤ -1.96 indicates transitions occurring significantly less often than expected. A square-root normalization of residual values was performed to facilitate graphical representation so that the new statistical cut-off was $\pm \text{"square-root"}(1.96) = \pm 1.40$. Adjusted Residuals of EB + P group and EB + DHT group are illustrated by means of histograms (Casarrubea et al., 2012).

2.7.2. T-pattern analysis

TPA is largely employed to study animal and human behavior (Magnusson et al., 2016; Casarrubea et al., 2018). This method was conceived to detect recurring sequences of events characterized by statistically significant constraints among the interval length separating them (Magnusson, 2000). To this purpose, we used Theme, a specifically developed software (PatternVision, Ltd, Iceland). The Theme algorithm performs comparisons of the distribution of each possible pair of behaviors along the observation window. When a recurring sequence of two given "A" and "B" behaviors is found, such a sequence, indicated with a textual string as "(A B)", basically represents a first level T-pattern encompassing two-events. Hence, the algorithm considers the "(A B)" pattern as an "A" or "B" term of other possible hierarchically higher T-patterns such as, for instance, "(A B C)". This bottom-up procedure continues up to any level and finishes when no more statistically significant relationships are found.

Theme software requires specific criteria to be selected according to the experimental set-up to search and detect T-patterns in the dataset. The following parameters were selected: significance level $p < 0.0001$; minimum sample performing a t-pattern in each group = 50 %; lumping factor = 0.80; Free algorithm procedure. For further information and details on T-pattern analysis see Casarrubea et al. (2018) and Magnusson et al. (2016).

3. Results

Preliminary analyses using a Mann-Whitney *U* test for non-parametric variables were conducted to test whether there were differences between the two negative controls (OVX rats treated with O + O and OVX rats treated with EB + O) in the main outcome measure (hops/

darts and solicitations). Confirming previous findings (Jones et al., 2013), no differences were detected between the two negative control groups ($U = 101.00$, $p = 0.10$, $r = 0.18$). Conversely, OVX rats treated with EB + DHT showed a significant increase in appetite behaviors as compared to EB + O ($U = 132.00$, $p < 0.0001$, $r = 0.85$). In order to simplify the presentation of the results, we decided to exclude the EB + O group from further analyses, and to consider only O + O as the negative control group.

A Kruskal-Wallis test was conducted in order to analyze overall differences among all the measures between the remaining groups, followed by post-hoc analyses using the Mann-Whitney U test (comparing the EB + DHT group to the 3 control groups), applying a Bonferroni correction. The corrected level of significance was set at $0.05/4 = 0.01$, whereas statistical trends were considered at $p < 0.05$. Effect sizes were calculated on the Mann-Whitney tests with the formula $r = Z/\sqrt{n}$. Finally, the percent distribution of total T-patterns was compared using the Chi-square test; measures of effect size were calculated as odds ratios.

3.1. Appetitive behaviors

A Kruskal-Wallis H test showed that there was a statistically significant difference in appetitive sexual behaviors (hops/darts and solicitations) among the different subgroups ($\chi^2(3) = 38.32$, $p < 0.0001$). As expected, Long-Evans OVX rats treated with EB + P showed a significant increase in appetite behaviors (hops/darts and solicitations) as compared to both oil groups ($U = 144.00$, $p < 0.0001$, $r = 0.85$; $U = 144.00$, $p < 0.0001$, $r = 0.85$ vs. O + O and O + DHT, respectively). Interestingly, a higher number of appetitive behaviors was observed in the O + DHT group compared to the O + O group ($U = 137.5$, $p < 0.0001$, $r = 0.78$; Fig. 1A). In addition, OVX rats treated with EB followed by DHT displayed significantly more appetitive behaviors compared to both oil groups (O + O: $U = 120.00$, $p < 0.0001$, $r = 0.85$; O + DHT: $U = 120.00$, $p < 0.0001$, $r = 0.84$), reaching a level that was similar to EB + P rats. In fact, no difference was observed between OVX females treated with EB + DHT and OVX rats treated with EB + P ($U = 37.00$, $p = 0.14$, $r = 0.32$) (Fig. 1A).

3.2. Female-male mounting

The number of female-male mounts was not affected by different hormonal treatments ($\chi^2(3) = 6.50$, $p = 0.09$; not shown).

3.3. Lordosis

As shown in Fig. 1B and C, statistically significant differences were detected in LR ($\chi^2(3) = 36.38$, $p < 0.0001$) or LQ ($\chi^2(3) = 37.65$, $p < 0.0001$) values among groups. OVX treated with EB + DHT displayed a significantly higher LR when compared to OVX treated with O + O ($U = 144.00$, $p < 0.0001$, $r = 0.88$) and O + DHT ($U = 144.00$, $p < 0.0001$, $r = 0.85$), whereas they did not differ from OVX rats treated with EB + P ($U = 57.00$, $p = 0.41$, $r = -0.18$; Fig. 1B).

Rats treated with EB + DHT also displayed a significant increase in LQ when compared to O + O ($U = 144.00$, $p < 0.0001$, $r = 0.88$) and O + DHT group ($U = 142.00$, $p < 0.0001$, $r = 0.83$) (Fig. 1C). No difference was found in LQ when comparing the EB + DHT and the EB + P groups ($U = 47.00$, $p = 0.16$, $r = -0.30$) (Fig. 1C).

3.4. Male behaviors

The number of male stimulations received (mounts, intromissions, and ejaculations) was also significantly different among groups (mounts, $\chi^2(3) = 31.10$, $p < 0.0001$, Fig. 1D; intromissions, $\chi^2(3) = 37.08$, $p < 0.0001$, Fig. 1E; ejaculations, $\chi^2(3) = 35.87$, $p < 0.0001$, Fig. 1F). Specifically, females treated with EB + DHT received from the male a higher number of mounts, intromissions, and

ejaculations than those treated with O + O ($U = 137.50$, $p < 0.0001$, $r = 0.78$; $U = 144.00$, $p < 0.0001$, $r = 0.89$; $U = 132.00$, $p < 0.0001$, $r = 0.79$ for mounts, intromissions, and ejaculations, respectively) or with O + DHT ($U = 134.50$, $p < 0.0001$, $r = 0.74$; $U = 144.00$, $p < 0.0001$, $r = 0.85$; $U = 129.00$, $p < 0.0001$, $r = 0.78$ for mounts, intromissions, and ejaculation, respectively; Fig. 1D–F). The effects of EB + DHT on male behavior were not significantly different from those observed in rats treated with E + P ($U = 50.00$, $p = 0.22$, $r = 0.26$; $U = 49.50$, $p = 0.19$, $r = 0.26$; $U = 44.50$, $p = 0.11$, $r = 0.07$ for mounts, intromissions, and ejaculation, respectively; Fig. 1D–F).

3.5. Visits to the male's side and time spent in the male's side

The number of visits to the male's side was significantly different among groups ($\chi^2(3) = 11.68$, $p = 0.009$). EB + DHT treatment was equivalent to EB + P in inducing visits to the male's side ($U = 63.50$, $p = 0.63$, $r = -0.10$; not shown). A tendency for EB + DHT to increase the number of visits when compared to O + O ($U = 113.00$, $p = 0.02$, $r = 0.48$; not shown) was observed. On the other hand, no difference emerged between EB + DHT and O + DHT ($U = 100.00$, $p = 0.11$, $r = 0.33$; not shown).

No difference in the total time spent in the male's compartment was found among the 4 groups ($\chi^2(3) = 3.56$, $p = 0.314$).

3.6. Female rejection responses

A Kruskal-Wallis test failed to detect significant differences among groups on number of female rejection responses ($\chi^2 = 2.06$, $p = 0.56$; Fig. 1G).

3.7. Dynamics of sexual behavior

The impact of different hormonal treatments on sexual behavior dynamics was assessed by using analyses based on transition matrices (Figs. 2 and 3) and an approach based on the evaluation of the temporal structure of behavior, known as T-pattern analysis (Figs. 4 and 5).

3.7.1. Analysis based on transition matrices

Concerning the analyses based on the elaboration of transition matrices, first we evaluated the probability of transition among sexual behaviors by analyzing the Transition Matrices (TM) in the experimental groups. As shown in the statistics-related inset Fig. 2 (bottom), the Kruskal-Wallis test detected differences among groups in the vast majority (13 of 15) of TMs. Post-hoc analysis did not find any significant difference among transitions observed in the two oil groups (O + O vs. O + DHT; Fig. 2, column A). In contrast, significant variations were observed in transitions of EB + DHT rats when compared to O + O or to O + DHT groups. In particular, we found a significantly higher probability of transition from female appetitive behaviors (hops and darts, H/D) to the receipt of male mounts/intromissions/ejaculations (M/I/E) in OVX treated with EB + DHT when compared either with O + O or O + DHT (Fig. 2, columns B and D respectively). An increased probability of transition from female lordosis (receptive behavior), mainly lordosis of magnitude 1 or 2 (LM1, LM2), to H/D and to M/I/E was also observed in OVX treated with EB + DHT when compared to both oil-groups (O + O and O + DHT, Fig. 2, columns B and D, respectively). Opposite transitions from the receipt of male M/I/E to lordosis of different magnitudes (LM1, LM2, LM3) were also more frequent in the EB + DHT group as compared to the two oil-groups (O + O and O + DHT, Fig. 2, columns B and D, respectively). Significant differences were observed also in EB + P as compared with the two oil groups (O + O and O + DHT, Fig. 2, columns C and E, respectively). Interestingly, OVX rats treated with EB + P also showed a higher, even though not statistically significant, frequency of all these patterns of behavioral transitions when compared to OVX rats treated with EB + DHT (Fig. 2, column F). The four path diagrams illustrated in the main

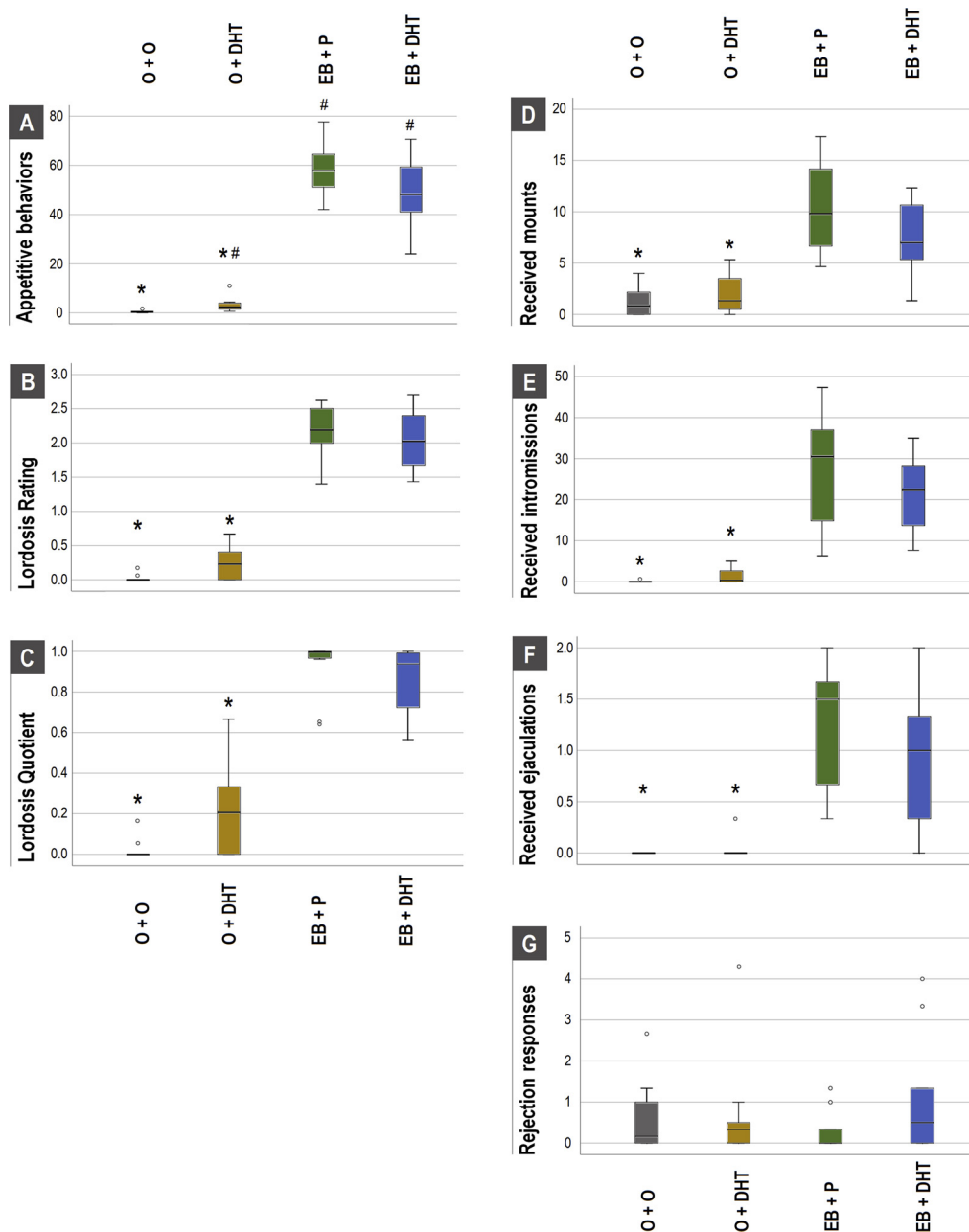


Fig. 1. Median frequency of appetitive behaviors (hops/darts and solicitations; panel A), lordosis measures (panels B and C), received mounts (panel D), intromissions (panel E), or ejaculations (panel F) and defensive behaviors (panel G) of ovariectomized Long-Evans rats following various hormone treatment combinations. Data were analyzed using Kruskal-Wallis to detect differences between groups, and significant effects were followed up using Mann-Whitney U, with a corrected p-value set at $p < 0.01$. Boxes represent interquartile range, and whiskers each represent the top and bottom 25 % of scores. O: sesame oil, EB: estradiol benzoate, 10 mcg; P: progesterone, 500 mcg; DHT: dihydrotestosterone, 500 mcg). ° = Outlier. * = Different from EB + DHT. # = Different from O + O. N = 12/group.

panel of Fig. 2 graphically summarizes these results, representing the probability of transitions among behaviors in the experimental groups. It is evident that the highest probabilities of transition do occur among behavioral components belonging to EB + P and EB + DHT groups.

In order to refine comparison between EB + DHT and EB + P rats, we also performed an analysis based on Adjusted Residual Matrices (ARMs; Vanderschuren et al., 1995), which allows for a qualitative assessment of sexual behavior. Histograms in Fig. 3 are a graphical representation of ARMs: positive bars represent transitions occurring significantly more often than expected; negative bars represent transitions occurring significantly less often than expected. The vast majority of behavioral transitions (75 over 81) occurred with similar residuals in EB + DHT and EB + P subgroups. In fact, in both groups, appetitive behaviors appear to be followed more often than expected by appetitive behaviors themselves or by male behaviors, which in turn are followed by lordosis. Lordoses showed different residuals depending on their magnitude. In both groups, LM0 showed more frequent than expected transitions towards female defenses, whereas LM1-3 showed towards

appetitive behaviors. In addition, only LM3 displayed positive residuals in the direction of Leaving (L; exit from the male's side of the cage).

Only slight differences were found between the residuals of the EB + DHT as compared to EB + P rats. Only EB + DHT rats exhibited a lower-than-expected transition from the receipt of male M/I/E to defensive behaviors (FD), while EB + P did not (Fig. 3). EB + P rats specifically showed a higher-than-expected transition from lordosis posture of the lowest magnitude (LM0) to leaving the male's side of the cage (L) and a higher frequency of transition from lordosis of magnitude (LM3) to female defensive (FD) behaviors (Fig. 3).

3.7.2. T-Pattern analysis

Multivariate T-Pattern analysis has been employed to detect the temporal structure of behavior. Fig. 4 illustrates, for each group, the behavioral sequences as terminal strings (that is, the textual expression of the events in sequences). For each sequence, length (Fig. 4, "Length" column) and number of occurrences (Fig. 4, "Occs" column) are indicated as well. Appetitive components of female sexual behavior ("H/

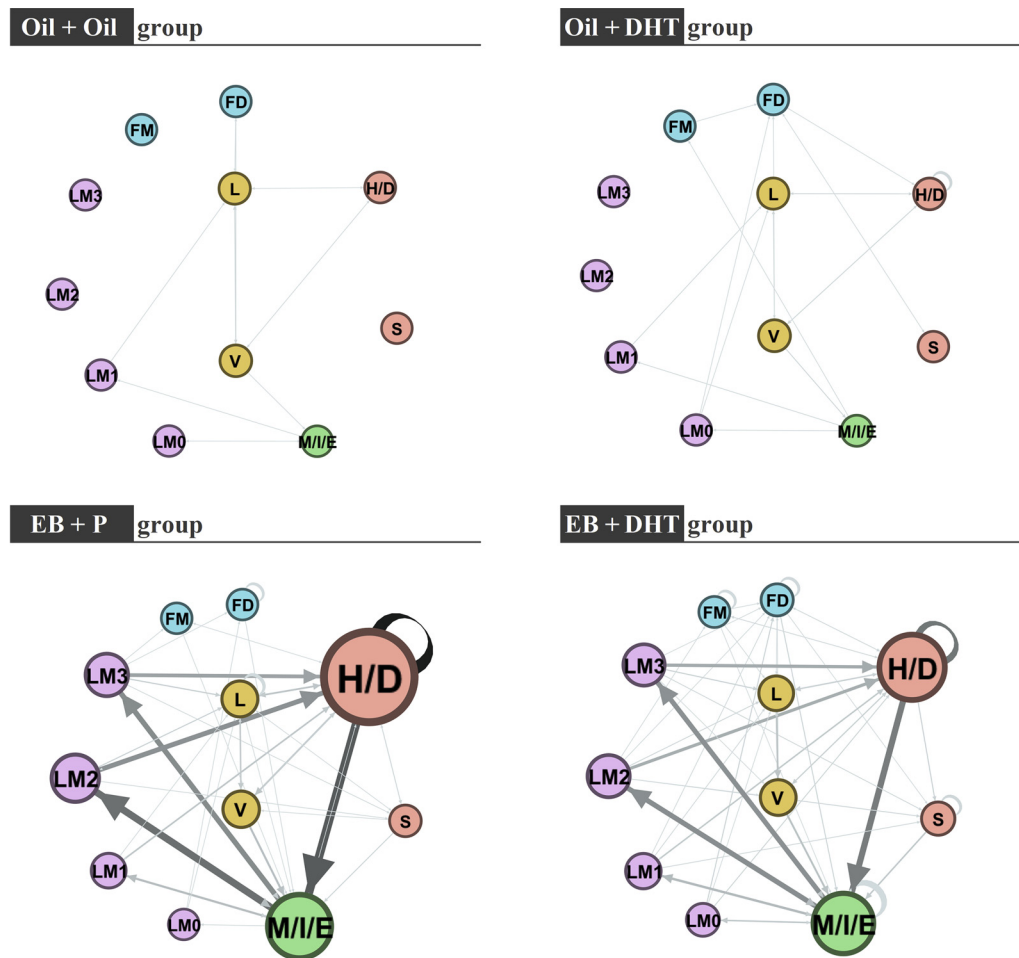


Fig. 2. Path diagrams representing transition probabilities among the behavioral components for each group of the study. The arrows of different thickness illustrate the likelihood of transition. Circles encompass the components of the behavioral repertoire: “V” = Visiting and “L” = Leaving the male’s compartment of the chamber; “H/D” = Hops/Darts and “S” = Solicitations are appetitive components of female sexual behavior; “M/I/E” = Mounts/Intromissions/Ejaculations stand for male copulatory behaviors; “LM 0 – 3” = Lordosis Magnitude, from 0 to 3; “FM” = Female mounting Male; “FD” = Female Defences. Circle size is related to the overall occurrences of each behavioral node. Top - Left: Oil + Oil group, $n = 11$. Top - Right: Oil + DHT group, $n = 12$. Bottom - Left: EB + P group, $n = 12$. Bottom - Right: EB + DHT group, $n = 12$. Below, statistical differences among the behavioral transitions illustrated in the figure. Non-parametric Kruskal-Wallis test was followed by Dunn’s post-hoc to test differences among groups. Bonferroni correction was applied to reduce the risk of type-I error.

Behavioral transition			Kruskal-Wallis Test ($\alpha = 0.003$, Bonferroni’s correction)		Dunn’s post-hoc test ($\alpha = 0.008$, Bonferroni’s correction)					
			$\chi^2 (3)$	p	column A	B	C	D	E	F
					Oil+Oil vs Oil+DHT	Oil+Oil vs EB+DHT	Oil+Oil vs EB+P	Oil+DHT vs EB+DHT	Oil+DHT vs EB+P	EB+DHT vs EB+P
H/D	to	H/D	38.86	< 0.003	$p = 1.000$	$p < 0.008$	$p < 0.008$	$p = 0.010$	$p < 0.008$	$p = 0.761$
H/D	to	V	20.29	< 0.003	$p = 1.000$	$p = 0.040$	$p < 0.008$	$p = 0.927$	$p = 0.016$	$p = 0.675$
H/D	to	L	13.03	= 0.005	n/a	n/a	n/a	n/a	n/a	n/a
H/D	to	M/I/E	34.40	< 0.003	$p = 1.000$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p = 1.000$
LM1	to	H/D	26.21	< 0.003	$p = 1.000$	$p < 0.008$	$p = 0.012$	$p < 0.008$	$p = 0.009$	$p = 1.000$
LM2	to	H/D	34.53	< 0.003	$p = 1.000$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p = 1.000$
LM2	to	M/I/E	36.66	< 0.003	$p = 1.000$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p = 1.000$
LM3	to	H/D	27.60	< 0.003	$p = 1.000$	$p = 0.003$	$p < 0.008$	$p = 0.002$	$p < 0.008$	$p = 1.000$
LM3	to	M/I/E	25.50	< 0.003	$p = 1.000$	$p = 0.004$	$p < 0.008$	$p = 0.003$	$p < 0.008$	$p = 1.000$
V	to	M/I/E	16.82	< 0.003	$p = 1.000$	$p = 0.014$	$p = 0.003$	$p = 0.172$	$p = 0.054$	$p = 1.000$
L	to	H/D	16.06	< 0.003	$p = 1.000$	$p = 0.339$	$p < 0.008$	$p = 1.000$	$p = 0.017$	$p = 0.345$
L	to	V	8.29	= 0.040	n/a	n/a	n/a	n/a	n/a	n/a
M/I/E	to	LM1	29.10	< 0.003	$p = 1.000$	$p < 0.008$	$p = 0.015$	$p < 0.008$	$p = 0.029$	$p = 0.813$
M/I/E	to	LM2	36.62	< 0.003	$p = 1.000$	$p = 0.002$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p = 1.000$
M/I/E	to	LM3	29.82	< 0.003	$p = 1.000$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p < 0.008$	$p = 1.000$

D”, hops/darts and “Solicit.”, solicitations) are highlighted in red. In the two oil groups (O + O; O + DHT) the only behavioral sequences detected were characterized by the visit-leave pattern (Fig. 4; “Visit Leave” string). On the other hand, both in EB + DHT and EB + P, several hierarchically higher-order patterns have been detected, thus suggesting a more complex temporal organization of sexual behavior. EB + DHT rats performed 33 different T-patterns distributed and composed as follows: 17 encompassing two events in their structure, 9

with three events, 3 with four events, and 4 with five events. In contrast, EB + P showed 43 different patterns with even a higher number of events in their structure as compared to EB + DHT (15 with two events, 6 with three events, 9 with four events, 4 with five events, 4 with six events, 3 with seven events and 2 with eight events; Fig. 4). Fig. 5 illustrates the percent distribution of T-Patterns containing specific behavioral components detected in EB + P and in EB + DHT groups. As assessed by means of Chi-square test, significant

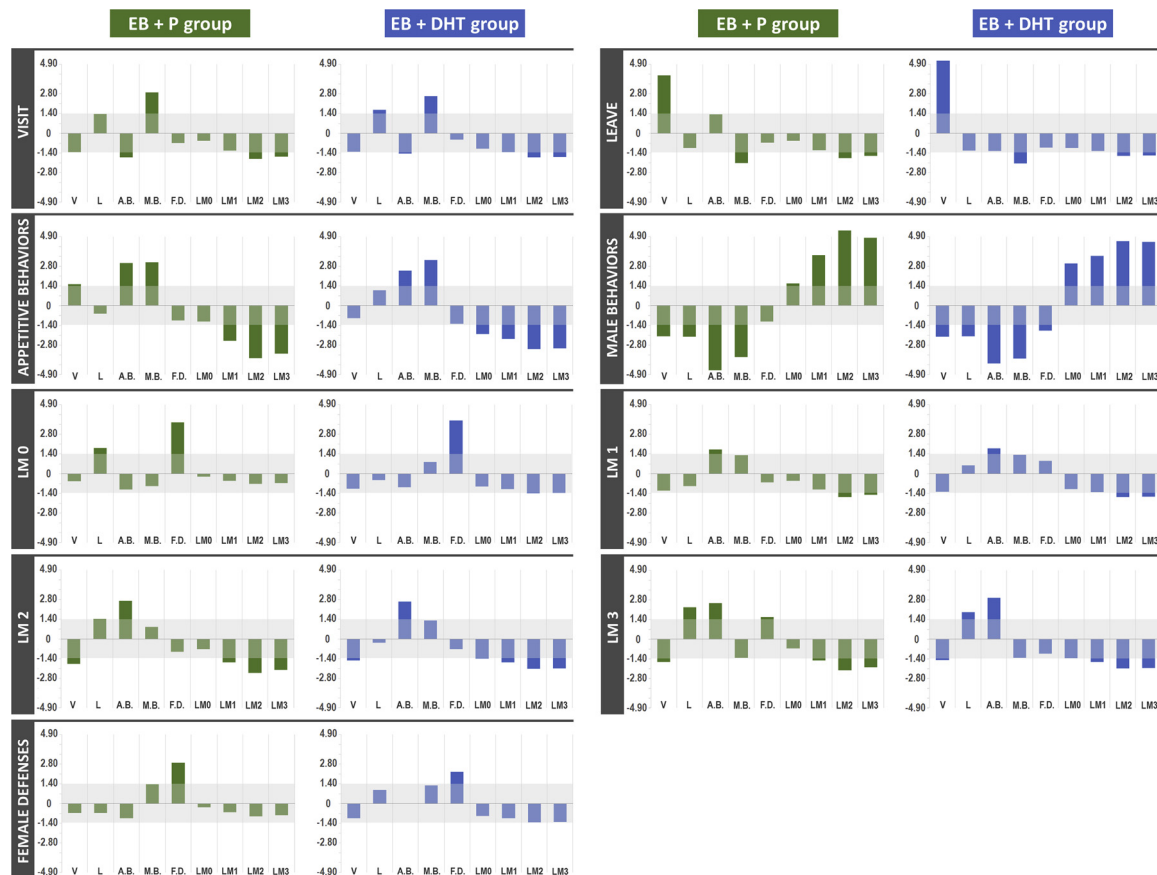


Fig. 3. Adjusted residuals representing the association strength of transitions among the behavioral components for the EB + P group ($n = 12$) and the EB + DHT group ($n = 12$). The behaviors on the left of each box (white letters in dark background) represent behavioral components that occurred in sequence before the components indicated at bottom of each X-axis. Y-axes: values of adjusted residuals. Positive bars: transitions occurring more often than expected. Negative bars: transitions occurring less often than expected. According to the Z-table the bars outreaching grey areas indicate a statistical significance ($p < 0.05$). “V” = Visiting and “L” = Leaving the male’s compartment of the chamber; “A.B.” = Appetitive Behaviors such Hops/Darts and Solicitations; “M.B.” = Male copulatory Behaviors (Mounts/Intromissions/Ejaculations); “FD” = Female Defences; “LM 0 – 3” = Lordosis Magnitude, from 0 to 3.

($p < 0.0005$) differences have been detected for T-patterns containing female behaviors only (64.9 % vs. 57.9 %), male behavior only (15.1 % vs. 21.8 %), Appetitive behaviors (47.4 % vs. 35.8 %), Appetitive behaviors + other male behaviors (13.2 % vs. 3.7 %). Finally, significant differences have been detected in higher order patterns (i.e. T-patterns containing 3 or more events in sequence) containing appetitive behaviors (65.9 % vs. 38.8 %).

Interestingly, in the EB + P group more than half of the different types of T-patterns included LM3, while this behavior was present only in one type of T-pattern in the EB + DHT group, namely the one coupled with male ejaculation (“Ejac. LM3”) (Fig. 4). In contrast, solicitation was present exclusively in T-patterns of the EB + DHT group: “Solicit. LM2”, “Solicit. (H/D H/D)”, “(H/D H/D) Solicit”, “[(H/D H/D) Solicit.] (H/D H/D)”, “(H/D H/D) [Solicit. (H/D H/D)]”, “Mount Solicit.”, “[Solicit. (Intro. Intro.)]”, “[Solicit. (Intro. Intro.) (H/D H/D)]”, “[LM2 (Mount Mount) (H/D H/D)]”. On the other hand, solicitation was completely absent in T-patterns of the EB + P group. EB + DHT also specifically displayed T-patterns of higher order encompassing appetitive sexual behavior-only events: “[(H/D H/D) Solicit.] (H/D H/D)” and “(H/D H/D) [Solicit. (H/D H/D)]” (Fig. 4).

4. Discussion

This study demonstrates that the administration of the non-aromatizable androgen DHT enhanced female sexual activity in OVX, EB-primed rats by significantly increasing both appetitive and receptive behaviors when compared to negative controls (O + O and O + DHT),

at comparable levels with the positive control (EB + P); furthermore, DHT enhanced appetitive behaviors when administered alone, compared to O + O. The current study was also uniquely designed to examine sexual behavior dynamics by analyzing the various stages of sexual intercourse as they unfolded over time. By using sequential analyses related to the elaboration of transition matrices we found that the administration of 10 μ g EB followed by 500 μ g DHT 4 h before testing significantly increased the probability of transition from either female appetitive or receptive behaviors to successful copulation, expressed by the receipt of male mounts/intromissions/ejaculations. These facilitatory effects of EB + DHT were as prominent as those observed after the canonical stimulatory treatment EB + P, used as a positive control. However, when we assessed hidden repetitive behavioral sequences through T-pattern analysis, some significant differences emerged between EB + DHT and EB + P groups: EB + DHT was the only treatment to enhance repetitive behaviors characterized by Solicitation, used by females to entice vigorous copulatory activity from males (Erskine, 1989). Our data finally confirm (Baum et al., 1974; Gladue, 1984) that DHT significantly triggers the emergence of both appetitive and receptive behaviors, suggesting that aromatization is not a mandatory prerequisite for the positive action of androgens on sexual activity.

OVX Long-Evans rats treated with EB + DHT displayed a significant increase in hops/darts and solicitations, which are commonly chosen as the main indicator of sexual motivation and appetitive behavior in this animal setting (Jones et al., 2015, 2017). In particular, these behaviors are aimed at enforcing the rate of sexual contact, thus reflecting the

Oil + Oil group				Oil + DHT group			
#	T-pattern string	Length	Occs.	#	T-pattern string	Length	Occs.
1	(Visit Leave)	2	11	1	(Visit Leave)	2	11

EB + P group				EB + DHT group			
#	T-pattern string	Length	Occs.	#	T-pattern string	Length	Occs.
1	(H/D H/D)	2	695	1	(H/D H/D)	2	381
2	(LM2 LM2)	2	182	2	(LM2 LM2)	2	107
3	(LM3 LM3)	2	114	3	(Visit Leave)	2	54
4	(Visit Leave)	2	64	4	(Leave Visit)	2	46
5	(Leave Visit)	2	61	5	(Leave Leave)	2	41
6	(LM3 Leave)	2	37	6	(LM1 LM1)	2	33
7	(LM1 LM1)	2	36	7	(LM1 LM2)	2	20
8	(LM1 LM2)	2	31	8	(LM2 LM1)	2	17
9	(LM2 Leave)	2	22	9	(Solicit. LM2)	2	12
10	(LM3 (Leave Visit))	3	33	10	(Leave (Visit Leave))	3	42
11	((Leave Visit) LM3)	3	27	11	(Solicit. (H/D H/D))	3	17
12	((LM2 LM2) LM3)	3	17	12	((H/D H/D) Solicit.)	3	16
13	((LM1 LM2) H/D H/D)	4	24	13	((H/D H/D) Solicit.) (H/D H/D)	5	12
14	((Leave Visit) (LM3 Leave))	4	22	14	((H/D H/D) (Solicit. (H/D H/D))	5	11

TP containing male OR female behaviors only				TP containing male AND female behaviors			
#	T-pattern string	Length	Occs.	#	T-pattern string	Length	Occs.
15	(Intro. Intro.)	2	310	15	(Intro. Intro.)	2	241
16	((Mount Mount) (Intro. Intro.))	4	7	16	(Mount Mount)	2	63

#	T-pattern string	Length	Occs.	#	T-pattern string	Length	Occs.
17	(Mount LM3)	2	36	17	(Mount LM2)	2	53
18	(Mount LM1)	2	32	18	(Mount LM1)	2	43
19	(Ejac. LM3)	2	11	19	(Mount Solicit.)	2	15
20	(Ejac. LM2)	2	7	20	(LM2 Mount)	2	13
21	(LM3 Mount)	2	7	21	(LM1 Mount)	2	11
22	((H/D H/D) Mount)	3	40	22	(Ejac. LM3)	2	10
23	(Mount (LM3 LM3))	3	14	23	((Mount LM1) Mount)	3	25
24	((Mount LM3) Ejac.)	3	10	24	(LM1 (Mount LM1))	3	19
25	((H/D H/D) (Mount LM3))	4	31	25	(Mount (LM1 LM2))	3	18
26	((H/D H/D) Mount) LM3)	4	31	26	(Solicit. (Intro. Intro.))	3	18
27	((Mount LM3) (H/D H/D))	4	21	27	(LM2 (Mount Mount))	3	10
28	(Mount (LM2 LM2) LM3)	4	13	28	((LM1 Mount) LM1)	3	8
29	((H/D H/D) Mount) LM1)	4	13	29	((Intro. Intro.) (LM2 LM1))	4	9
30	(LM3 ((H/D H/D) Mount))	4	10	30	((LM2 Mount) (Intro. Intro.))	4	8
31	((Intro. Intro.) (LM2 LM2) LM3)	5	13	31	((Intro. Intro.) (LM2 Mount))	4	6
32	((H/D H/D) Mount) LM3) Ejac.)	5	12	32	((Solicit. (Intro. Intro.)) (H/D H/D))	5	11
33	((Mount LM3) (H/D H/D)) LM3)	5	11	33	((LM2 (Mount Mount)) (H/D H/D))	5	7
34	((H/D H/D) Mount) LM1) LM3)	5	10				
35	((H/D H/D) (Mount LM3) (H/D H/D))	6	18				
36	((Mount LM3) (H/D H/D)) (Intro. Intro.))	6	18				
37	((LM3 ((H/D H/D) Mount)) (LM2 LM2))	6	9				
38	((Intro. Intro.) (LM3 ((H/D H/D) Mount)))	6	8				
39	((Mount LM3) (H/D H/D)) (Intro. Intro.)) LM3)	7	9				
40	((LM3 ((H/D H/D) Mount)) (LM2 LM2)) Ejac.)	7	8				
41	((H/D H/D) (Mount LM3) (H/D H/D)) LM3)	7	7				
42	((H/D H/D) ((Mount LM3) (H/D H/D)) (Intro. Intro.))	8	15				
43	((Intro. Intro.) (LM3 ((H/D H/D) Mount)) (LM2 LM2))	8	7				

Fig. 4. Results from T-pattern analysis for each group of the study. T-pattern string: textual representation of T-patterns of different composition. Numbers on the right of each string indicate their length (i.e. the number of behavioral components included in that sequence) and the overall occurrences (Occs.). Parentheses indicate the hierarchical organization of T-patterns. TP = T-pattern. “H/D” = Hops/Darts and “Solicit.” = Solicitations indicate appetitive components of female sexual behavior and are highlighted in red. “Intro.” = Intromission; “Ejac.” = Ejaculation. “LM 0 – 3” = Lordosis Magnitude, from 0 to 3. Top - Left: Oil + Oil group, n = 11. Top - Right: Oil + DHT group, n = 12. Bottom - Left: EB + P group, n = 12. Bottom - Right: EB + DHT group, n = 12. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

willingness of the female to initiate and engage in sexual interaction. Interestingly, in our study the positive effect of DHT on these measures was as potent as that observed with P over the EB pretreatment. Jones and colleagues, by using a different testing apparatus (bilevel pacing chamber), recently showed that 200 µg T propionate administered 4 h prior to testing in OVX, EB-primed rats facilitated hops/darts and lordosis ratings (Jones et al., 2017). These findings confirmed earlier studies demonstrating a positive involvement of aromatizable androgens in female rat sexual behavior, and specifically in appetitive behaviors (Gladkova and Karpenko, 1986; Jones et al., 2012). Hereby, we demonstrated for the first time that also a non-aromatizable androgen, DHT, exerts a potent facilitatory effect on the full array of female sexual behavior in the EB primed rat, and is able to trigger appetitive measures when given alone. A previous study on the effects of DHT in rodents was also promising, demonstrating that 500 µg DHT added to EB increased masculine sexual behaviors (female mounting of a receptive female) in OVX Wistar rats (Baum et al., 1974). In our study we did not assess female-female mounting, because it has been considered more an expression of dominance in social hierarchy than a display of sexual motivation (Fang and Clemens, 1999).

Similar to the effects for appetitive behaviors, OVX rats treated with EB + DHT received more mounts, intromissions and ejaculations compared to both oil control groups, and up to similar levels to those undergoing EB + P replacement. As already described in another experimental setting (Jones et al., 2017), this increase in males' responsiveness might be a direct consequence of females displaying more hops/darts and solicitations and thus being more attractive to the males who then are enticed to chase and mount them accordingly. In the

present study, we also evaluated female-male mounting, failing to observe a significant stimulation by DHT alone or EB + DHT. However, this parameter has been strongly linked to sexually sluggish or non-copulating males, as females mount a male when he is nonresponsive (Afonso and Pfau, 2006).

Administration of DHT in rats primed with EB also resulted in both enhanced lordosis magnitudes (expressed by LR) and the lordosis to mount ratio (expressed by LQ) relative to negative controls. Lordosis, a stationary dorsiflexion of the spine that allows the male to mount and intromit, is the hallmark of receptive behavior in female rats (Beach, 1976). The specific involvement of DHT in facilitating not only appetitive behaviors but also lordosis emphasizes its central effects and supports the view that the neural pathways underlying appetitive and receptive phases of rat sexual intercourse are deeply intertwined (Pfau, 2006). Indeed, earlier works hypothesized that receptive behavior was specifically stimulated by P administration (Pfaff et al., 1994). In particular, as reviewed by Pfau, treating OVX females with EB alone produces only a moderate activation of lordosis in response to male stimulation, whereas its full expression normally depends on additional activation by P (Pfau, 2006). Therefore, our finding that LR and LQ were increased by EB + DHT up to levels equivalent to the EB + P regimen is of particular interest. Even though the possibility of a direct action of DHT on its receptor might be an interpretation of the present findings, we cannot exclude a cross reactivity between DHT and the P receptor, due to the high sequence homology between their respective receptor ligand-binding domains (Gao et al., 2005).

Another important novelty of the present study was the employment of Matrix and T-pattern analyses to deeply investigate the effect of DHT



Fig. 5. Percent distribution of T-patterns detected in EB + P group (n = 12) and EB + DHT group (n = 12). TP: T-pattern. Chi-square test, * = $p < 0.0005$.

on the dynamics of sexual behavior. Indeed, even though such methodologies are considered valuable tools for behavioural testing in rodent models (Casarrubea et al., 2018; Santangelo et al., 2018), they have never been applied in studies focusing on sexuality. Interestingly, Matrix and T-pattern analyses were able to reveal several analogies and some divergences in the sexual behavior dynamics observed after EB + DHT or EB + P administration. By using Matrix analysis we found that both groups performed frequent transitions from hops/darts to copulation (receipt of mounts/intromissions/ejaculations together with lordosis), and vice-versa. This finding confirms the presence of a recurring behavioral loop in the performance of sexual intercourse in rats (Pfaus, 2006). Moreover, the adjusted residuals plot highlighted some aspects related to the influence of lordosis magnitude on the organization of sexual behavior which were identical in the EB + P and EB + DHT groups. In both groups, the absence of lordosis (LM0) was associated with female defenses, indicating a stop signal on sexual intercourse. In contrast, lordosis of every magnitude (LM1 to LM3) was more frequently-than-expected followed by appetitive behaviors, thus reinforcing the sexual activity cycle. Noteworthy, only LM3 displayed positive residuals in the direction of leaving, indicating an alternative behavioral negative feedback. Although both LM0 and LM3 were associated with a negative feedback on sexual behavior, the underlying motivational mechanism might be divergent. In fact, as proposed in Fig. 6, LM0 might be reasonably related to a rejection of sexual activity (low desire), whilst LM3 could mirror the achievement of sexual satisfaction. Expanding this concept, not only the magnitude of lordosis reflex appears as a marker of the female rat disposition within the sexual activity cycle, as previously described in bilevel chambers (Pfaus et al., 1999), but its systematic evaluation from a behavioural dynamic

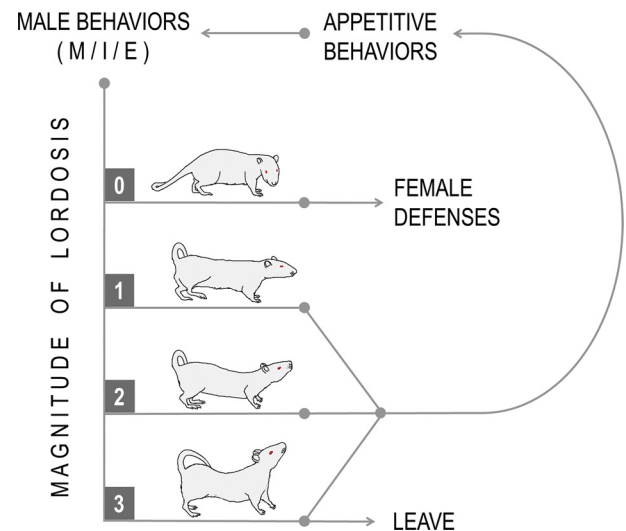


Fig. 6. Graphical representation of the magnitude of lordosis in the behavioral loop of rat sexual intercourse. Both LM0 and LM3 were associated with a negative feedback on sexual behavior; however, LM0 might be reasonably related to a rejection of sexual activity (low desire), whilst LM3 could mirror the achievement of sexual satisfaction. LM = lordosis magnitude.

perspective allowed us to suggest a formalization of its significance in the unilevel bisected testing apparatus. Indeed, while LM0 terminates sexual interaction, LM3 alternatively leads to appetitive behaviors (i.e. hops/darts), thus reinforcing the stimulus towards the male and starting

a new copulation bout, or is followed by the female leaving the male's side (Fig. 6). This can be interpreted as a display of contentment following male's ejaculation, in response to the male's post-ejaculatory refractory period, preceding a new ejaculatory series. However, if ejaculation did not occur, leaving the male's side also allows the female to put as much distance as possible between herself and the male, to eventually make a full solicitation.

Additionally, T-pattern analysis revealed the recurrence of hidden sequences of sexual-related behaviors only in EB + DHT and EB + P groups, and not in the two oil groups (O + O and O + DHT). T-pattern analysis is indeed a technique that allows for the assessment of the structure of behavior with a more complete representation, while encompassing sequences of behaviors that would be invisible to conventional analysis. In particular, T pattern-analysis demonstrated that EB + DHT was associated with hidden sequences containing Solicitation, that appears as a wider, longer and more robust movement than hops/darts and may therefore be considered as the strongest proxy of appetitive behavior. Interestingly, in the EB + DHT group hidden appetitive behaviors were not only present in strings containing male behaviors (mounts, intromissions or ejaculations), but they also appeared as standing-alone in complex (length ≥ 3) strings. In contrast, in the EB + P group complex hidden sequences included only appetitive behaviors associated with male mounts, intromissions, or ejaculations. Therefore, T-pattern analyses of hidden behavioral activity suggest that DHT modifies male-female sexual interplay by triggering appetitive behaviors either immediately linked or not to the copulatory act. Ultimately, T-pattern analyses revealed subtle peculiarities of sexual behavior in rats treated with EB + DHT, suggesting that these statistical techniques might be employed to thoroughly explore the effect of hormonal manipulations in this and other animal models.

Although evidence exists suggesting that DHT may exert its effects on sexual response by acting on the brain, the complex underlying mechanisms have not been clarified. ARs have a similar, broad distribution in the brain of rodents, primates and adult humans (Pelletier, 2000) with the highest levels described in the hypothalamic nuclei involved in modulating neuroendocrine and reproductive functions, including the ventromedial hypothalamus (VMH), arcuate nucleus, periventricular nucleus and medial preoptic area (mPOA) (Handa and McGivern, 2002). In these and other regions, such as the mesolimbic reward system, androgens may modulate sexual function mainly acting on dopaminergic neurotransmission (Sanderson et al., 2008; Hermans et al., 2010). It is worth emphasizing that, beyond a central action, several lines of evidence from preclinical and clinical studies demonstrate that a peripheral role of androgens in genitourinary physiology is also crucial. It has been widely reported that androgens, via their actions on AR, are critical for modulating hemodynamics and maintaining structural and functional integrity in female genital tissues (reviewed by Traish et al., 2018). In particular, in vitro animal studies showed that androgens positively regulate the relaxant machinery in smooth muscle cells, both in the clitoris (Comeglio et al., 2016) and in the vagina (Kim et al., 2004). Although these mechanisms are related to the peripheral arousal response, their potential involvement in the present experiments has to be taken into account. Future studies on similar animal models should address histological and functional changes of genital tissues following the administration of different hormone regimens, including DHT.

A few limitations of the present work have to be recognized. First, rats treated with E + O were not included in the investigation of sexual behavior dynamics, as they were dropped from further analyses once significant differences with the O + O group on the main outcome measure (appetitive behaviors) had been excluded. Second, the lack of a naturally cycling, sexually receptive group may be considered as a limitation. Finally, while hypothesizing a central action of DHT on the brain, we cannot rule out a peripheral stimulating effect, which could have been blunted, for example, by anesthetization of the clitoris and external vagina. In this regard, in the previously mentioned study by

Baum et al. (1974), 200 mcg DHT administered daily for 17 days was found to increase both clitoral size and the growth of cornified papillae on the glans clitoris in Wistar OVX rats. In this study, lidocaine paste was applied on the clitoris and external vagina prior to behavioral tests in order to avoid peripheral stimulating effects, and compared with vaseline; interestingly, local anesthetization did not change the findings on the facilitation of mounting behavior in EB + DHT vs. EB only rats (Baum et al., 1974).

A strength of the study is the methodological design. First, by using the uni-level chamber, an apparatus specifically designed to allow the female to pace the copulatory contact, we selectively focused on female sexual behavior. Secondly, the other main novelty of our data lies in the study of behavioral dynamics, and in particular T-patterns, used for the first time to investigate qualitative effects of hormonal stimulation in this animal model. From a clinical perspective, our findings can provide useful input for developing new treatments for women with Hypoactive Sexual Desire Disorder (HSDD) targeting selective androgen signaling, in particular in women undergoing surgical or iatrogenic menopause. Indeed, the complications of surgical or iatrogenic menopause often affect young women and represent an important risk factor for HSDD, with a strong negative impact on quality of life (West et al., 2008). As reported by the recent Global Consensus Position Statement (Davis et al., 2019), there is an unmet need for the approval of testosterone treatments in postmenopausal women. It has also to be considered that testosterone therapy is aggravated by the risk of estrogen-dependent adverse events, due to aromatization; it follows that menopausal hormone-dependent cancers survivors, representing a population enriched in HSDD, are completely out of the indication for testosterone therapy. In this context, if a favorable action of DHT on sexual behavior is confirmed, it could be considered as the base for safe therapeutic options for HSDD in these populations.

In conclusion, in the present work we observed a significant facilitative effect of DHT on appetitive sexual behaviors (hops/darts and solicitations) and indices of sexual receptivity (lordosis rating, lordosis quotient, received mounts, intromissions, and ejaculations) in OVX, EB-primed Long-Evans rats. Most importantly, the array of sexual behaviors displayed by the EB + DHT group reached levels equivalent to that of the positive control (EB + P). Therefore, a direct stimulation by androgens on the female sexual response, independent of aromatization, has to be hypothesized. The study of behavioral dynamics, in particular T-patterns, demonstrated a specific stimulus of DHT both on appetitive behaviors linked to copulation and on repeated partner stimulations, independent of the stimulation-copulation loop. Future studies should address the molecular mechanism underpinning these effects.

Funding

This research was supported by a project grant from the Canadian Institutes of Health Research to JGP (IGH 162264), a studentship from Consejo Nacional de Ciencia y Tecnología (CONACyT) of Mexico to BLF and a PRIN ("Progetti di Rilevante Interesse Nazionale": 2017S9KTNE) to LV.

Declaration of Competing Interest

None.

Acknowledgements

The authors would like to thank Dr. Sherri Lee Jones for sharing research data.

References

Afonso, V.M., Pfau, J.G., 2006. Hormonal and experiential control of female-male

- mounting in the female rat. *Horm. Behav.* 49, 30–37. <https://doi.org/10.1016/j.yhbeh.2005.04.013>.
- Askew, E.B., Gampe Jr, R.T., Stanley, T.B., Faggart, J.L., Wilson, E.M., 2007. Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. *J. Biol. Chem.* 281, 25801–25816. <https://doi.org/10.1074/jbc.M703268200>.
- Bachmann, G., 2001. Physiologic aspects of natural and surgical menopause. *J. Reprod. Med.* 46 (3 Suppl), 307–315 Review.
- Barnett, S.A., 1967. Attack and defense in animal societies. *UCLA Forum Med. Sci.* 7, 35–56.
- Baum, M.J., Södersten, P., Vreeburg, J.T., 1974. Mounting and receptive behavior in the ovariectomized female rat: influence of estradiol, dihydrotestosterone, and genital anesthetization. *Horm. Behav.* 5, 175–190.
- Beach, F.A., 1976. Prolonged hormone deprivation and pretest cage adaptation as factors affecting the display of lordosis by female rats. *Physiol. Behav.* 16, 807–808.
- Beyer, C., Jaffe, R.B., Gay, V.L., 1972. Testosterone metabolism in target tissues: effects of testosterone and dihydrotestosterone injection and hypothalamic implantation on serum LH in ovariectomized rats. *Endocrinology* 91, 1372–1375.
- Boling, J.L., Blandau, R.J., 1939. The estrogen-progesterone induction of mating responses in the spayed female rat. *Endocrinology* 25, 359–371.
- Cabilio, S., 1996. Behavioral Observation Program.
- Casarrubea, M., Sorbera, F., Crescimanno, G., 2009. Multivariate data handling in the study of rat behavior: an integrated approach. *Behav. Res. Methods* 41, 772–781. <https://doi.org/10.3758/BRM.41.3.772>.
- Casarrubea, M., Sorbera, F., Santangelo, A., Crescimanno, G., 2012. The effects of diazepam on the behavioral structure of the rat's response to pain in the hot-plate test: anxiolysis vs. pain modulation. *Neuropharmacology* 63 <https://doi.org/10.1016/j.neuropharm.2012.03.026>. 310–221.
- Casarrubea, M., Magnusson, M.S., Anguera, M.T., Jonsson, G.K., Castañer, M., Santangelo, A., Palacino, M., Aiello, S., Faulisi, F., Raso, G., Puigarnau, S., Camerino, O., Di Giovanni, G., Crescimanno, G., 2018. T-pattern detection and analysis for the discovery of hidden features of behaviour. *J. Neurosci. Methods* 310, 24–32. <https://doi.org/10.1016/j.jneumeth.2018.06.013>.
- Comeglio, P., Cellai, I., Filippi, S., Corno, C., Corcetto, F., Morelli, A., Maneschi, E., Maseroli, E., Mannucci, E., Fambrini, M., Maggi, M., Vignozzi, L., 2016. Differential effects of testosterone and estradiol on clitoral function: an experimental study in rats. *J. Sex. Med.* 13, 1858–1871. <https://doi.org/10.1016/j.jsxm.2016.10.007>.
- Davis, S.R., van der Mooren, M.J., van Lunsen, R.H., Lopes, P., Ribot, C., Rees, M., Moufarge, A., Rodenberg, C., Buch, A., Purdie, D.W., 2006. Efficacy and safety of a testosterone patch for the treatment of hypoactive sexual desire disorder in surgically menopausal women: a randomized, placebo-controlled trial. *Menopause* 13, 387–396 Erratum in: *Menopause* 2006, 13, 850.
- Davis, S.R., Baber, R., Panay, N., Bitzer, J., Perez, S.C., Islam, R.M., Kaunitz, A.M., Kingsberg, S.A., Lambrinoudaki, I., Liu, J., Parish, S.J., Pinkerton, J., Rymer, J., Simon, J.A., Vignozzi, L., Wierman, M.E., 2019. Global consensus position statement on the use of testosterone therapy for women. *J. Clin. Endocrinol. Metab.* 104, 4660–4666. <https://doi.org/10.1210/je.2019-01603>.
- Davison, S.L., Bell, R., Donath, S., Montalto, J.G., Davis, S.R., 2005. Androgen levels in adult females: changes with age, menopause, and oophorectomy. *J. Clin. Endocrinol. Metab.* 90, 3847–3853.
- Erskine, M.S., 1989. Solicitation behavior in the estrous female rat: a review. *Horm. Behav.* 23, 473–502 Review.
- Fang, J., Clemens, L.G., 1999. Contextual determinants of female-female mounting in laboratory rats. *Anim. Behav.* 57, 545–555. <https://doi.org/10.1006/anbe.1998.1025>.
- Gao, W., Bohl, C.E., Dalton, J.T., 2005. Chemistry and structural biology of androgen receptor. *Chem. Rev.* 105, 3352–33570. <https://doi.org/10.1021/cr020456u>.
- Gelez, H., Greggain-Mohr, J., Pfau, J.G., Allers, K.A., Giuliano, F., 2013. Flibanserin treatment increases appetitive sexual motivation in the female rat. *J. Sex. Med.* 10, 1231–1239. <https://doi.org/10.1111/jsm.12094>.
- Giuliano, F., Pfau, J., Srilatha, B., Hedlund, P., Hisasue, S., Marson, L., Wallen, K., 2010. Experimental models for the study of female and male sexual function. *J. Sex. Med.* 7, 2970–2995. <https://doi.org/10.1111/j.1743-6109.2010.01960.x>. Erratum in: *J. Sex. Med.* 2010, 7, 3804.
- Gladkova, A.I., Karpenko, N.A., 1986. [Effect of testosterone and dihydrotestosterone on the sexual behavior of female rats]. *Farmakol. Toksikol.* 49, 33–37.
- Gladue, B., 1984. Dihydrotestosterone stimulates mounting behavior but not lordosis in female rats. *Physiol. Behav.* 33, 49–53.
- Handa, R.J., McGivern, R.F., 2002. Androgens and brain function: behavioral perspectives. In: Chawnsang, Chang (Ed.), *Androgens and Androgen Receptor*. Kluwer Academic Publishers, San Diego, USA, pp. 325–344. https://doi.org/10.1007/978-1-4615-1161-8_15.
- Hardy, D.F., Debold, J.F., 1971. Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. *Physiol. Behav.* 7, 643–645.
- Hermans, E.J., Bos, P.A., Ossewaarde, L., Ramsey, N.F., Fernández, G., van Honk, J., 2010. Effects of exogenous testosterone on the ventral striatal BOLD response during reward anticipation in healthy women. *Neuroimage* 52, 277–283. <https://doi.org/10.1016/j.neuroimage.2010.04.019>.
- Jones, S.L., Ismail, N., King, L., Pfau, J.G., 2012. The effects of chronic administration of testosterone propionate with or without estradiol on the sexual behavior and plasma steroid levels of aged female rats. *Endocrinology* 153, 5928–5939. <https://doi.org/10.1210/en.2012-1578>.
- Jones, S.L., Farrell, S., Gregory, J.G., Pfau, J.G., 2013. Sensitization of sexual behavior in ovariectomized rats by chronic estradiol treatment. *Horm. Behav.* 64, 8–18. <https://doi.org/10.1016/j.yhbeh.2013.04.012>.
- Jones, S.L., Germé, K., Graham, M.D., Roy, P., Gardner Gregory, J., Rosenbaum, S., Parada, M., Pfau, J.G., 2015. Vaginal stimulation attenuates the sensitization of appetitive sexual behaviors by estradiol benzoate in the ovariectomized rat. *Horm. Behav.* 75, 70–77. <https://doi.org/10.1016/j.yhbeh.2015.08.003>.
- Jones, S.L., Ismail, N., Pfau, J.G., 2017. Facilitation of sexual behavior in ovariectomized rats by estradiol and testosterone: a preclinical model of androgen effects on female sexual desire. *Psychoneuroendocrinology* 79, 122–133. <https://doi.org/10.1016/j.psyneuen.2017.02.018>.
- Kim, N.N., Min, K., Pessina, M.A., Munarriz, R., Goldstein, I., Traish, A.M., 2004. Effects of ovariectomy and steroid hormones on vaginal smooth muscle contractility. *Int. J. Impot. Res.* 16, 43–50. <https://doi.org/10.1038/sj.ijir.3901138>.
- Kow, L.M., Pfaff, D.W., 1975. Induction of lordosis in female rats: two modes of estrogen action and the effect of adrenalectomy. *Horm. Behav.* 6, 259–276.
- Leiblum, S.R., Koochaki, P.E., Rodenberg, C.A., Barton, I.P., Rosen, R.C., 2006. Hypoactive sexual desire disorder in postmenopausal women: US results from the Women's International Study of Health and Sexuality (WISHeS). *Menopause* 13, 46–56. <https://doi.org/10.1097/01.gme.0000172596.76272.06>.
- Magnusson, M.S., 2000. Discovering hidden time patterns in behavior: T-patterns and their detection. *Behav. Res. Methods Instrum. Comput.* 32, 93–110.
- Magnusson, M.S., Burgoon, J.K., Casarrubea, M., 2016. *Discovering Hidden Temporal Patterns in Behavior and Interaction*. Neuromethods Vol. 111. Springer, New York, NY.
- Panay, N., Al-Azzawi, F., Bouchard, C., Davis, S.R., Eden, J., Lodhi, I., Rees, M., Rodenberg, C.A., Rymer, J., Schwenkhagen, A., Sturdee, D.W., 2010. Testosterone treatment of HSDD in naturally menopausal women: the ADORE study. *Climacteric* 13, 121–131. <https://doi.org/10.3109/13697131003675922>.
- Pelletier, G., 2000. Localization of androgen and estrogen receptors in rat and primate tissues. *Histol. Histopathol.* 15, 1261–1270. <https://doi.org/10.14670/HH-15.1261>.
- Pfaff, D.W., 1980. *Estrogens and Brain Function*. Springer, New York.
- Pfaff, D.W., Schwartz-Giblin, S., McCarthy, M.M., Kow, L.M., 1994. Cellular and molecular mechanisms of female reproductive behavior. In: Knobil, E., Neil, J.D. (Eds.), *The Physiology of Reproduction*. Raven Press, New York, USA, pp. 107–220.
- Pfau, J.G., 2006. Of rats and women: preclinical insights into the nature of female sexual desire. *Sex. Relation. Ther.* 21, 463–476. <https://doi.org/10.1080/14681990600967011>.
- Pfau, J.G., Smith, W.J., Coopersmith, C.B., 1999. Appetitive and consummatory sexual behaviors of female rats in bilevel chambers. *Horm. Behav.* 35, 224–240.
- Pfau, J.G., Kippin, T.E., Coria-Avila, G., 2003. What can animal models tell us about human sexual response? *Annu. Rev. Sex Res.* 14, 1–63.
- Pfau, J.G., Jones, S.L., Flanagan-Cato, L.M., Blaustein, J.D., 2015. Female sexual behavior. In: Plant, T.M., Zeleznik, A.J. (Eds.), *Knobil and Neill's Physiology of Reproduction* (Fourth Edition). Academic Press, pp. 2287–2370.
- Sanderson, N.S., Le, B., Zhou, Z., Crews, D., 2008. Preoptic neuronal nitric oxide synthase induction by testosterone is consistent with a role in gating male copulatory behavior. *Eur. J. Neurosci.* 27, 183–190. <https://doi.org/10.1111/j.1460-9568.2007.05989.x>.
- Santangelo, A., Bortolato, M., Mosher, L.J., Crescimanno, G., Di Giovanni, G., Cassioli, E., Ricca, V., Casarrubea, M., 2018. Behavioral fragmentation in the DICT-7 mouse model of Tourette's syndrome. *CNS Neurosci. Ther.* 24, 703–711. <https://doi.org/10.1111/cns.12789>.
- Simon, J., Braunstein, G., Nachtigall, L., Utian, W., Katz, M., Miller, S., Waldbaum, A., Bouchard, C., Derzko, C., Buch, A., Rodenberg, C., Lucas, J., Davis, S., 2005. Testosterone patch increases sexual activity and desire in surgically menopausal women with hypoactive sexual desire disorder. *J. Clin. Endocrinol. Metab.* 90, 5226–5233. <https://doi.org/10.1210/jc.2004-1747>.
- Spuijdt, B.M., Gispén, W.H., 1984. Behavioral sequences as an easily quantifiable parameter in experimental studies. *Physiol. Behav.* 32, 707–710.
- Traish, A.M., Vignozzi, L., Simon, J.A., Goldstein, I., Kim, N.N., 2018. Role of Androgens in Female Genitourinary Tissue Structure and Function: Implications in the Genitourinary Syndrome of Menopause. *Sex. Med. Rev.* 6, 558–571. <https://doi.org/10.1016/j.sxm.2018.03.005>.
- Van Den Berg, C.L., Van Ree, J.M., Spuijdt, B.M., 1999. Sequential analysis of juvenile isolation-induced decreased social behavior in the adult rat. *Physiol. Behav.* 67, 483–488.
- van Lier, H., Coenen, A.M., Drinkenburg, W.H., 2003. Behavioral transitions modulate hippocampal electroencephalogram correlates of open field behavior in the rat: support for a sensorimotor function of hippocampal rhythmic synchronous activity. *J. Neurosci.* 23, 2459–2465.
- Vanderschuren, L.J.M.J., Spuijdt, B.M., Hol, T., Niesink, R.J.M., Van Ree, J.M., 1995. Sequential analysis of social play behavior in juvenile rats: effects of morphine. *Behav. Brain Res.* 72, 89–95.
- West, S.L., D'Aloisio, A.A., Agans, R.P., Kalsbeek, W.D., Borisov, N.N., Thorp, J.M., 2008. Prevalence of low sexual desire and hypoactive sexual desire disorder in a nationally representative sample of US women. *Arch. Intern. Med.* 168, 1441–1449. <https://doi.org/10.1001/archinte.168.13.1441>.