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Estradiol, reproductive cycle and preference behavior in a northern swordtail

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ABSTRACT

Estrogen is associated with female sexual behaviors, particularly receptive behaviors during the reproductive cycle. Less is known about the relationship between estrogen and female preference behaviors that may precede receptivity and copulation. Separating the mechanisms underlying preference from receptivity is often confounded by the tightly coupled cycle- or estrogen-dependent expression of female sexual behaviors. Here we utilize a live-bearing poeciliid (*Xiphophorus nigrensis*), a model species for studying the evolution of female mate choice that can store sperm over multiple brood cycles. We assayed estradiol along with preference, receptivity and locomotor behaviors in gestating females and then re-tested these females on days 1, 7, 14, 21, and 28 post-parturition. With *a posteriori* reproductive cycle assessment, we asked whether reproductive state predicts differences in (i) estradiol levels, and (ii) behaviors (preference, receptivity, and general locomotor activity). We then examined if estradiol levels (independent of reproductive state) explain any variation in these behaviors.

We found that endogenous estradiol levels vary across the reproductive cycle: gestating females had lower estradiol levels than those undergoing vitellogenesis/fertilization. In contrast, receptivity and preference behaviors did not vary over the reproductive cycle. Estradiol levels did not predict variation in receptive behavior, but were associated with increased locomotion. While individual female preference behaviors were consistent across the reproductive cycle, there was a small *negative* relationship between estradiol and preference behaviors explaining between 3% and 10% of the inter-female variation in preference behavior. Our data indicate *X. nigrensis* females may exhibit a facultatively dissociated reproductive system.

1. Introduction

Female reproductive cycles exist to coordinate the expression of sexual behavior and can be regulated by a variety of external and internal cues (reviewed [36]). In many taxa, steroid hormones are associated with a reproductive cycle as a mechanism to coordinate the timing of female reproductive effort with a maximum chance for reproductive success. This classic paradigm is known as an associated reproductive system wherein ova production, steroid hormones and sexual behaviors cycle together [10]. For instance, in the rat, female sexual behaviors are strongly associated with reproductive cycle status, and behavioral estrus (i.e. the willingness to perform proceptive/solicitation and receptive behaviors towards males) is expressed primarily during the proestrus/estrus stages of the ovarian cycle when estrogen levels are high, ovulation is imminent, and the female is willing to accept copulation (reviewed in [15]). In other systems such as the red-sided garter snake, sexual behaviors and steroid hormones are decoupled. These systems display a dissociated reproductive pattern wherein steroid hormone levels and ova production do not coincide with sexual behavior, and mating behavior can occur with very low levels of circulating steroid hormones (e.g. [10]).

The relationship between hormones and other critical behaviors leading to reproduction has been under-studied. Specifically, the role that hormones play in female mate discrimination or preferences for specific male phenotypes has only recently received attention [1]. In associative reproductive systems, changes in hormone levels have been shown to correlate with changes in receptivity, permissiveness and discrimination behaviors in female anurans [28,29], while in mice, peptide hormones may play a role in governing female response to dominant males [30]. However, in these systems the role of steroid hormones in female preferences may act primarily in a motivational aspect of mate choice: driving females to prefer to associate with opposite sex partners rather than mediating a discriminatory function amongst different males [1,7,37].

Little is known about the social and physiological influences underlying female preference in a dissociated reproductive system. The de-synchronization of reproductive behavior and gamete production can arise from physical constraints (e.g. in garter snakes, [16]; and sea perch, [54]) or social environment (e.g. sperm competition, see [4]). In many poeciliid fish species, a single mating bout

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can fertilize multiple subsequent broods, and broods with evidence of multiple paternity appear to be quite common [5,8,40,48]. Poeciliids are ovoviviparous and breed throughout the year in both natural and laboratory conditions [34]. Temporal patterns of brood production vary across species, but in swordtails (genus *Xiphophorus*), multiple ova cohorts at different levels of development are present in the ovary, although only one primary brood is fertilized and actively gestating at a time [50]. Whether driven by environment or social pressures, any physiological modification that allows gamete storage can then allow the de-coupling of the suites of behaviors associated with mate preference and sexual behavior from the steroid hormone levels necessary for gamete production. Such systems may favor expression of preference behaviors independent of reproductive cycle: allowing females to discriminate and mate amongst potential male partners at any stage of ova development.

In many spawning fish such as the goldfish, prostaglandins are the hormonal trigger for female sexual behavior (reviewed in [35]). In live-bearing fish, however, estrogen plays a key role in inducing receptivity. Much early poeciliid research focused on female reproductive cycling [24,40,47,50–52] and the endocrine factors underlying female receptivity [25,26]. Steroid hormone levels cycle along with brood development: estrogen is highest during fertilization/parturition phases of the reproductive cycle and then declines during gestation [22,52]. In some poeciliids, female receptivity is tightly coupled to reproductive cycle, and females are generally most receptive during the brief period after parturition when the next brood of ova is ready to be fertilized [24,25]. However, it is less clear whether female preference behavior is necessarily confined to discrete phases of the reproductive cycle or is estrogen-dependent.

In recent decades, poeciliids have also become a model system in sexual selection for female mate choice studies [2,12,18, 32,42,43], and preferences for discrete male phenotypes are very tractable in laboratory conditions. Preference (e.g. association bias, [33,53]) and receptivity (e.g. glides, [11]) behavioral measures in laboratory dichotomous mate choice experiments have proven to be reliable indicators of reproductive choices and copulation events, respectively. In *Xiphophorus nigrensis*, males come in genotypically-determined size classes with larger male classes that develop swords, colorful ornamentation, and court females, and small class non-ornamented males that use a female chase strategy to acquire matings [19,41]. Most females show a strong and consistent preference for the largest size class males over the smallest size class [11,43].

Here we ask if preference behavior is dependent on reproductive cycle stage in *X. nigrensis* females. We also test whether individual variation in the expression of preference, receptivity or general activity is linked to endogenous estrogen (17β-estradiol) levels in intact *X. nigrensis* females.

2. Materials and methods

We measured estradiol levels and quantified male-oriented behaviors in gestating female *X. nigrensis* fish and then at weekly intervals post-parturition. We then analyzed the association between reproductive cycle status and/or estradiol levels on individual variation in preference, receptive and general locomotor behaviors.

2.1. Collection and monitoring of test animals

Pregnant *X. nigrensis* females ($N = 21$) were obtained from semi-wild populations held at Brackenridge Field Laboratories (University of Texas) or from populations originally wild-caught at the Nacimento de Rio Choy in San Luis Potosi, Mexico. In the laboratory, fish were isolated and kept at a 12:12 photoperiod in a temperature-controlled (~27 °C) room. Initial pregnancy status was estimated by visual assessment of the size of the distended abdomen and brood patch (a visible pregnancy sign; [3,39]), and females were checked daily for parturition. Female standard lengths (SL; body length from the tip of the snout to the anterior edge of the caudal fin) ranged from 21.1 to 44.3 mm. Female estradiol (E_2) levels and preference behaviors were tested before parturition, and then tested again on Days 1, 7, 14, 21, and 28 post-parturition (parturition = Day 0). Three females gave birth before they could be given a pre-parturition trial, so those three had a total of 5 trials (all post-parturition), while the rest ($N = 18$) had a total of 6 trials. All trials took place between August 2007 and June 2008.

2.2. Reproductive cycle assessments

The presence or absence of stored sperm in poeciliids appears to determine whether sexually isolated females follow parturition with another brood development cycle (red circle in Fig. 1A) or enter an indeterminate stage with intermittent phases of vitellogenesis and unfertilized egg reabsorption (blue circle in Fig. 1A). With no *a priori* knowledge of whether females had stored sperm in this study, we could only assess reproductive cycle status *a posteriori*. Therefore, we monitored all 21 females for a second parturition. All trials in which females were known to be actively gestating a brood were assigned as gestation trials, and so include not only all the original pre-parturition trials but also those trials taking place during the gestation period for females who produced a second brood ($N = 6$; referred to as 'sperm-present' females, Fig. 1B). We estimated the gestation period as 27 days per our temperature and lighting regimen (see also [47]). Therefore, we subtracted 27 days from any second parturition dates and assigned trials taking place during this period as gestation category trials. Trials taking place in the window following the initial parturition date and prior to the 27-day gestation period were assigned to a vitellogenesis/fertilization category because the vitellogenesis/egg maturation and fertilization phases cannot be distinguished *a posteriori*. The cycle lengths for the 6 "sperm-present" category females were 28, 39, 42, 41, 56, and 62 days. The variation in cycle length is assumed to occur with the timing of egg maturation and fertilization, not gestational length [8,47]. The remaining 15 females did not produce a second brood during the experiment and were assigned to a putatively sperm-depleted 'indeterminate' category (Fig. 1B). The indeterminate group is likely to be heterogeneous in that it represents females undergoing vitellogenesis as well as the reabsorption stages, and so this group contains females at very different reproductive conditions.

2.3. Sexual isolation

Of the 21 females in the study, most ($N = 17$) were isolated from males <7 days prior to onset of the experiment and were termed 'short-term isolates' (including all 6 sperm-present females). Four females were isolated from males >60 days prior to the onset of the experiment and were labeled 'long-term isolates'. Long-term isolation had no effect on our behavioral measures, but did have a significant effect on E_2 levels ($t = 2.577$; $p = .019$; data not shown). Therefore, the long-term isolate females were removed from the reproductive cycle status and individual variation in E_2 and behavior analyses. However, including them (full data set $N = 21$ versus $N = 17$ short-term isolate data set) produces similar results in both reproductive status and individual variation analyses (data not shown).

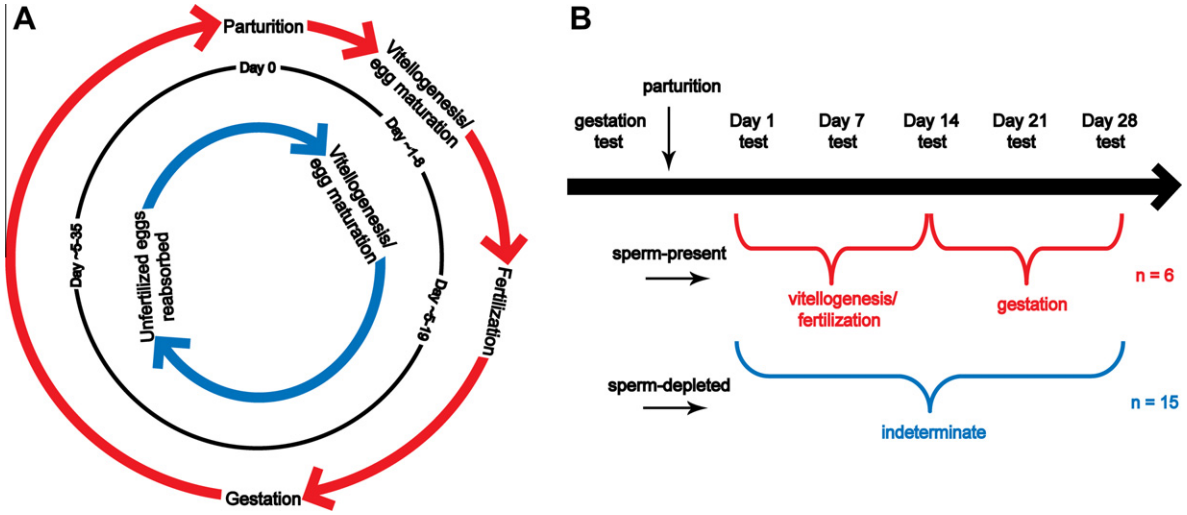

Fig. 1. Reproductive cycle in sexually isolated female *Xiphophorus nigrensis* fish. (A) Circle depicts two possible paths through the *Xiphophorus nigrensis* reproductive cycle. The red outer circle represents the path for females who have fertilized a subsequent brood from stored sperm. This path includes vitellogenesis/egg maturation, fertilization and gestation. The blue inner circle represents the path for females who did not fertilize a subsequent brood, and so can be considered sperm-depleted. This path includes vitellogenesis/egg maturation but no fertilization or gestation phases. Instead, cohorts of ova are re-absorbed. Timing estimates for the various phases of the reproductive cycle overlap to reflect the natural variation in exact cycle length across fish. (B) Assigning reproductive cycle status (brood production category). Females were tested pre-parturition and then again on days 1, 7, 14, 21, and 28 post-parturition. All pre-parturition trials were assigned as gestation trials. For the post-parturition trials, females were designated as either sperm-present ($N = 6$) or sperm-depleted ($N = 15$) based on whether they gave birth to a subsequent brood. For those who did develop another brood, trials were assigned as either gestation or vitellogenesis/fertilization categories (see Section 2). All post-parturition trials for the sperm-depleted females were assigned into the indeterminate category. Reproductive cycle lengths for the sperm-present category fish were quite variable, so the bracket positions designating trial dates as either vitellogenesis/fertilization or gestation categories are intended for illustrative purposes and do not imply all Day 1/7 trials were vitellogenesis/fertilization nor all Day 21/28 trials were gestation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Behavior trials

Females were placed into a non-contact dichotomous mate choice setup as described in [13]. Females were exposed to a large and small male stimulus pair (LS). Males were isolated from semi-wild BFL populations ($N = 9$ large class males; defined as SL > 31 mm, actual sizes ranged from SL 32.4 to 50.7 mm; $N = 8$ small class males; defined as SL < 25 mm, actual sizes ranged from SL 19.3 to 22.8 mm; see [19] for size class designations). Each female was exposed to the same LS stimulus pair for each test across the reproductive cycle (with 17 unique LS pairs used in total), with the exception of three females whose small males were replaced because two small males died during the course of the experiment. The testing aquarium was comprised of a center compartment and two side compartments divided by UV-transparent Plexiglas dividers. The center compartment consisted of 3 equal subzones: left and right association zones and a central neutral zone containing a plastic plant. Lighting conditions recreated the natural lighting conditions found in their native Rio Choy, San Potosi habitat in Mexico [12].

A male of each pair was placed in each of the end compartments and acclimated for 30 min. The female was placed in an opaque cylinder in the neutral zone and allowed to acclimate to the testing tank for 5 min without visual contact with the males. Once the cylinder was lifted, the female was allowed to acclimate to the testing conditions for 1 additional minute. We quantified and videotaped female behaviors during the 30 min trial. The males were switched midway through the trial to control for female side bias. We assessed female preference by three measures (association bias, glide bias, and a time + behavior composite preference score); receptivity by counting total glide displays; and general locomotor activity by counting total transits across the tank (see Table 1 for definitions).

2.5. Hormone assays

Estradiol (E_2) levels were measured using a non-invasive holding water assay. All materials were rinsed in 100% ethanol (EtOH) prior to use for each fish. Females were placed into a 250 ml glass beaker containing 150 ml of reservoir water for 1 h. Reservoir

Table 1
Xiphophorus nigrensis behaviors.

Behavior measure	Definition
Preference behaviors	Biased behaviors toward the preferred male; (preferred male = male a, where time with male a > time with male b); includes both time and behavior measures
Association bias	Proportion of time a female spends in the association zone with male a*; (time with male a/(time with male a + male b))
Glide bias	Proportion of glide displays performed toward male a*; (glides toward male a/(total glides performed to male a + male b))
Preference score	Composite preference score: association bias + log ((1 + # glides to male a*)/total transits)
Receptivity behavior	Behaviors directed towards a male that may precede putative copulation events during barrier free trials Ref. [11]
Total glides	Glides are a social display behavior wherein the female orients toward the male and then swims away from him in a slow swim, turns, and then returns to the barrier; total glides are the total number of glide displays a female performs during the trial and can be directed toward either male
General activity	Non-social general locomotor activity during the trial
Total transits	Number of times the female crosses out of an association zone and into the neutral zone

*Male a, where time with male a > time with male b.

water is aerated and temperature-controlled dechlorinated tap water (Prime, Seachem), and is the source for all home and experimental tank waters. To reduce stress on the fish, a brown paper towel was placed around the beaker throughout the 1-h immersion. Confinement within the measurement beaker produced no noticeable stress-related behavioral changes in our fish (personal observation), however, this procedure can cause a stress response in some fish [14,55]. Due to the small size of our fish we could not concurrently measure cortisol and estradiol levels from the same sample, but future studies should include cortisol measures to assess stress in our immersion assays. Following immersion, females were placed immediately into the behavior trial. Water samples were stored in polypropylene bottles at −20 °C until E_2 extraction. Prior to storage, water samples were examined for visible feces or body matter, and samples with solids were then filtered with Whatman P5 filter paper (Fisher Scientific). Pilot experiments indicated that filtration did not affect E_2 measurement ($N = 4$; Related-Samples Wilcoxan Signed Ranks test, $p = .273$). Control reservoir samples (no fish exposure) were taken throughout the experiment ($N = 14$). There was no change in control reservoir water measurements over time ($r = .156$, $R^2 = .024$, $F = .298$, $p = .595$).

Steroid hormones were extracted from water samples using C18 Solid Phase Extraction columns (Sep-Pak® Plus C18 cartridge 55–105 μm; Waters Corporation, Milford, MA). The columns were attached to a 12-port vacuum manifold (Alltech Associates, Inc., Deerfield, IL), and steroid hormones extracted using a protocol modified from [20]. Columns were first primed with 6 ml 100% EtOH and then rinsed with 6 ml Nanopure (Barnstead, Thermo Scientific) water prior to sample extraction. Samples were pulled through the columns followed by another 6 ml of Nanopure water to remove any contaminating particles, and then stored at −20 °C until elution. Steroid hormones were eluted from the columns into 13 × 100 mm glass collection tubes (Fisher Scientific) with 4 ml 100% EtOH. The collection tubes were then placed in a 40 °C water bath and the EtOH evaporated under a stream of nitrogen gas. The resulting residue was resuspended in 350 μl Assay Buffer 3 (Assay Designs, Ann Arbor, MI), and for each sample, a 150 μl aliquot was run through the Correlate-EIA 17β-estradiol Enzyme Immunoassay Kit (Assay Designs) according to the manufacturers protocol. The EIA kit uses a rabbit polyclonal antibody to 17β-estradiol and has a sensitivity of 28.5 pg/ml. The use of the Assay Designs EIA kit for water-based E_2 measurement has been previously validated in a cichlid fish [20]. For the swordtail EIA, we verified that the regression slopes between the kit standard curve and a pooled sample serial dilution (6 points: 1:1, 1:4, 1:16, 1:64. 1:256, 1:1024) were parallel (ANCOVA test for homogeneity of slopes, $F = .497$, $p = .501$). Samples were run in duplicate. Previous optimization experiments indicated that 75 μl/well was the optimal amount of sample to result in pre-normalized E_2 concentrations well within the manufacturer's standard curve sensitivity (data not shown). Duplicate well values were then averaged for the final E_2 concentration/sample, which was then normalized by dividing the measured E_2 concentration by the fish weight. Hormone samples were run on eight 96-well EIA assay plates: inter-assay CV was 9.1%, and intra-assay CV was 3.3%.

2.6. Water immersion assay validation

Individual *X. nigrensis* females are too small to draw sufficient blood for traditional blood plasma-based E_2 assays. Therefore, to get repeated E_2 measures on individual fish we optimized a non-invasive water immersion protocol. Fish release free steroids from the gills that have been shown to mimic plasma steroid values [44–46]. To validate our assay, we pooled sexually mature females (nine groups, six individuals/group) and compared blood plasma and holding water E_2 levels using the same Correlate-EIA 17β-estradiol Enzyme Immunoassay Kit for each method. Validation water assays were conducted as described above except the hormone pellet was resuspended in 300 μl Assay Buffer 3 rather than 350 μl. For plasma assays, blood was drawn from each female via dorsal aortal puncture within 60 min of water immersion. Whole blood was spun 15 min at 2000 rpm. Plasma was removed and stored at −80 °C. Plasma was then pooled from 6 individuals (1.1 μl plasma/individual) for use in the assay. We then ran a linear regression on the normalized averaged waterborne E_2 levels for each group of 6 and the pooled plasma E_2 concentrations. E_2 levels were significantly correlated between pooled serum measures and pooled holding water measures (Fig. 2; Pearsons $r = .798$, $R^2 = .637$; $F = 12.30$, $p = .010$).

2.7. Statistics

To analyze changes in E_2 levels and behaviors across the reproductive cycle, each trial was assigned a brood production category (Fig. 1). We analyzed this dataset in two ways: (1) Females of determined reproductive status ($N = 6$ 'sperm-present' females) were analyzed with paired Wilcoxon test between vitellogenesis/fertilization and gestation cycle phases. For these comparisons, the initial pre-parturition gestation trial scores were used as the "gestation" category trial and "vitellogenesis/fertilization" category was an average of Day 1 and Day 7 scores. (2) Short-term isolation females ($N = 17$ females isolated from males <7 days prior to initial parturition, including females of known reproductive status ($N = 6$) and ($N = 11$) indeterminate category females) were evaluated with repeated measures correlational analysis (Hierarchical Linear Modeling (HLM 6), Scientific Software International (SSI); http://www.ssicentral.com/) between the 3 cycle categories (vitellogenesis/fertilization, gestation and indeterminate).

In the full repeated measures data set (HLM analyses), each fish was designated as a level 2 unit such that the individual trial data (level 1 units) were nested within each fish. We examined whether reproductive cycle status or E_2 could explain variation in female preference behavior at both the within (level 1) and between (level 2) female levels. We first looked at the explanatory power of reproductive cycle status for predicting E_2 levels or behavior. We created models with E_2 estimates or relevant behaviors (i.e. three preference behaviors, receptivity, general locomotion) as outcome variables and reproductive cycle status as the fixed effect

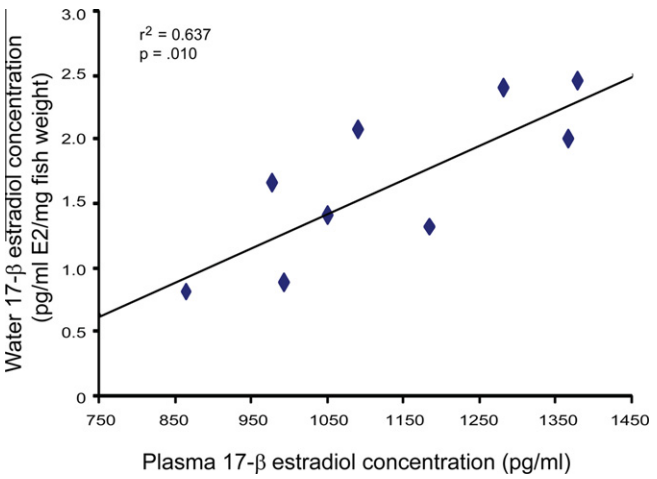

Fig. 2. Pearsons correlation and linear regression between water and plasma 17β-estradiol (E_2) levels for pooled *X. nigrensis* samples. Each pooled sample ($N = 9$) contained pooled serum from 6 individuals. Water E_2 levels are presented as pg/ml E_2 concentration/mg fish weight. This to indicate that the *y*-axis values were normalized by dividing measured E_2 in holding water by body weight.

explanatory/predictor variable. We then looked at the explanatory power of endogenous E_2 on individual variation of these same behaviors by constructing a series of models with receptivity, general locomotion and each of the three preference behaviors as outcome variable and E_2 level as the fixed effect predictor variable. We also explored the relationship between E_2 and receptivity and preference behaviors within single sampling points (pre-parturition and Day 1 post-parturition) using standard linear regressions. These two sampling points were chosen because they consist of females under the most similar reproductive states – all females were gestating during the pre-parturition trial, and Day 1 served as a reset post-parturition for all females regardless of the presence of stored sperm.

In order to assess goodness of fit for the models, we used the Full Maximum Likelihood estimation setting in HLM. Model fit was tested with a Chi Dist test, where input was the difference in model deviance scores (χ^2) between the unrestricted null model (i.e. with no explanatory variable) and the restricted model (i.e. reproductive cycle status or E_2 as explanatory variables) and the degrees of freedom were equal to the difference in the number of estimated parameters between the null and restricted models.

The traditional least squares regression R^2 coefficient of determination cannot be calculated in maximum likelihood iterative estimations such as HLM [21]. Instead, we assessed the explanatory power of models containing reproductive cycle status or E_2 (level 1 explanatory variables) by calculating pseudo R^2 values for within- and between-unit variances [21,49]. In Table 2, pseudo R^2 across all trials (the within-unit variance) addresses how well reproductive cycle status or E_2 explains the outcome variable (i.e. the 5 measured behaviors) across all trials, where within pseudo R^2 = (null level 1 error – restricted level 1 error)/null level 1 error). Pseudo R^2 between fish explains the amount of variance explained by our predictor variables between fish, where pseudo R^2 between = (null level 2 error – restricted level 2 error)/null level 2 error). An uninterpretable negative pseudo R^2 value results if the inclusion of a predictor variable increases the variance component over the null model [17,21], and is denoted by N/A in Table 2.

3. Results

3.1. Reproductive cycle

Within the six females with a determinant reproductive state (sperm-present group), females had lower levels of E_2 during their initial (pre-parturition) gestation trial than in trials during their vitellogenesis/fertilization phase (pre-parturition gestation mean = 4.89 ± 2.14 pg/ml; vitellogenesis/fertilization mean = 11.01 ± 8.96 pg/ml; Wilcoxan Signed Ranks $Z = -1.992$; $p = .046$, Fig. 3A). In contrast, none of the preference measures, receptivity displays, or general locomotor activity changed over the cycle in these females (Fig. 3B–F).

Looking across all 17 short-term isolated females, an overall HLM model containing reproductive cycle status as a predictor variable explained significantly more of the variance in estradiol levels than did the estradiol null model (Chi Dist $p = 0.043$). In contrast, overall models containing reproductive cycle status as a predictor for the three preference measures, receptivity, and general locomotion did not explain more variance than their respective null models (Table 2). Across the three reproductive cycle categories (vitellogenesis/fertilization, gestation, or indeterminate), reproductive cycle status continues to significantly predict E_2 levels (HLM; $t = -2.086$; $p = .040$; Fig. 4A). Similar to the analysis restricted to the sperm-present females, reproductive cycle status does not predict any of the preference behaviors (Fig. 4B, D and F), nor does it predict receptivity or general locomotion (Fig. 4C and E).

In deconstructing the variance components of the model, reproductive cycle status explains little trial-level variance. In fact, for all behaviors except for preference score, models including reproductive cycle status actually increased the variance over the null model (pseudo R^2 across all trials), resulting in an undefined, negative pseudo R^2 value (N/A, Table 2). Reproductive cycle status also had little explanatory power at the between-fish level (Table 2). There was no relationship between E_2 levels, preference behaviors, receptivity, or general activity by trial day (data not shown).

Table 2
Model fit and explained variance between models. For each behavior, model fit (difference in model deviance score; χ^2) and pseudo R^2 was assessed between the null and a restricted model with either reproductive cycle status or E_2 as a level 1 predictor variable for each behavior. In the χ^2 column, values represent the improvement in model deviance score from the null for each behavior. The pseudo R^2 across all trials column represents the within-unit variance (i.e. how well the predictor variable explains the outcome variable) while pseudo R^2 between fish column represents the between-unit variance (i.e. how much of the between fish variance in a particular behavior is accounted for by the predictor variable). N/A represents an undefined negative pseudo R^2 value such that the restricted model explained less variance than the null model. In the model equations, β_{00} = intercept; β_{10} = level 1 regression coefficient; r_0 = level 2 error, and e = level 1 error.

Model (all comparisons to null) $\beta_{00} + (0) * \text{repstatus} + r_0 + e$ $\beta_{00} + (0) * E_2 + r_0 + e$	χ^2	Pseudo R^2 across all trials	Pseudo R^2 between fish
Association bias			
$\beta_{00} + \beta_{10} * \text{repstatus} + r_0 + e$	0.079	N/A	0.020
$\beta_{00} + \beta_{10} * E_2 + r_0 + e$	6.750*	0.071	0.030
Glide bias			
$\beta_{00} + \beta_{10} * \text{repstatus} + r_0 + e$	0.871	N/A	0.024
$\beta_{00} + \beta_{10} * E_2 + r_0 + e$	7.033*	0.070	0.097
Prefscore			
$\beta_{00} + \beta_{10} * \text{repstatus} + r_0 + e$	1.880	0.042	N/A
$\beta_{00} + \beta_{10} * E_2 + r_0 + e$	13.926**	0.155	0.069
Total glides			
$\beta_{00} + \beta_{10} * \text{repstatus} + r_0 + e$	1.185	N/A	0.091
$\beta_{00} + \beta_{10} * E_2 + r_0 + e$	1.124	0.015	N/A
Total transits			
$\beta_{00} + \beta_{10} * \text{repstatus} + r_0 + e$	0.004	N/A	0.004
$\beta_{00} + \beta_{10} * E_2 + r_0 + e$	7.942*	0.070	0.149

* Indicates the model deviance Chi Dist test is significant $p < .01$.
** Indicates Chi Dist test is significant $p < .001$.

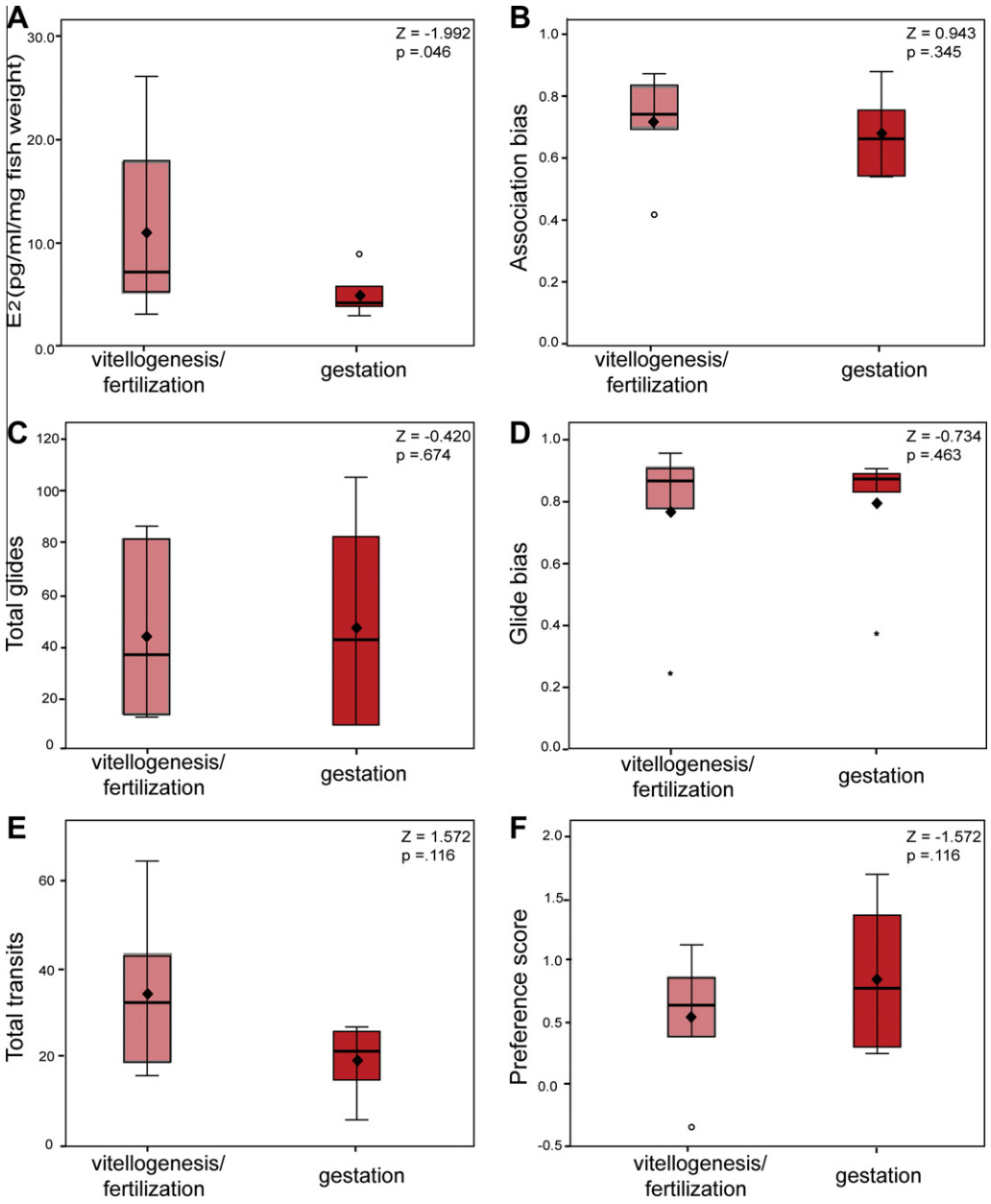

Fig. 3. Estradiol and behavior across the reproductive cycle: females with known post-parturition reproductive status. Paired Wilcoxon signed ranks tests indicate E_2 levels were significantly associated with reproductive cycle status (A). Estradiol levels (A) are presented as pg/ml E_2 concentration normalized by body weight (pg/ml E_2/mg fish weight). Three preference measures (association bias (B), glide bias (D), and preference score (F) as well as receptivity (total glides; C) and general locomotion (total transits; E) were not correlated with reproductive cycle status. $N = 6$. Box plots indicate 1st and 3rd quartiles, the line indicates the median, and the whiskers indicate high and low values. Outliers (1.5 interquartile range from the box end) are indicated by an open circle and extreme values (3 interquartile range from the box end) are indicated by an asterisk. See Table 1 for behavior definitions.

3.2. E_2 and individual variation in behavior

As with reproductive cycle status, we assessed overall model fit for E_2 on behavior (Table 2). Inclusion of E_2 as an explanatory variable creates a model with significantly better fit for locomotion and all 3 preference behaviors (Chi Dist $p < .01$; Table 2), but does not produce a better fit for receptivity measure (Chi Dist $p = .289$). The significant relationship between E_2 and preference behavior is a negative one. Females with higher E_2 levels were significantly less likely to express preference behaviors (Fig. 5A–C). Association bias ($t = -2.646$; $p = .010$), glide bias ($t = -2.704$; $p = .009$), and preference score ($t = -3.887$; $p < .001$) were negatively correlated with E_2 levels (Fig. 5A–C). Receptivity (total glides) was not related to E_2 levels (Fig. 5D), but general locomotion (total transits) was positively correlated with E_2 levels ($t = 2.887$; $p = .005$; Fig. 5E). In addition, body size did not predict preference behaviors, receptivity or general locomotion when it was included as an additional fixed effect variable in the estradiol models (data not shown).

Although these models explain significantly more variation than the null model, pseudo R^2 analyses indicates that E_2 is actually a relatively weak explanatory factor in the measured *X. nigrensis* behaviors (Table 2). For example, while endogenous E_2 levels are significant predictors of preference behaviors, they actually explain a relatively small amount of the variation in individual preference behavior, ranging from 3% to 9.7% (pseudo R^2 between fish) or 7–15.5% (pseudo R^2 across trials) depending on the measure (Table 2).

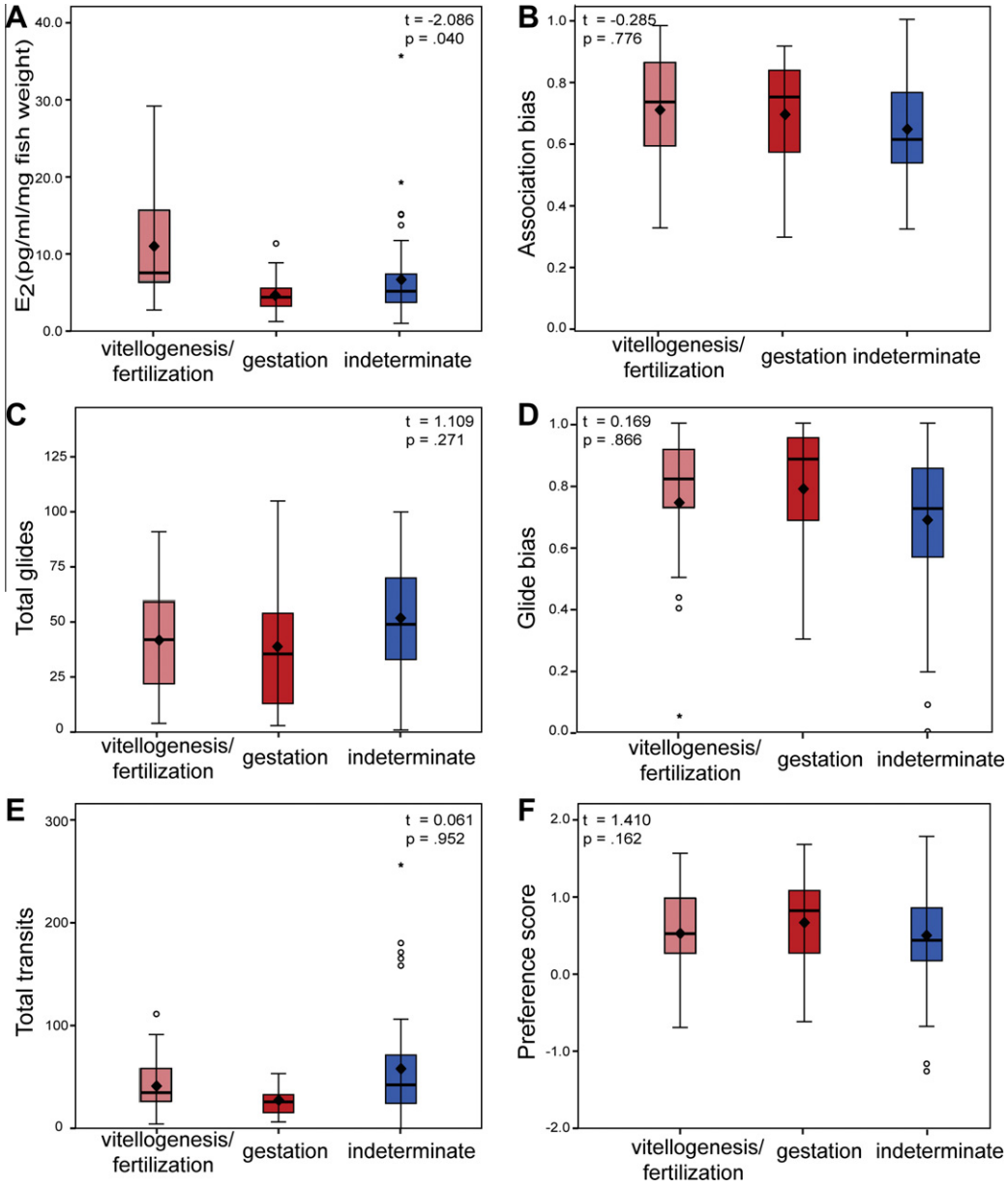

Fig. 4. Estradiol and behavior across the reproductive cycle: repeated measures analysis with females undergoing vitellogenesis/fertilization, gestation, or indeterminate reproductive cycle phases. Reproductive cycle status significantly predicts E_2 levels (A), but does not predict association bias (B), glide bias (D), preference score (F), receptivity displays (total glides; C) or general locomotion (total transits; E). $N = 17$. E_2 normalization is described in figure legends 2 and 3. Box plot descriptions are given in figure 3 legend.

Within-sampling point linear regression analyses of the relationship between E_2 and behaviors at pre-parturition and Day 1 post-parturition showed consistent patterns with the HLM repeated measure analyses. Similar to the HLM correlation results, there was a significant negative relationship between E_2 and the three preference measures (association bias, $r = -.633$, $R^2 = .400$, $F = 7.342$, $p = .020$; glide bias, $r = -.665$, $R^2 = .442$, $F = 8.706$, $p = .013$; preference score, $r = -.680$, $R^2 = .462$, $F = 9.451$, $p = .011$; data not shown), no relationship for receptivity and significant positive relationship with locomotion (total transits $r = .595$, $R^2 = .!354$, $F = 6.029$, $p = .032$; data not shown). In the Day 1 sampling point, although the direction of the relationships for all 5 behaviors was the same as the HLM repeated measures and pre-parturition point results, estradiol levels predicted only glide bias behavior ($r = -.564$, $R^2 = .263$, $F = 4.632$, $p = .051$).

Three females (see Fig. 5) had particularly high E_2 levels. To verify that the relationships between E_2 and our measured behaviors were not being overly influenced by these high E_2 trials, we re-ran the analyses with trials of females with >20 pg/ml/normalized by body weight removed. All patterns were consistent with the full data set analyses except total glides, which was now negatively correlated with E_2 ($t = -2.275$; $p = .026$; data not shown).

4. Discussion

Like many other vertebrates, estradiol levels in female *X. nigrensis* fish vary according to a female's stage in her reproductive cycle. However, unlike many other taxa, female preference and receptivity behaviors did not vary by female reproductive stage. This disassociation between preference behavior and reproductive stage may

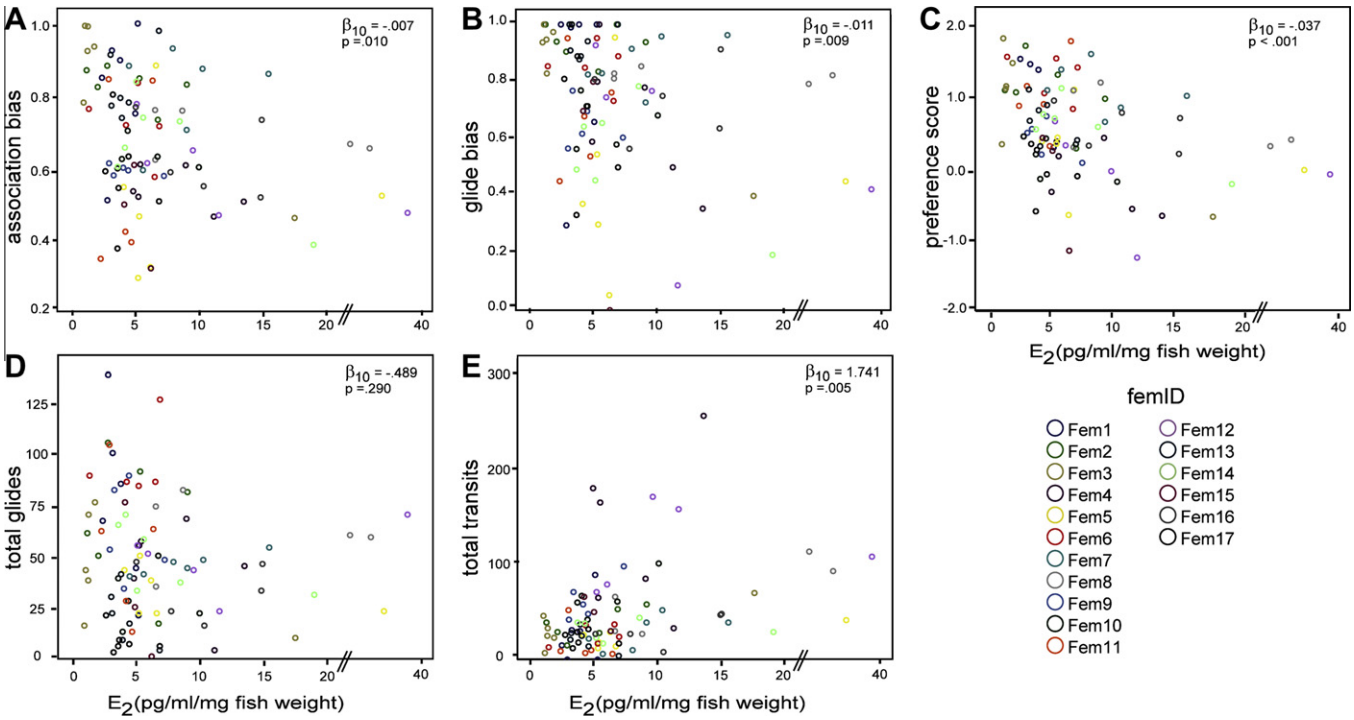

Fig. 5. Estradiol and individual variation in behavior. Pre-behavior trial E_2 levels are negatively correlated with association bias (A), glide bias (B), and preference score (C). E_2 is not correlated with receptivity displays (D), but is positively correlated with general locomotor activity (E). For all graphs, each fish is represented by color (see legend for fish ID) such that each trial from a particular fish is the same color within each graph. $N = 17$. For each scatter plot, β_{10} values are the regression coefficient (slope) for E_2 on each behavior. E_2 normalization is described in figure legends 2 and 3.

partly explain why endogenous levels of E_2 only explained a small portion of the variation in inter-female preference behaviors (3–10% depending on the measure; Table 2).

For *X. nigrensis* females, there are two paths through the reproductive cycle based on sperm presence and fertilization (Fig. 1; [47]). If sperm is present, then mature eggs are fertilized and the developing embryos gestate until parturition. In the absence of fertilization, mature eggs are re-absorbed and a new batch develops for potential fertilization in the next cycle [8,47]. These two paths are represented in the current work by the sperm-present and indeterminate groups (Fig. 1). Females undergoing vitellogenesis/fertilization phases of the reproductive cycle had higher E_2 levels than females undergoing gestation phases (Figs. 3 and 4), and so follow the pattern of E_2 release found in other naturally cycling poeciliids [22,52]. The indeterminate females also had low E_2 levels even in the absence of active gestation. However, the E_2 levels for these indeterminate female trials were also quite variable – presumably reflecting the heterogeneous nature of this group (females in vitellogenesis, egg maturation or reabsorption phases). Importantly, in *X. nigrensis* the reproductive cycle is not a progression of days but rather a cycle of brood production, and may explain why there was no pattern in E_2 levels by trial date.

In contrast to associated reproductive systems such as those found in the best-studied rodents and anurans, the potential decoupling of ova production/development and fertilization achieved through sperm storage can also de-couple preference behaviors from cycle status. For example, in the tungara frog, a well-studied associated-type system, there are cyclic patterns in female preference behavior that appear to be mediated by changes in steroid hormone profiles [28], and treatment with exogenous E_2 is sufficient to induce phonotaxis behavior in post-mated frogs [6]. In tungara females, multiple aspects of female mate choice (receptivity, permissiveness and discrimination) vary across different reproductive phases [29], indicating a cycle of female preference and receptivity that is tightly linked to ova production/spawning readiness and steroid hormone profiles. In contrast, preference behavior in *X. nigrensis* females was not confined to a discrete period of the reproductive cycle, and could be elicited repeatedly over time by the presence of a favored male. In the current work, preference behaviors were not dependent on higher E_2 levels found during the vitellogenesis/fertilization stages, and in fact E_2 had a weak but significant *negative* relationship with all three preference measures when cycle status is disregarded (Fig. 5 and see below). Ongoing experiments involving the manipulation of E_2 levels within *X. nigrensis* females will serve as a critical test for the cycle-independent expression of preference behavior in this species.

Somewhat unexpectedly, receptive displays showed no change over the reproductive cycle (Figs. 3 and 4). In most poeciliids, receptivity is highest during a discrete phase post-parturition when the next brood of eggs is completing maturation and ready to be fertilized [24,25]. Under our testing conditions, there was no change in receptivity behavior, even in those females who were tested during known vitellogenesis/fertilization versus gestation stages of the cycle. Instead, while total glide displays were somewhat variable across trials and between females, receptivity displays were generally robust (overall average 44 glides/30 min trial) across repeated trials regardless of reproductive cycle status or E_2 level (Figs. 3–5). Presumably, our females remained highly receptive throughout a reproductive cycle because they were prevented from copulation. Copulation is known to suppress female receptive behavior, and physical stimulation associated with paced mating bouts shortens behavioral estrus in rats [9]. In the guppy, copulation also inhibits receptivity displays [24]. This effect can be mitigated by gonopodectomy of the males [25], but females still tend to habituate in contact trials regardless of whether the male can make gonopodial contact with the female.

There is considerable variation in the extent to which female sexual behavior is dependent on ova development and hormonal

cycling, particularly in disassociated systems [10,27]. In *X. nigrensis* females, the temporal and functional coupling between preference and the reproductive cycle is relaxed, and we can investigate the role that estradiol may play in individual variation in preference behavior regardless of cycle status. While reproductive cycle status did not predict preference behaviors, estradiol levels did predict some of the variation in preference behavior.

Estradiol levels were significant negative predictors of all three preference measures (Fig. 5 and Table 2), but they explained very low levels of the variation in any of the preference measures, particularly at the between-fish level. Females with higher estradiol levels exhibited less bias towards their favored males, whether measured as time (association bias) or behavior (glide bias) or as a composite measure (preference score). There are two interpretations to this relationship. First, females with higher estradiol levels may be less discriminatory. If females with higher estradiol spend less time with a particular male, they might also be more likely to sample both males. However, this interpretation seems somewhat unlikely since estradiol did not increase total association time with males – our measure of motivation to associate with males regardless of choice. In fact, females with higher estradiol levels actually spent significantly less of their total trial time associating with males (total association; HLM, $t = -2.325$, $p = .022$; data not shown) than females with lower estradiol levels. Second, females with higher estradiol levels were consistently more active than females with lower estradiol levels (Fig. 5 and Table 2). Therefore, the estrogenic effect on preference may have been indirect. Females with higher estradiol levels going into a trial were simply more active and so did not stay in close proximity to a potentially preferred male long enough to perform consistently biased behaviors toward a particular stimulus, although there was no significant relationship between general locomotion and preference behaviors (data not shown). This hypothesis is consistent with what is known about estrogen and activity in other animals. Estradiol levels and activity are linked in rodents (for reviews see [23,31]. Female rodents are more active than males, and females are more active during proestrus (while estradiol levels are highest) than other times in the cycle (reviewed in [31]). In addition, ovarectimized females are less active than intact, naturally cycling females, and estrogen replacement in ovarectimized or ERα knockout females increases activity [38].

X. nigrensis females exhibit a facultatively dissociated reproductive system wherein steroid hormones, ova production and sexual behavior can be uncoupled. Our data suggests that reproductive cycle status does not impact preference behavior. However, there is a significant (but weak) effect of estradiol levels on the expression of preference and locomotion behavior independent of reproductive cycle. Of note, our study tracked a proxy for circulating levels of estradiol and not localized brain levels. This may be a contributing factor to the substantially lower explanatory power that circulating estradiol levels exhibited for the inter-female variation in preference score (7%) relative to candidate preference gene expression levels (76–79%) in the brains of *X. nigrensis* females (e.g. *neuroserpin*, *neuroligin*; [13]). A critical next step will be to manipulate and/or block estradiol levels (e.g. treating *X. nigrensis* females with exogenous estradiol or aromatase inhibitor) to identify whether there is a concomitant change in preference behavior. To further determine how E_2 action within the brain relates to *X. nigrensis* preference behaviors, we are currently conducting research to assess the relationship between preference behaviors, E_2 and gene expression within the brain, including the relationship between components of the steroid signaling system (estrogen receptors, aromatase) and candidate preference-associated genes.

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