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Himani Nautiyal
University of Petroleum and Energy Studies
Kuldeep K. Roy
University of Petroleum and Energy Studies
Shubham Dwivedi
Shubham.dwivedi@ddn.upes.ac.in

University of Petroleum and Energy Studies

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Exploring Developmental, Neurobehavioral, and Transcriptional Effects of Early Embryonic Testosterone Propionate Exposure in Zebrafish Larvae

Himani Nautiyal [a], Kuldeep K. Roy [a], Shubham Dwivedi [a]*

[a] *Department of Pharmaceutical Sciences, School of Health Sciences and Technology, UPES, Dehradun, 248001*

Himani Nautiyal

ORCID ID: 0000-0002-5928-4066

Kuldeep K. Roy

ORCID ID: 0000-0001-9623-0617

*Corresponding Author: Shubham Dwivedi

ORCID ID: 0000-0003-2817-7775

Email: Shubham.dwivedi@ddn.upes.ac.in; Shubham.ms.pc@gmail.com

ABSTRACT:

Maternal hyperandrogenism associated with polycystic ovary syndrome, alters the fetal hormonal milieu and has been epidemiologically associated with neurodevelopmental vulnerabilities. However, the mechanisms linking early androgen excess to altered neurodevelopmental trajectories remain poorly understood. Early embryonic development represents a critical period during which steroid hormone signalling contributes to neuronal development. In the present study, we investigated whether early embryonic testosterone propionate exposure alters developmental and behavioral phenotypes and is accompanied by changes in the expression of gene related to steroid hormone receptors, MAPK signalling, and neurotrophic processes in zebrafish larvae. Mortality and morphological assessments were performed to identify the concentration range for subsequent behavioral and molecular analyses. TP exposure altered spontaneous motor activity at 24 hpf and affected wall sticking behavior, vertical distribution, and light-dark preference at 5 and 7 dpf. Molecular analysis at 7dpf showed reduced transcript expression of androgen receptor *(AR),* estrogen receptor alpha *(ESR1),* mitogen-activated protein kinase *(MAPK1/MAPK14/p38*) and brain-derived neurotrophic factor (*BDNF)*. These findings indicate that early embryonic testosterone propionate exposure is associated with developmental and stage-specific behavioral alterations accompanied by altered expression of genes related to steroid hormone signaling, MAPK, and neurotrophic signaling in zebrafish larvae. Our study provides a basis for further investigation of the molecular mechanisms through which developmental androgen exposure may contribute to neurodevelopmental vulnerability.

Keywords: Zebrafish; Embryonic Exposure; Developmental Toxicity; Larval Behavior; Neurodevelopment; Gene Expression

1. INTRODUCTION

Maternal hyperandrogenism, a characteristic endocrine feature of polycystic ovarian syndrome (PCOS), has been associated with altered fetal neurodevelopment [1, 2]. Elevated maternal androgen levels may influence the intrauterine hormonal environment, thereby interacting with processes that regulate early embryonic development [3]. Such influences may be particularly relevant during early development, when neurogenesis, neuronal differentiation, and synaptic organization are sensitive to hormonal cues [4, 5]. Alterations in the developmental hormonal milieu have therefore been suggested as one of the several environmental factors, that may influence neurodevelopmental trajectories [6].

Epidemiological and clinical studies have reported associations between maternal hyperandrogenic conditions, including PCOS, and neurodevelopmental outcomes in offspring [7, 8]. Altered androgen exposure during critical developmental windows has been proposed to influence neuronal differentiation, synaptic development, and subsequent behavioral outcomes [9] [5, 10]. Although these associations provide a basis for investigating developmental androgen exposure experimentally, epidemiological studies cannot readily distinguish direct effects of altered androgen signalling from other maternal, genetic or environmental factors. Experimental models are therefore required to examine the developmental responses associated with androgen exposure under controlled conditions.

Steroid hormones exert their effects through receptor-mediated transcriptional programs and downstream intracellular signalling cascades that contribute to neuronal maturation and circuit development. Androgen receptor *(AR)* is a ligand dependent transcription factor that regulates gene expression through interactions with regulatory genomic elements [11]. Estrogen receptor alpha *(ESR1)* is similarly a ligand-activated transcription factor whose interaction with chromatin contributes to regulation of gene transcription [12]. Both are important steroid hormone receptors involved in transcriptional regulation during neural

development [11, 13]. Alterations in steroid receptor signalling may consequently influence developmental processes that depends on hormonal regulation.

MAPK signalling pathways, including the ERK and p38 branches, participate in diverse cellular and developmental processes, including proliferation, and maturation of neural and other cells types [14, 15]. Recent studies further suggest that ERK signalling contributes to developmental differentiation supporting its broader role in developmental cellular regulation and synaptic function [14, 16]. Brain-derived neurotrophic factor (BDNF) acts as a neuronal survival, and synaptic function regulator [17]. BDNF/TrkB signalling engages intracellular pathways that contribute to neuronal maturation and synaptic plasticity [18].

Our previous integrative bioinformatics analysis of PCOS and ASD associated transcriptomic datasets identified *AR, ESR1, MAPK1* and *MAPK14* among the prioritized hub genes and highlighted signaling pathways related to steroid hormone regulation, intracellular signalling [19], and these findings provided a molecular rationale for experimental validation of the expression of these genes in developmental androgen exposure.

Zebrafish *(Danio rerio)* provide a tractable vertebrate model to interrogate these mechanisms due to their conserved neuroendocrine pathways, rapid ex utero development, and amenability to behavioral and molecular assays [20, 21]. Recent studies have demonstrated the utility of zebrafish for investigating developmental endocrine disruption and neurobehavioral toxicity, including the integration of developmental, behavioral, and molecular endpoints [22]. Their external embryonic development permits controlled exposure during defined developmental windows, while larval behavioral assays provide functional endpoints for assessing responses during early neurodevelopment [23].

In the present study, we investigated the developmental, behavioral, and transcriptional responses of zebrafish to early embryonic exposure to testosterone propionate (TP).

Survival and morphological endpoints were assessed to characterize developmental toxicity and identify concentrations for subsequent behavioral and molecular analyses. Spontaneous tail coiling activity was assessed at 24 hours post fertilization (hpf). While other behavioral responses were examined at 5 and 7 days post fertilization (hpf). At 7 dpf, transcript expression of *AR, ESR1, BDNF, MAPK1* and *MAPK14* was evaluated to determine whether behavioral responses were accompanied by changes in genes related to steroid hormone signalling, intracellular signaling and neurotrophic processes. This experimental framework was used to characterize developmental responses associated with early TP exposure and to examine the molecular changes accompanying the observed behavioral phenotypes. This model provides a controlled experimental framework to investigate how developmental androgen exposure associated with maternal hyperandrogenism may contribute to neurodevelopmental vulnerability.

2 Methodology

2.1 Zebrafish Husbandry and Embryo Collection

Adult wild-type zebrafish (*Danio rerio*, AB strain) were maintained in a recirculating aquatic system at 28.5 ± 0.5ºC under a 14:10 h light-dark cycle. Water quality was maintained at pH 7.0-7.5, conductivity 300-500 µS/cm, and dissolved oxygen > 6mg/L. Adult fish were fed twice daily with commercial dry flakes (Tetra Bits, India) and live Artemia.

Fertilized embryos were obtained by natural pairwise spawning and collected within 1 h post-fertilization (hpf). Embryos were examined under stereomicroscope and staged according to the literature [24]. Only morphologically normal fertilized embryos with regular cleavage were selected for experiment. Unfertilized eggs were discarded and selected embryos were maintained in E3 embryonic medium containing NaCl (5 mM), KCl (0.17 mM), $CaCl_2$ (0.33 mM), and $MgSO_4$ (0.33 mM). All experimental procedures involving zebrafish were approved by the Institutional Animal Ethical Committee (IAEC)

(UPES/IAEC/2023/2/01) and were performed in accordance with the relevant guidelines and regulation of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India. As the study involved non-invasive exposure and behavioral assessment of zebrafish embryos/larvae without surgical procedures or intentional tissue injury, analgesic intervention was not required.

2.2 Range finding assay: mortality and developmental defects

Testosterone propionate (TP; Sisco Research Laboratories Pvt. Ltd., India; product no. 71344; extrapure, 98%; CAS No. 57-85-2; molecular formular $C_{22}H_{32}O_3$; molecular weight 344.50g/mol) was used as an experimental androgenic compound. TP was freshly prepared in dimethyl sulfoxide (DMSO) and subsequently diluted with E3 embryo medium to obtain the required exposure concentrations. The final concentration of DMSO was maintained at 0.01% (v/v) in experimental vehicle and treatment groups.

A preliminary concentration range-finding experiment was performed using TP concentrations ranging from 0.1-300 µM. Embryos were exposed under static conditions from approximately 4 hpf, and mortality was assessed at 24, 48, 72 and 96 hpf by stereomicroscopic observation (Figure 1). Three independent biological replicates were performed, with 20 embryos per concentration in each replicate. Mortality at each assessment time point was recorded as the number of newly dead embryos, and cumulative mortality at 96 hpf was calculated based on the total number of dead embryos during the 24 - 96 hpf, observation period.

Mortality was determined based on overall viability of embryos as observed under a stereomicroscope. The range- finding experiment was performed according to the principles described in OECD test guideline 236; however, it was not designed as a full OECD Test Guideline 236 fish embryo acute toxicity test. Cumulative mortality at 96 hpf was corrected for background mortality observed in the DMSO vehicle control group before dose response analysis. Corrected mortality values were calculated by subtracting

the mortality observed in DMSO vehicle control group from the mortality observed at each TP treated group. The corrected 96 hpf mortality data were fitted using a variable-slope non-linear regression model to estimate the medial lethal concentration (LC_{50}) and its 95% confidence interval in GraphPad Prism.

Developmental abnormalities, including pericardial edema, spine curvature, uninflated swim bladder, and yolk-sac edema were assessed at 96 hpf among surviving embryos exposed to 1-100 µM TP. Quantifiable morphological abnormalities were observed within this concentration range, whereas concentrations above 100 µM were associated with early lethality.

The malformation rate (%) was calculated using the following formula

$$\%\ \text{Malformation rate} = \frac{\text{Number of malformed embryos}}{\text{Number of surviving embryos}} X100$$

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test ($p < 0.05$) [25].

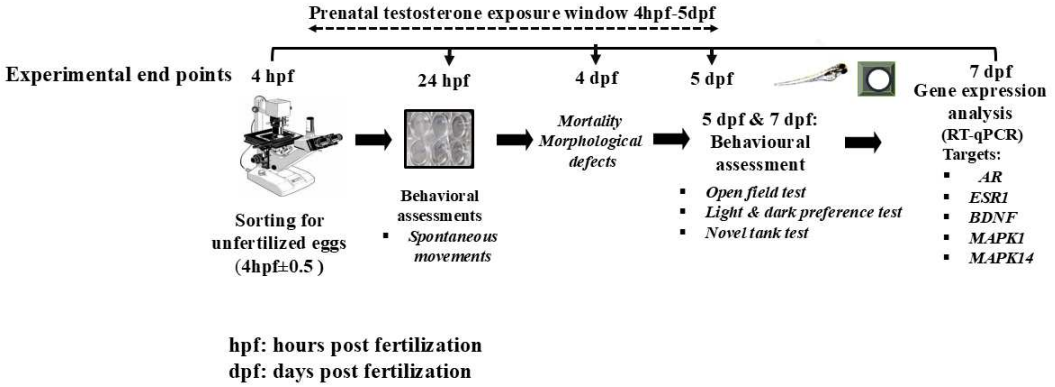


Figure 1. Schematic representation of experimental design. Adult wild type zebrafish were paired for spawning in a 1:1 breeding setup, and fertilized embryos were collected and

screened at 4 ± 0.5 hpf. Selected embryos were exposed to testosterone propionate (TP), under static exposure condition. A concentration range- finding experiment was conducted using 0.1-300 µM TP, with exposure from approximately 4 hpf to 96 hpf. Mortality was assessed at 24,48,72, and 96 hpf, and cumulative mortality at 96 hpf was used for LC_{50} estimation. Developmental morphological abnormalities were assessed separately at 96 hpf following exposure to 1-100 µM TP. Based on the range finding results, 1,3,10 and 30 µM TP were selected for subsequent behavioral experiments, with exposure maintained from approximately 4 hpf - 5 dpf. Spontaneous embryonic tail coiling activity was assessed at 24 hpf, Larval behavioral assessments, including open field, light-dark preference, and novel tank assays were performed at 5- and 7-days post fertilization (dpf). For behavioral testing, viable larvae without visible developmental abnormalities were used. At 7 dpf, larvae were collected for quantitative reverse-transcription PCR (RT-qPCR) analysis of *AR, ESR1, BDNF, MAPK1*, and *MAPK14* expression. The biological replicate was considered the experimental unit for statistical analysis, and technical replicates were averaged before statistical testing. The schematic summarizes the timing and sequence of exposure, phenotypic assessment, behavioral assessment and molecular analysis.

2.3 Behavioral assessments

For the behavioral experiments, embryos were exposed to 1,3,10 and 30 µM TP from 4 hpf through 5 dpf in 6-well plates (30 embryos/well in 6 ml) at 28 ± 1°C under static exposure conditions. On 5 and 7 dpf, larvae were transferred to fresh E3 medium 2 hours prior to the behavioral assessment. For behavioral testing, viable larvae without visible developmental abnormalities were used. Behavioral analyses were conducted at the stage-specific time points (5 and 7 dpf) to characterize behavioral responses at two developmental stages. Behavioral assessments included wall sticking behavior, vertical distribution in a novel environment, and light-dark preference. All behavioral assays were performed under standardized environmental conditions. Behavioral scoring was manually

performed by observer blind to the treatment to ensure unbiased scoring. The behavioral scoring framework was adapted from previously validated zebrafish neurodevelopmental protocols with minor modifications to accommodate the current experimental design [26-28].

2.3.1 Spontaneous movements

Spontaneous embryonic movements were assessed at 24 hpf by manually quantifying all visible tail-coiling events under a stereomicroscope for 1 minute observation period [28-30]. A tail-coiling event was defined as a complete contraction followed by relaxation of the trunk and tail. Both robust swim bouts and subtle twitch-like contractions were included in the analysis. Three independent biological replicates, with three technical replicates per biological replicate and 10 embryos per technical replicate. The number of tail-coiling events was recorded for each embryo, and the mean number of tail-coiling events per embryo per minute was calculated for each technical group. Technical replicate means were subsequently averaged to obtain a single value for each biological replicate prior to statistical analysis.

2.3.2 Open field test

The open field assay was performed to assess larval peripheral-zone occupancy as a measure of thigmotactic and exploratory behavior. Ten larvae were transferred to a 90-mm petri dish containing E3 medium and allowed to acclimate for 2 minutes. Larval behavior was subsequently recorded for 30 minutes. The outer 1 cm of the petri dish was defined as the peripheral zone, with the remaining area considered the central zone. Manual scoring quantified the number of larvae spent in the peripheral zone (outer 1 cm region) relative to the central zone [27, 31]. Increased wall-sticking behavior was considered an indicator of altered thigmotactic and exploratory behavior. The percentage of larvae in the periphery was calculated using the formula:

$$\% \text{ larvae in periphery} = \frac{\text{Number of larvae in periphery}}{\text{Total number of larvae in the arena}} X100$$

2.3.3 Novel tank test

Vertical exploratory behavior was assessed at 5 and 7 dpf using a modified larval diving assay based on previously described zebrafish behavioral paradigms [32, 33]. Five larvae were gently transferred into a 100 ml glass beaker containing approximately 80 ml of E3 medium and allowed to acclimate for 1 min. The beaker was externally marked to visually delineate the upper and lower zones, and the number of larvae occupying the lower zone was manually recorded at 1 min intervals during a 5 min observation period. The mean percentage of larvae occupying the lower zone over the observation period was calculated for each technical replicate. Three technical replicates were averaged to obtain a single value for each biological replicate. Three independent biological replicates were analyzed at 5 dpf and five at 7 dpf, and the resulting biological replicate values were used for statistical analysis.

2.3.4 Light and dark test

The light-dark preference was assessed at 5 and 7 dpf using a 24-well plate containing 2 ml of E3 embryo medium per well. Ten wells were utilized for each technical replicate, with one larva placed individually in each well. Light and dark regions were visually differentiated using alternating black and white stripes, as described previously [26]. Following 2 minutes acclimation, larval position was manually recorded at 1 min interval for 10 min.

Preference Index was calculated using the formula:

$$\text{Preference Index} = \frac{\text{Larvae in Light} - \text{Larvae in Dark}}{\text{Total larvae in the arena}}$$

The mean preference index over the 10 min observation period was calculated for each technical replicate. The three technical replicates were then averaged to obtain a single

value for each biological replicate. Three independent biological replicates were analyzed at 5 dpf and five at 7 dpf, and the resulting values were used for statistical analysis.

After the behavioral assessments at 7 dpf, larvae were euthanized by rapid freezing. Larave maintained in E3 embryo medium were subjected to rapid colloing in an ice bath without direct contact with ice until complete cessations of movement was observed. The larvae were subsequently collected for molecular analysis.

2.4 Quantitative RT- PCR analysis of gene expression

Total RNA was extracted from pooled zebrafish larvae (n = 50 larvae per biological replicate) using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA concentration and purity were determined using an Agilent BioTek Synergy H1 multimode microplate reader USA. For cDNA synthesis, 1µg of total RNA was reverse transcribed using the Verso cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed using TB green Premix Ex Taq II (Takara Bio, Japan) on a Bio-Rad CFX96 real time PCR detection system. Gene specific primers for *ESR1, AR, MAPK1, MAPK14*, and *BDNF* were synthesized by integrated DNA Technologies (IDT, India), with primer sequences provided in the supplementary file (Table S-1). β-actin was used as the reference gene. All Reactions were performed in the technical triplicate, and three independent biological replicates were analyzed. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method and expressed relative to the corresponding control group.

2.5. Statistical analysis

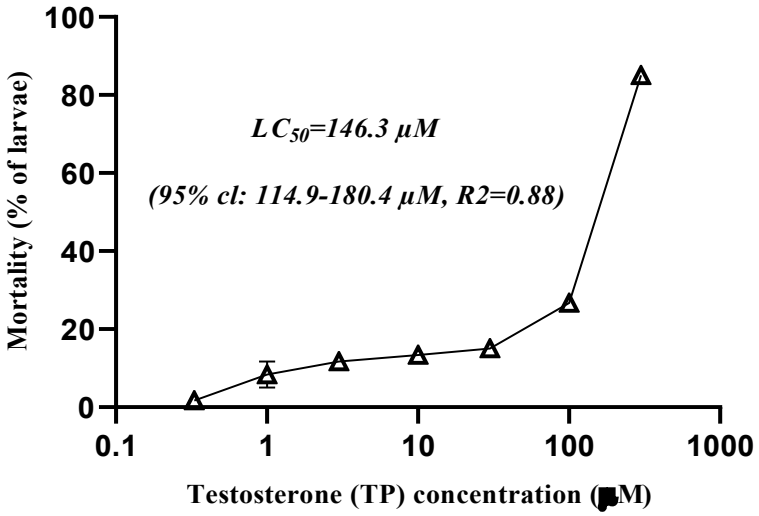
All statistical analyses were performed using GraphPad Prism (version; 8.0.2, GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM, with the biological replicate considered the experimental unit. Technical replicates were averaged within each biological replicate before statistical analysis. For mortality analysis, cumulative mortality

at 96 hpf was corrected for background mortality observed in the vehicle control group and analyzed using a variable-slope model nonlinear regression model to estimate the median lethal concentration (LC50) and its 95% confidence interval. For morphological and behavioral endpoints, differences among treatment groups were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test against the vehicle control. For gene expression analyses, treatment groups were compared with the untreated control group using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. A p value < 0.05 was considered statistically significant.

3 Results

3.1 Mortality assessment

Zebrafish embryos exposed to testosterone propionate (TP) from approximately 4 hpf to 96 hpf were evaluated for mortality across three independent replicates, with 20 embryos per concentration in each replicate. Mortality increased with increasing TP concentration, with the highest mortality observed at 300 µM. The cumulative mortality at 96 hpf was corrected for background mortality in the DMSO vehicle- control group, were analyzed using a variable- slope nonlinear regression model (Figure 2). The estimated LC_{50} value was 146.3 µM (95% cl: 114.9-180.4 µM, R^2=0.88) as determined using GraphPad Prism.



278

279 **Figure 2.** Determination of LC_{50} of testosterone (TP) at 96 hpf in zebrafish larvae. Embryos were exposed to
280 increasing concentrations of testosterone (0.1-300µM) from 4 hpf to 96 hpf. Mortality was assessed at 24,
281 48, 72, and 96 hpf, and cumulative mortality at 96 hpf was corrected for background mortality observed in
282 the DMSO vehicle control group. The corrected mortality data were fitted using a variable slope nonlinear
283 regression model. A concentration-dependent increase in mortality was observed, with a sharp rise in higher
284 doses. The LC_{50} value is calculated as 146.3 (95% cl: 114.9-180.4 µM; R^2=0.88). Data presented as mean ±
285 SEM, from three independent biological replicates (n = 3).

286 ## 3.2 Experimental model optimization

287 The mortality profile, together with the developmental responses observed during the
288 range-finding experiment, was used to define the exposure range for subsequent
289 experiments. Quantifiable developmental abnormalities were observed within the 1-100
290 µM TP range, whereas concentrations above 100 µM were associated with early lethality.
291 The 1-30 µM TP range showed substantially lower mortality and was therefore selected for
292 subsequent behavioral assessments. For molecular analysis, 3 and 10 µM TP were selected
293 from this lower exposure range.

294 ## 3.3. Developmental morphological effect of early embryonic TP Exposure

Developmental morphological abnormalities were assessed at 96 hpf following exposure to TP at concentration ranging from 1-100 μM. Four morphological endpoints evaluated included pericardial edema, yolk sac edema, uninflated swim bladder, and spinal curvature (Figure 3). The incidence of morphological abnormalities varied across the TP exposure concentration.

The incidence of uninflated swim bladder increased with increasing TP concentration, with mean incidence of 3.88%, 26.22% and 52.50 % at 10, 30, and 100 μM, respectively. compared with the vehicle control group. Significant increases were observed at 30, and 100 μM (adjusted $p < 0.0001$ for both), whereas the differences at 10 μM were not statistically significant (adjusted $p < 0.717$). Pericardial edema showed a similar concentration related pattern, with mean incidences of 1.32%, 2.63%, 13.68%, 17.57% and 33.25% at 1,3,10,30 and 100 μM respectively. Compared with the vehicle control group, pericardial edema was significantly increased at 10, 30 and 100 μM (adjusted $p < 0.0001$ for each), while no significant difference was observed at 1 or 3 μM.

Spinal curvature, mean incidence were 4.06%, 7.83%, 13.11%, 19.64% and 48.50% at 1, 3, 10, 30, and 100 μM, respectively. Significant increases compared with the vehicle-control group were observed at 30 μM (adjusted $p = 0.0031$) and 100 μM (adjusted $p < 0.0001$), The difference at 1,3, and 10 μM were not statistically significant (adjusted $p = 0.9052, 0.4216, 0.0615$ respectively).

Yolk sac edema was not prominent at 1 and 3 μM but increased at higher concentrations, with mean incidences of 5.13%, 10.26%, and 15.50% at 10, 30 and 100 μM, respectively. Compared with the vehicle-control group, significant increases were observed at 10 μM (adjusted $p = 0.0493$), 30 and 100 μM (adjusted $p < 0.0001$ for both). The most pronounced morphological abnormalities were observed at 30 and 100 μM TP, while lower concentrations produced fewer or no significant changes depending on the endpoint assessed.

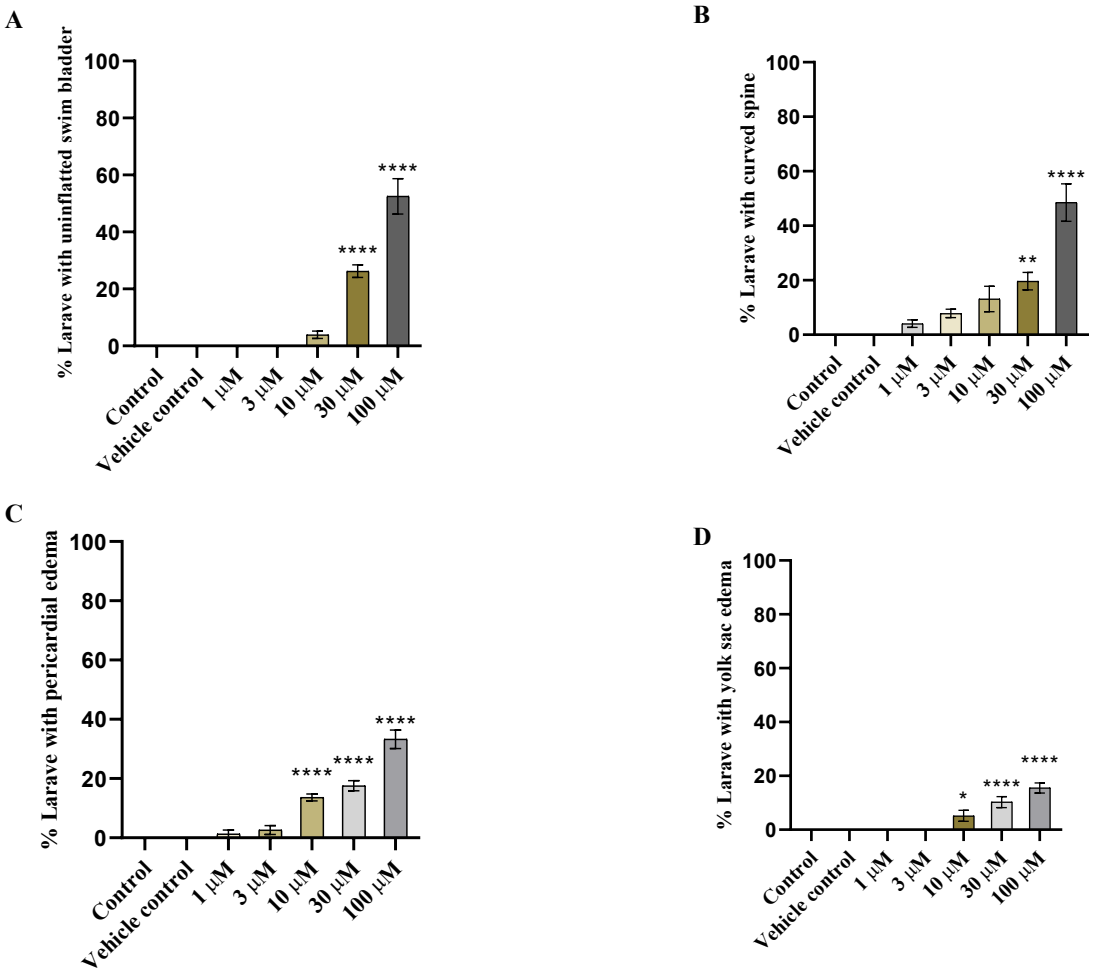


Figure 3. Morphological abnormalities induced by testosterone exposure in zebrafish larvae at 96 hpf. Percentage of larvae exhibiting (A) Uninflated swim bladder, (B) spinal curvature (C) pericardial edema, (D) yolk sac edema following exposure to increasing concentrations of testosterone (1-100 μM) from 4 hpf to 5dpf. Each condition contained 20 embryos per replicate (N = 4 biological replicates). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test vs vehicle control ($p < 0.05$, $p < 0.01$, $p < 0.0001$). Morphological defects or abnormalities increased progressively with concentration of testosterone, confirming a dose-dependent developmental impact.

3.4 Behavioral effects of early embryonic TP exposure

Behavioral responses to early embryonic TP exposure (1- 30 μM) were assessed using spontaneous movement at 24 hpf and open-field, light- dark preference, and novel tank assay at 5 and 7 dpf. Spontaneous movement was assessed in live larvae at 24 hpf, while behavioral testing was performed at 5 and 7 dpf using morphologically normal larvae

3.4.1 Spontaneous movement

Spontaneous tail-coiling frequency was assessed at 24 hpf as an early behavioral endpoint of TP exposure (Figure 4). Increased spontaneous movement, with a significant increase observed at 30 μM compared with DMSO (adjusted $p = 0.0342$), whereas no significant differences were detected at 1, 3 or 10 μM TP exposure. Spontaneous tail coiling at 24 hpf is widely used as an early measure of motor activity during zebrafish development [34]. Spontaneous coiling at 24 hpf is a sensitive early indicator of motor-network excitability and is widely used in developmental neurotoxicity screening [34].

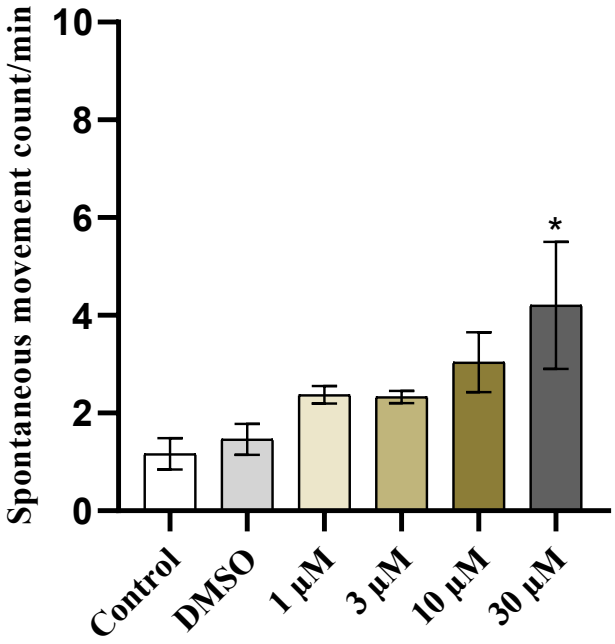


Figure 4. Spontaneous movement in zebrafish embryos with TP exposure at 24 hpf: Spontaneous coiling activity was assessed in zebrafish embryos at 24 hpf after exposure to 1-30 µM TP. Data are presented as mean ± SEM from three independent biological replicates, with three technical replicates per biological replicates. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test against the DMSO ($p < 0.05$).

3.4.2 Open field test

The open- field assay evaluated the wall sticking behavioral activity in TP-exposed larvae. At 5 dpf, TP exposure altered wall-sticking behavior in the open field assay compared with the DMSO vehicle control. Significant increases were observed at 1,3,10, and 30 µM (adjected $p < 0.0001$). (Figure 5 A-B). At 7dpf, a similar pattern was observed with significantly increased wall sticking behavior at 3, 10, and 30 µM TP compared with the DMSO control (adjusted $p = 0.0325$, 0.0036 and 0.0001 respectively), whereas 1 µM group did not differ significantly from the DMSO control (adjusted $p= 0.4094$). Data are presented as mean ± SEM of biological replicates (N = 3), with technical replicates

averaged prior to statical analysis. The enhanced edge preference indicates altered exploratory behavior and may reflect early changes in neural circuit regulation of environmental explorations following early embryonic testosterone exposure.

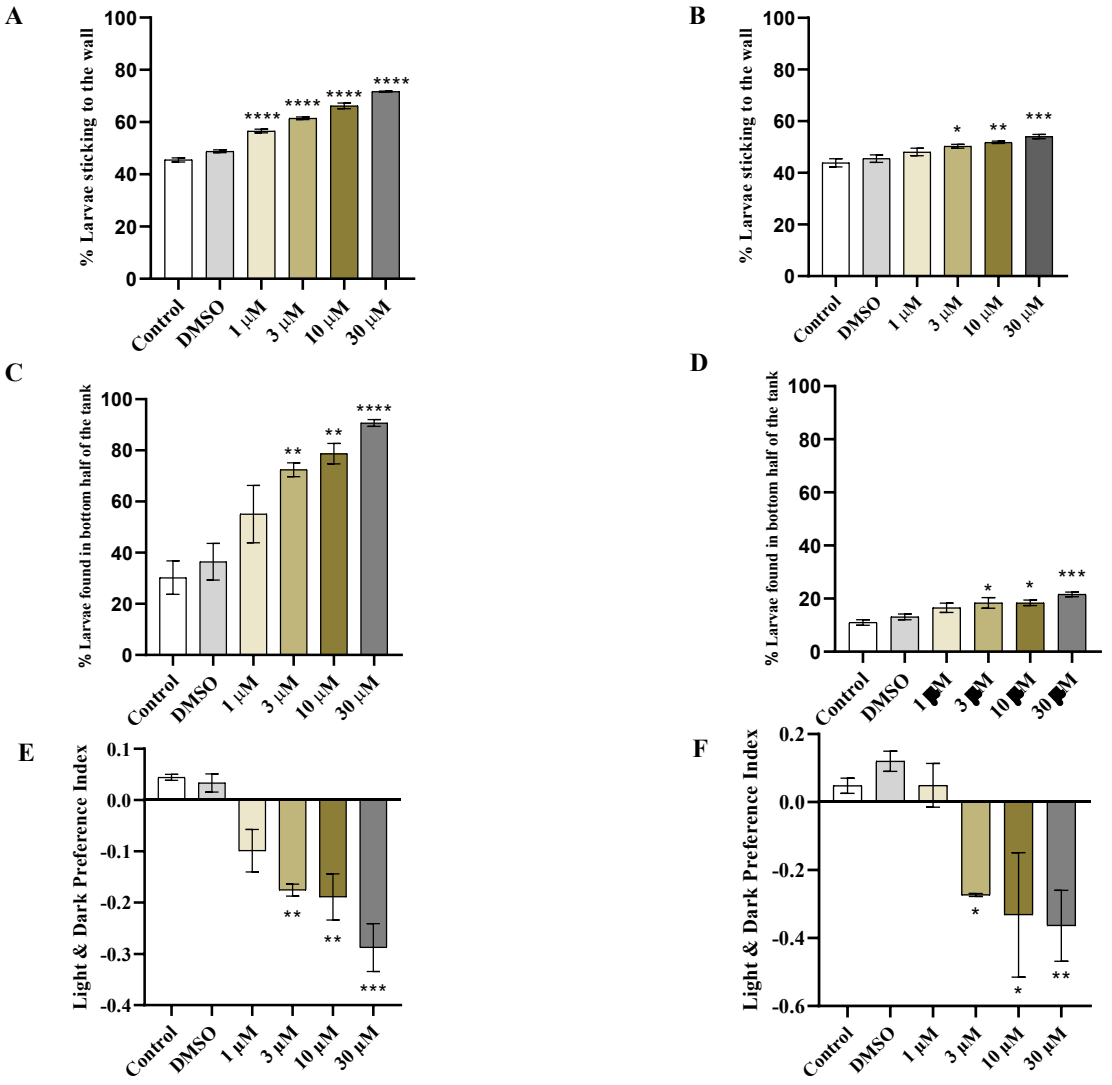
3.4.3 Novel tank test

Novel tank test was assessed by measuring the percentage of larvae occupying the lower half of the tank during the observation period (Figure 5 C-D). TP exposure increased lower half of occupancy compared with DMSO control group. Significant increases were observed at 3 μM (adjusted p= 0.0074), 10 μM (adjusted p= 0.0023) and 30 μM (adjusted p= 0.0003), whereas the difference at 1 μM was not significant (adjusted p= 0.2037).

At 7 dpf, TP exposure increased bottom-dwelling behavior compared with the DMSO vehicle control. Significant increases were observed at 3 μM and 10 μM (adjusted p= 0.0466) and 30 μM (adjusted p= 0.0009), whereas the difference at 1 μM was not significant (adjusted p= 0.2973).

3.4.4 Light and dark test

Light-dark preference behavior was assessed using the preference index (PI), calculated as (L-D)/ total larvae in the arena, where L and D represent the number of larvae in the light and dark zones, respectively **(**Figure 5 E-F). Negative PI values indicate a preference for the dark zone. TP exposure reduced PI across the tested concentrations. Compared with the DMSO vehicle control, the differences were significant at 3 μM (adjusted $p = 0.0029$, 10 μM (adjusted $p = 0.0018$) and 30 μM (adjusted $p < 0.0001$), whereas the difference at 1 μM was not significant (adjusted $p = 0.0534$). At 7 dpf, larvae exposed to 1 μM TP showed no significant difference (adjusted $p = 0.9731$). In contrast, TP exposure at 3, 10, and 30 μM resulted in significantly lower PI values compared with DMSO vehicle control, with adjusted p values of 0.0226, 0.0078, and 0.0043, respectively.



392

393 **Figure 5.** Behavioral changes in zebrafish larvae following testosterone propionate (TP) exposure
394 at 5 dpf and 7 dpf. (A-B) Open field test: Analysis of wall sticking behavior (5 dpf: N = 3
395 independent biological replicates; 7dpf: N= 5 independent biological replicates), with each
396 biological replicate consisting of n = 3 technical replicates and 10 larvae per plate. (C-D) Novel
397 tank assay: Analysis of bottom-dwelling behaviors (5 dpf: N = 3 independent biological replicates;
398 7 dpf: N = 5 independent biological replicates), with each biological replicate consisting of n = 3

technical replicates and 5 larvae per beaker. (E-F) Light-dark preference test: Analysis of light-dark preference index (5 dpf: N = 3 independent biological replicates; 7 dpf: N = 5 independent biological replicates), with each biological replicate consisting of n = 3 technical replicates and 10 wells per technical replicate and one larva per well. Data are presented as mean ± SEM and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test, where $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to control group.

3.5 Gene expression analysis

Relative gene expression was assessed using the $2^{-\Delta\Delta Ct}$ method, with expression normalized to the untreated control group [35, 36]. Quantitative RT-PCR analysis showed significant reductions in the expression of steroid receptor related and neurodevelopment associated genes following TP exposure (Figure 6). Quantitative RT-PCR analysis showed significant reductions in the expression of steroid receptor- related and neurodevelopment - associated genes following TP exposure. *AR, ESR1, BDNF* and *MAPK14* expressions were significantly reduced at both 3 and 10 µM TP compared with the untreated control (adjusted $p < 0.0001$). *MAPK1* expression was significantly reduced at 3 µM (adjusted $p < 0.001$) and 10 µM (adjusted $p < 0.01$).

TP exposure significantly reduced the expression of *AR, ESR1, BDNF, MAPK1*, and *MAPK14* compared with the untreated control, with the magnitude of reduction varying among the genes and TP concentrations.

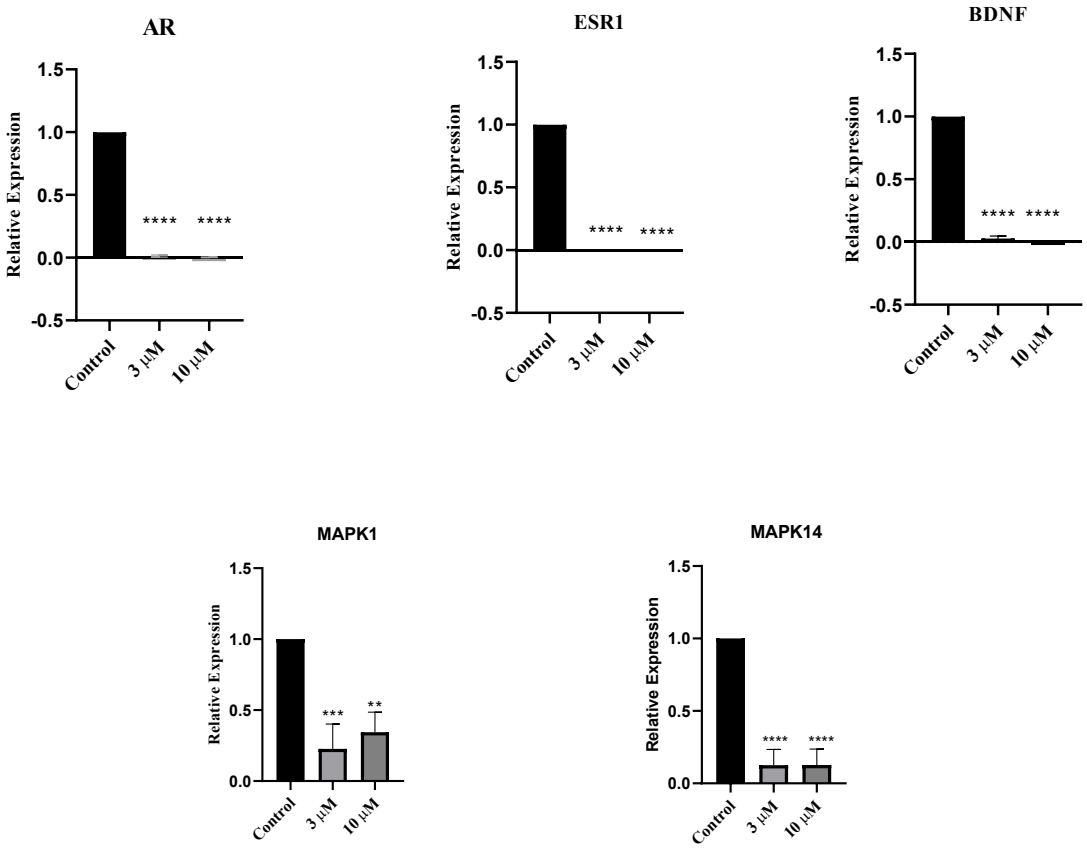


Figure 6. Gene expression changes in zebrafish larvae with testosterone at 7 -dpf. Relative mRNA expression levels of *AR, ESR1, BDNF MAPK1*, and *MAPK14* were quantified by RT qPCR from 3 replicates of study (n =3x50). The results are reported as mean ±S.E.M and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism software, where $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to control group.

Discussion

Testosterone signalling is a significant regulator of vertebrate development, and changes in androgen exposure during early developmental period have been associated with differences in developmental and behavioral phenotypes [37, 38]. Several epidemiological studies have also indicated an association between maternal androgen excess and altered

neurodevelopmental outcomes in offspring [39-42]. Rodent studies have also reported behavioral alterations associated with prenatal androgen exposure [43-45]. However, the biological mechanisms linking early androgen excess to altered brain development remain poorly understood [46]. These previous findings strengthen the biological plausibility of androgen-mediated developmental risk pathway [43, 45, 47]. Zebrafish is an excellent vertebrate system for studying hormone-mediated neurodevelopment due to its conserved endocrine pathways and rapid brain development [48, 49].

In the present study, we examined the effects of early embryonic testosterone (TP) exposure on developmental morphology, behaviors, and gene expression levels. Our findings suggest that early embryonic testosterone exposure affected the developmental morphology at 96 hpf, different behavioral alterations including spontaneous embryonic movement at 24 hpf, larval behavioral responses at 5 and 7 dpf, and changes in the expression of genes related to steroid hormone signaling, neurotrophic regulation and intracellular signaling. [10, 37, 50, 51].

At 24 hpf, TP exposure significantly increased spontaneous tail coiling activity at 30μM compared with DMSO. Spontaneous coiling is one of the earliest observable motor behaviors in zebrafish embryos and reflects the developing sensorimotor system. The Spontaneous embryonic movement is an early functional behavior that emerges during zebrafish development and reflects the maturation of motor activity [52]. Previous studies have used spontaneous embryonic movement as an early behavioral indicator of developmental effects on motor function, supporting its use as an early behavioral endpoint [26, 53]. However, the present study did not directly measure neuronal activity or spinal-circuit function; therefore, the increased coiling frequency should not be interpreted as direct evidence of neuronal hyperexcitability. This early behavioral response was followed by more complex behavioral alterations in larvae at 5 and 7 dpf across the open field, novel tank test, and light - dark assays.

The open field assay showed altered wall-sticking behavior in TP exposures, with significant differences observed at several concentrations relative to DMSO. Increased occupancy of peripheral region may reflect an alteration in spatial exploration within the open arena. The light-dark preference testing provided a behavioral measure of zone preference, with lower preference index at the higher TP concentrations reflecting increased occupancy of the dark region. The novel tank assay similarly demonstrated altered vertical distribution, showing increased bottom occupancy by larvae both at 5 and 7 dpf. These behavioral assays showed consistent alterations in spatial distribution and zone preference across different environmental contexts in the developing sensory, motor and behavioral systems between these stages [54-56]. However, the magnitude of the response differed between 5 and 7 dpf. This difference should be interpreted in the context of ongoing behavioral maturation during larval development rather than as evidence of behavioral compensation.

The transcriptional analysis provided additional evidence of molecular changes associated with TP exposure. The expressions of *AR, ESR1,* and *BDNF* were significantly reduced, while *MAPK1,* and *MAPK14* also showed altered expression. *AR* and *ESR1* encode steroid hormone receptors that function as transcriptional regulators, and recent studies have further characterized the mechanisms through which androgen and estrogen receptors regulate gene expression [11, 12, 57]. The observed changes in *AR* and *ESR1* expression therefore suggest an alteration in steroid hormone receptor associated transcriptional responses subsequent TP exposure. *BDNF* has established role in neuronal development, differentiation and synaptic function, and recent work has further characterized *BDNF/TrkB* signalling during neural development [18, 58-62]. The reduction in *BDNF* transcript abundance observed in TP exposure, therefore provides a molecular finding that can be considered alongside the behavioral alterations observed in the larvae. Nevertheless, the present study measured transcript levels rather than *BDNF* protein or downstream

signaling activity, and the relationship between transcriptional changes and the behavioral phenotypes remains to be established experimentally.

Changes in *MAPK1* and *MAPK14* expression provide additional evidence of transcriptional changes associated with TP exposure. *MAPK/ERK* signalling is involved in cellular differentiation and developmental processes, including cellular regulation during development [15, 63, 64], while p38 MAPK signalling also contributes to neuronal cellular responses [65]. And related pathways participate in a wide range of developmental processes, including cellular differentiation and neuronal development. The observed changes in *MAPK1,* and *MAPK14* transcript abundance therefore broaden the molecular response identified in the present study beyond steroid receptor-related genes. The altered MAPK1 and MAPK14 transcript abundance provides molecular evidence of changes in genes associated with intracellular signaling, although transcript measurements alone cannot establish altered pathway activity.

This study has several limitations. The zebrafish model provides an experimentally tractable system for studying developmental responses to androgen exposure, but direct embryonic TP exposure does not reproduce the complex maternal and placental environment associated with maternal hyperandrogenic conditions in humans. The molecular analysis was also limited to selected genes and transcript-level measurement; therefore, changes in protein abundance, receptor activity and downstream signaling remain to be determined. These limitations identify important directions for future studies rather than diminishing the developmental, behavioral, and transcriptional responses observed in the present model.

Conclusion

In conclusion, early embryonic testosterone exposure was associated with developmental abnormalities, altered larval behavioral responses and transcriptional changes in zebrafish. Developmental exposure was associated with morphological abnormalities at higher

concentration, alterations in spontaneous motor activity at 24 hpf, and behavioral changes across the open field, light-dark and novel tank assays at 5 and 7 dpf. TP exposure also reduced the expression of *AR, ESR1, MAPK14, MAPK1* and *BDNF.* These findings indicate that early embryonic testosterone (TP) exposure can influence developmental, behavioral and molecular phenotypes in zebrafish. Further investigation at protein and pathway levels is required to determine the mechanisms linking altered androgen exposure with these developmental and behavioral responses.

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Data availability

The datasets generated in this study are available on request.

CRediT authorship contribution statement

Himani Nautiyal: Writing - original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Kuldeep K. Roy:** Writing - review & editing, Supervision **Shubham Dwivedi:** Conceptualization**,** Writing – review & editing, Supervision, Project administration.

Ethical statement

All experimental procedures were approved by the institutional Animal Ethical Committee (IAEC) proposal no. UPES/IAEC/2023/2/01 and conducted in accordance with Committee for the purpose of control and supervision of experiments on animals, CCSEA, Government of India.

Competing interest

Authors declare no competing interest.

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Reference

1. Darlas, M., S. Kalantaridou, and G. Valsamakis, *Maternal Hyperandrogenemia and the Long-Term Neuropsychological, Sex Developmental, and Metabolic Effects on Offspring*. Int J Mol Sci, 2025. **26**(5).
2. Kahn, L.G., et al., *Maternal polycystic ovarian syndrome and offspring psychopathology and neurodevelopment*. Hum Reprod, 2025. **40**(7): p. 1257-1265.
3. Bravo-Muñoz, F., et al., *"Risk of Autism Spectrum Disorder in Descendants of Women With Gestational Diabetes and Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis"*. Autism Res, 2025. **18**(10): p. 1916-1934.
4. Gurule, S., et al., *Developmental programming of the neuroendocrine axis by steroid hormones: Insights from the sheep model of PCOS*. Frontiers in Endocrinology, 2023. **Volume 14 - 2023**.
5. Briones-Valdivieso, C., et al., *Hormonal and sex-specific functional genomic pathways of genetic risk candidates in autism spectrum disorder: evidence of sex-over-chance effects*. Hormones and Behavior, 2025. **174**: p. 105795.
6. Wang, Z., et al., *Androgen levels in autism spectrum disorders: a systematic review and meta-analysis*. Front Endocrinol (Lausanne), 2024. **15**: p. 1371148.
7. Zhang, Y., et al., *Association between maternal polycystic ovary syndrome and attention-deficit/hyperactivity disorder in offspring aged 3-6 years: A Chinese population-based study*. Front Public Health, 2022. **10**: p. 1032315.
8. Yesildemir, O. and M.N. Celik, *Association between pre- and postnatal exposure to endocrine-disrupting chemicals and birth and neurodevelopmental outcomes: an extensive review*. Clin Exp Pediatr, 2024. **67**(7): p. 328-346.
9. Love, C., et al., *Prenatal environmental risk factors for autism spectrum disorder and their potential mechanisms*. BMC Med, 2024. **22**(1): p. 393.

10. Worsham, W., S. Dalton, and D.A. Bilder, *The Prenatal Hormone Milieu in Autism Spectrum Disorder*. Front Psychiatry, 2021. **12**: p. 655438.
11. Effah, W., et al., *Advances in the understanding of androgen receptor structure and function and in the development of next-generation AR-targeted therapeutics*. Steroids, 2024. **210**: p. 109486.
12. Soota, D., et al., *RNA fine-tunes estrogen receptor-alpha binding on low-affinity DNA motifs for transcriptional regulation*. Embo j, 2024. **43**(21): p. 5186-5210.
13. Altıntaş, U.B., et al., *Decoding the epigenetics and chromatin loop dynamics of androgen receptor-mediated transcription*. Nat Commun, 2024. **15**(1): p. 9494.
14. Baek, H.B. and S. Arur, *RAS/ERK signaling and PLK1: Coordinating developmental regulation and disease mechanisms*. Curr Opin Cell Biol, 2025. **95**: p. 102544.
15. Chen, F., et al., *Developmental regulation of Erk signaling by mitotic kinases*. Sci Adv, 2026. **12**(2): p. eadq7469.
16. Rosina, E., et al., *Disruption of mTOR and MAPK pathways correlates with severity in idiopathic autism*. Transl Psychiatry, 2019. **9**(1): p. 50.
17. Kaminskaya, Y.P., et al., *Brain-Derived Neurotrophic Factor (BDNF) in the Frontal Cortex Enhances Social Interest in the BTBR Mouse Model of Autism*. Biochemistry (Mosc), 2024. **89**(8): p. 1509-1518.
18. Barde, Y.A., *The physiopathology of brain-derived neurotrophic factor*. Physiol Rev, 2025. **105**(4): p. 2073-2140.
19. Nautiyal, H., et al., *Deciphering the molecular connections between polycystic ovarian syndrome and autism spectrum disorder using bioinformatic analysis*. Horm Behav, 2026. **177**: p. 105859.
20. Sakai, C., S. Ijaz, and E.J. Hoffman, *Zebrafish Models of Neurodevelopmental Disorders: Past, Present, and Future*. Front Mol Neurosci, 2018. **11**: p. 294.
21. Rea, V. and T.J. Van Raay, *Using Zebrafish to Model Autism Spectrum Disorder: A Comparison of ASD Risk Genes Between Zebrafish and Their Mammalian Counterparts*. Front Mol Neurosci, 2020. **13**: p. 575575.
22. Jindal, G., et al., *Developmental and neurobehavioral toxicity of di-(2-ethylhexyl) phthalate (DEHP) in zebrafish larvae*. Comp Biochem Physiol C Toxicol Pharmacol, 2025. **296**: p. 110254.
23. Truong, L., et al., *Zebrafish behavioral assessment as a first-tier whole-organism NAM for developmental neurotoxicity: A multi-laboratory evaluation*. Neurotoxicology, 2026. **116**: p. 103504.
24. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish*. Dev Dyn, 1995. **203**(3): p. 253-310.
25. Wang, R., et al., *Evaluation of the Developmental Toxicity Induced by E804 in Zebrafish Embryos*. Front Pharmacol, 2020. **11**: p. 32.
26. Chen, J., et al., *Developmental and behavioral alterations in zebrafish embryonically exposed to valproic acid (VPA): An aquatic model for autism*. Neurotoxicol Teratol, 2018. **66**: p. 8-16.

27. Dwivedi, S., et al., *Larval zebrafish model for studying the effects of valproic acid on neurodevelopment: An approach towards modeling autism*. J Pharmacol Toxicol Methods, 2019. **95**: p. 56-65.
28. Camussi, D., et al., *Delving into the Complexity of Valproate-Induced Autism Spectrum Disorder: The Use of Zebrafish Models*. Cells, 2024. **13**(16).
29. Chen, J., et al., *Trimethyltin chloride (TMT) neurobehavioral toxicity in embryonic zebrafish*. Neurotoxicol Teratol, 2011. **33**(6): p. 721-6.
30. Licitra, R., et al., *Cannabidiol Mitigates Valproic Acid-Induced Developmental Toxicity and Locomotor Behavioral Impairment in Zebrafish*. Journal of Biological Regulators and Homeostatic Agents, 2023. **37**: p. 4935-4946.
31. Hagen, E.V., et al., *Exploring the impact of acute solvent exposure on larval zebrafish behaviour*. Frontiers in Behavioral Neuroscience, 2025. **Volume 19 - 2025**.
32. Shinkai, T., et al., *Refinement of the novel tank diving test: toward standardized and robust analysis of anxiety-like behavior in zebrafish*. Frontiers in Behavioral Neuroscience, 2025. **Volume 19 - 2025**.
33. Johnson, A., et al., *Examining behavioural test sensitivity and locomotor proxies of anxiety-like behaviour in zebrafish*. Scientific Reports, 2023. **13**(1): p. 3768.
34. de Oliveira, A.A.S., T.A.V. Brigante, and D.P. Oliveira, *Tail Coiling Assay in Zebrafish (Danio rerio) Embryos: Stage of Development, Promising Positive Control Candidates, and Selection of an Appropriate Organic Solvent for Screening of Developmental Neurotoxicity (DNT)*. Water, 2021. **13**(2): p. 119.
35. Livak, K.J. and T.D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method*. Methods, 2001. **25**(4): p. 402-408.
36. Mech, A.M., et al., *Behavioral and Gene Regulatory Responses to Developmental Drug Exposures in Zebrafish*. Front Psychiatry, 2021. **12**: p. 795175.
37. Kelava, I., et al., *Androgens increase excitatory neurogenic potential in human brain organoids*. Nature, 2022. **602**(7895): p. 112-116.
38. Zadmajid, V., S. Shahriar, and D.A. Gorelick, *Testosterone acts through the membrane protein GPRC6A to cause cardiac edema in zebrafish embryos*. Development, 2024. **151**(23).
39. Teede, H.J., et al., *Recommendations From the 2023 International Evidence-based Guideline for the Assessment and Management of Polycystic Ovary Syndrome*. J Clin Endocrinol Metab, 2023. **108**(10): p. 2447-2469.
40. Palm, C.V.B., et al., *Prenatal Androgen Exposure and Traits of Autism Spectrum Disorder in the Offspring: Odense Child Cohort*. J Autism Dev Disord, 2023. **53**(3): p. 1053-1065.
41. Kosidou, K., et al., *Maternal polycystic ovary syndrome and the risk of autism spectrum disorders in the offspring: a population-based nationwide study in Sweden*. Mol Psychiatry, 2016. **21**(10): p. 1441-8.
42. Tsompanidis, A., V. Warrier, and S. Baron-Cohen, *The genetics of autism and steroid-related traits in prenatal and postnatal life*. Front Endocrinol (Lausanne), 2023. **14**: p. 1126036.

43. Erdogan, M.A., M.F. Bozkurt, and O. Erbas, *Effects of prenatal testosterone exposure on the development of autism-like behaviours in offspring of Wistar rats*. Int J Dev Neurosci, 2023. **83**(2): p. 201-215.
44. Burke, F.F., et al., *Perinatal hyperandrogenization and immune activation in rodents model subtypes of autism*. Translational Psychiatry, 2026. **16**(1): p. 97.
45. Xiang, D., et al., *Berberine Ameliorates Prenatal Dihydrotestosterone Exposure-Induced Autism-Like Behavior by Suppression of Androgen Receptor*. Front Cell Neurosci, 2020. **14**: p. 87.
46. Georgousopoulou, V., et al., *Prenatal stress, hormones, and fetal brain development: gender differences*. Hormones, 2025.
47. Stathori, G., et al., *Endocrine-Disrupting Chemicals, Hypothalamic Inflammation and Reproductive Outcomes: A Review of the Literature*. International Journal of Molecular Sciences, 2024. **25**(21): p. 11344.
48. Hasan, A.K.M.M., S. Niyogi, and D.P. Chivers, *Bisphenol S toxicity in zebrafish: Endocrine, neurobehavioural, and metabolic disruptions*. Environmental Toxicology and Pharmacology, 2026. **123**: p. 104959.
49. Reum Kwon, B., et al., *Thyroid, neurodevelopmental, and kidney toxicities of common organic UV filters in embryo-larval zebrafish (Danio rerio), and their potential links*. Environment International, 2024. **192**: p. 109030.
50. Risal, S., et al., *Prenatal androgen exposure causes a sexually dimorphic transgenerational increase in offspring susceptibility to anxiety disorders*. Transl Psychiatry, 2021. **11**(1): p. 45.
51. Rotem, R.S., et al., *Associations of Maternal Androgen-Related Conditions With Risk of Autism Spectrum Disorder in Progeny and Mediation by Cardiovascular, Metabolic, and Fertility Factors*. Am J Epidemiol, 2021. **190**(4): p. 600-610.
52. Avitan, L., et al., *Spontaneous and evoked activity patterns diverge over development*. Elife, 2021. **10**.
53. Mathouchanh, M. and C.A. Lessman, *Effects of progestogen neurosteroids on locomotor activity in zebrafish embryos and larvae*. Fish Physiol Biochem, 2025. **51**(3): p. 105.
54. Rock, S., et al., *Detailed Analysis of Zebrafish Larval Behaviour in the Light Dark Challenge Assay Shows That Diel Hatching Time Determines Individual Variation*. Frontiers in Physiology, 2022. **Volume 13 - 2022**.
55. Muralidharan, A., A. Swaminathan, and A. Poulose, *Deep learning dives: Predicting anxiety in zebrafish through novel tank assay analysis*. Physiol Behav, 2024. **287**: p. 114696.
56. Wagle, M., et al., *Heritable natural variation of an anxiety-like behavior in larval zebrafish*. J Neurogenet, 2017. **31**(3): p. 138-148.
57. Chhabra, A., et al., *Estrogen receptors α/β exhibit distinct intermolecular interactions to mediate gene regulatory events*. Life Sci, 2026. **389**: p. 124249.
58. Lu, B., *BDNF and activity-dependent synaptic modulation*. Learn Mem, 2003. **10**(2): p. 86-98.

59. Wang, C.S., E.T. Kavalali, and L.M. Monteggia, *BDNF signaling in context: From synaptic regulation to psychiatric disorders*. Cell, 2022. **185**(1): p. 62-76.
60. Numakawa, T. and R. Kajihara, *Roles of MAPKs, Including Those Activated by BDNF/TrkB, and Their Contribution in Neurodegenerative Diseases*. Int J Mol Sci, 2026. **27**(2).
61. Chen, R., et al., *Function and application of brain-derived neurotrophic factor precursors (Review)*. Int J Mol Med, 2025. **56**(1).
62. Russell, K., et al., *Bdnf-Ntrk2 Signaling Promotes but is not Essential for Spinal Cord Myelination in Larval Zebrafish*. bioRxiv, 2025.
63. Ma, C., et al., *Fine-tuning ERK activity enables proliferation-differentiation balance during lineage specification of human embryonic stem cells*. PLoS Biol, 2026. **24**(3): p. e3003711.
64. Gagliardi, P.A. and O. Pertz, *The mitogen-activated protein kinase network, wired to dynamically function at multiple scales*. Curr Opin Cell Biol, 2024. **88**: p. 102368.
65. Naderi Yeganeh, P., et al., *Integrative pathway analysis across humans and 3D cellular models identifies the p38 MAPK-MK2 axis as a therapeutic target for Alzheimer's disease*. Neuron, 2025. **113**(2): p. 205-224.e8.