

**Individual anxiety assessment in zebrafish: development and validation of a sex-specific
Individual Anxiety Scoring System (IASS)**

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ABSTRACT

Anxiety disorders represent a significant global health challenge, imposing a substantial societal burden. Preclinical models such as the Novel Tank Test (NTT) are crucial for research but are limited to group-level comparisons, masking individual heterogeneity. To bridge this gap, we developed a sex-specific Individual Anxiety Scoring System (IASS) for zebrafish (*Danio rerio*), based on a foundational analysis of baseline anxiety and sex differences in a cohort of 240 individuals. Principal component analysis (PCA) was employed to identify key sex-specific behavioral parameters from the NTT, which were integrated into a single, quantitative anxiety score. In agreement analyses, the IASS achieved an agreement rate exceeding 96% with the traditional NTT in both sexes. It successfully quantified individual anxiety shifts and identified inter-individual variability in responses to caffeine exposure. Integrated transcriptomic and IASS score analysis using Weighted Gene Co-expression Network Analysis (WGCNA) identified anxiety score-associated gene modules enriched in known anxiety-related pathways, providing exploratory evidence that IASS scores are associated with anxiety-related transcriptional features. In summary, this work established the IASS as a framework for individual-level anxiety assessment that facilitates the integration of behavioral phenotypes with molecular features and provides insights that may be obscured by conventional group-level analyses.

Keywords: Individual Anxiety Scoring System; Zebrafish; Novel Tank Test; Principal Component Analysis; Heterogeneity; Weighted Gene Co-expression Network Analysis



INTRODUCTION

Anxiety disorders represent a significant global health challenge, reaching 470 million (361–597 million) in 2023 according to The Lancet (Santomauro et al., 2026)—a striking rise of 158.6% from 1990—and impose a substantial societal burden (Javaid et al., 2023). Clinically, approximately 40–50% of patients do not respond effectively to initial first-line pharmacotherapy due to individual heterogeneity (Bokma et al., 2019; Roy-Byrne, 2015). This therapeutic challenge is compounded by the inability of preclinical models to quantitatively assess individual anxiety levels—a critical barrier to mechanistically informed drug development (Cryan & Sweeney, 2011).

Individual behavioral heterogeneity—persistent even among genetically identical subjects under standardized conditions (Bierbach et al., 2017; Kong et al., 2022)—constitutes a fundamental challenge in anxiety research. Cross-species evidence demonstrates that anxiety susceptibility varies with strain (Egan et al., 2009; Marchette et al., 2018; Maximino et al., 2013), sex (An et al., 2011), genetic background (Volgin et al., 2018), context (Soto et al., 2024; Tunbak et al., 2020), and trait sensitivity (Buenhombre et al., 2023), thereby influencing pharmacological responses. Furthermore, inter-individual anxiety differences are also associated with variations in anti-predator responses, metabolic rates, environmental stress tolerance (Li et al., 2025), and cognitive performance (Gibelli et al., 2019). High-anxiety rodents exhibit enhanced diazepam sensitivity compared to low-anxiety counterparts (Kalisch et al., 2004), while zebrafish (*Danio rerio*) show ethanol response stratification by baseline activity (Davis et al., 2021; Tran et al., 2016). Crucially, such biologically grounded variability mirrors clinical subpopulations requiring personalized therapies, yet remains obscured by population-averaged analyses.

Preclinical animal models are pivotal for bridging basic research and clinical applications and are indispensable for elucidating neurobiological mechanisms (Grillon et al., 2019).



Zebrafish serve as an excellent model organism in behavioral neuroscience (Chahardehi et al., 2023; Shams et al., 2018), exhibiting conserved drug responsivity critical for anxiety research (Bencan et al., 2009; Bogachev et al., 2023). The Novel Tank Test (NTT), is recognized as a classic method for assessing anxiety in zebrafish (Haghani et al., 2019). However, similar to other traditional analytical frameworks, its statistical approach remains constrained to comparing group means of isolated parameters (e.g., time spent in the top zone, freezing duration), thereby preventing direct comparison of anxiety levels between individuals. This limitation precludes direct integration with individual-level molecular multi-omics data (Sarkar et al., 2022) and creates significant bottlenecks in anxiety research.

To overcome these limitations, we sought a data-driven approach to integrate these multiple behavioral parameters into a unified metric. Dimensionality reduction techniques, particularly Principal Component Analysis (PCA), have proven powerful in dissecting complex behavioral constructs in neuroscience, such as relationships between anxiety-like behaviors and motor abilities in rodents (Clément et al., 2007; Feyissa et al., 2017) and in revealing strain-specific behavioral differences (Audira et al., 2020), variations in anxiety behavior patterns (Blaser et al., 2010), and pollutant-induced anxiety (Chen et al., 2017) in zebrafish. This established utility supported our hypothesis that PCA could be effectively repurposed to quantify individual anxiety levels from NTT-derived behaviors—a novel application of this approach.

Therefore, this study aimed to establish an Individual Anxiety Scoring System (IASS) in zebrafish, addressing two critical limitations in anxiety animal models: the inability of traditional group-mean paradigms to resolve inter-individual heterogeneity and the lack of standardized tools for aligning behavioral phenotypes with molecular mechanisms at single-subject resolution. Building on PCA's proven efficacy in cross-disciplinary composite scoring, we developed a sex-stratified IASS and evaluated the agreement between IASS scores and conventional NTT-based classifications, as well as its responsiveness to caffeine-induced



behavioral changes and its ability to identify heterogeneous responses among individuals. Separately, we employed WGCNA to explore the transcriptional relevance of the IASS through enrichment analysis of anxiety-related gene modules.

METHODS

Animals and housing

Wild-type zebrafish (AB strain) were acquired in two batches from Nanjing EzeRinka, China. Each batch originated from synchronized matings of parental zebrafish that were full or half siblings. All zebrafish were experimentally naïve, with no history of mating or prior experimental exposure. They were housed in an automated aquatic system (Haisheng, China) with a water pH of 7.0 ± 0.2 and conductivity of 550 ± 10 $\mu\text{S}/\text{cm}$ at a constant temperature of $28 \pm 0.5^\circ\text{C}$. The light-dark cycle was set to 14 hours of light and 10 hours of darkness. The zebrafish were fed twice daily with fresh *Artemia* nauplii. All breeding and experimental procedures were approved by the Animal 118 Ethics Committee of Nankai University (Approval No. 2021–SYDWLL–000459).

Novel Tank Test (NTT)

A total of 240 zebrafish (with a male-to-female ratio of 1:1 and aged 6–8 months) were selected randomly. After a two-week acclimatization period, the zebrafish were subjected to the NTT protocol (Figure 1A), which was conducted following established methods described in the literature (Cachat et al., 2010). Briefly, each zebrafish was placed into a pre-treatment beaker and then transferred to a trapezoidal container (28 cm top length $\times$ 21.6 cm bottom length $\times$ 15.8 cm high $\times$ 7 cm wide) filled with 1200 mL of system water (system water and room temperature: $28 \pm 0.5^\circ\text{C}$) for behavioral observation. The tank was conceptually divided into two virtual zones: Top and Bottom. The duration of the experiment was set to 5 minutes, starting when the zebrafish entered the Bottom. Under standard conditions, the system water in the testing

container was replaced after every five zebrafish. In cases where a test zebrafish exhibited pronounced anxiety-like behavior, the system water was replaced immediately.

The movement of each zebrafish was recorded using the ZebraTower (ViewPoint, France) and analyzed with ViewPoint Application Manager software (Tracking mode and Quantization mode, v5.18.0.0/v5.31.0.300, ViewPoint, France). Eight behavioral parameters were assessed, with their definitions and software threshold settings detailed in Supplementary Table S1: Latency to enter the top (Latency, s), Number of entries to the top (Num_top), Time spent in the top (Time_top, s), Distance traveled in the top (Dis_top, m), Total distance traveled (Dis_total, m), Freezing duration (Dur_fre, s), Burst duration (Dur_bur, s), and Average velocity in movement (V_mov, m/s).

Sex-specific behavioral parameters screening for the IASS

Seven behavioral parameters (Latency, Num_top, Time_top, Dis_top, Dis_total, Dur_fre, Dur_bur) were used to generate seven distinct parameter combinations (7P, 6P-1, 6P-2, 5P-1, 5P-2, 4P-1, 4P-2; Supplementary Table S2). PCA was performed on each parameter set. For each analysis, a linear regression line was fitted for the pre_High_Anx_Beh subgroup (defined by Num_top=0), and the corresponding formula and R^2 value were calculated. The optimal combination was selected based on the highest cumulative variance explained by the first two principal components (PCs, PC1 and PC2) and the associated R^2 value.

PCA was conducted on the NTT behavioral parameters set using PCA app (Origin v.2019b). Prior to PCA, the dataset was evaluated using Kaiser-Meyer-Olkin (KMO) and Bartlett's Test of Sphericity. Parameter combinations did not meet the pre-set criteria (KMO>0.6, Bartlett's Test $P<0.05$) were excluded.

IASS model construction and anxiety score calculation

Model construction and anxiety score definition: Sex-specific IASS models were developed independently for female and male zebrafish using a cohort of 120 individuals each. PCA was



performed on the five selected behavioral parameters using Origin (v.2019b). PCs with eigenvalues > 1 and cumulative explained variance > 80% were retained (the following formulas are illustrated using the first two retained PCs as an example). For each model, we obtained the mean (μ), standard deviation (σ), the matrix L corresponding to the retained PCs, eigenvalues (λ) and the percentages of variance explained by the retained PCs.

The matrix L , exported from Origin and labeled as "Loadings", mathematically corresponds to the unit eigenvector matrix of the covariance matrix.

The projection matrix P for the first two retained PCs (PC1 and PC2) was constructed by scaling each column of the unit eigenvector matrix L with the inverse square root of the corresponding eigenvalue, yielding:

$$P = P_{5 \times 2} = \begin{bmatrix} P_{11} & P_{12} \\ P_{21} & P_{22} \\ P_{31} & P_{32} \\ P_{41} & P_{42} \\ P_{51} & P_{52} \end{bmatrix}, \quad P_{ij} = L_{ij} / \sqrt{\lambda_j} \quad (i = 1, \dots, 5; j = 1, 2) \quad (1)$$

where L_{ij} denotes the element in the "Loadings" matrix exported from Origin, representing the unit eigenvector coefficient of the i^{th} behavioral parameter on the j^{th} retained principal component; λ_1 and λ_2 are the eigenvalues corresponding to PC1 and PC2, respectively.

The retained PC scores calculated using the projection matrix P were subsequently combined into an individual anxiety score (AS) as a variance-weighted sum:

$$AS = (PC_1 \times w_1) + (PC_2 \times w_2) \quad (2)$$

where PC_1 and PC_2 are the scores for the retained PCs, and the weights w_1 and w_2 are the corresponding weights based on their explained variance, calculated as:

$$w_i = \text{Var}(PC_i) / (\text{Var}(PC_1) + \text{Var}(PC_2)), \quad i = 1, 2 \quad (3)$$

This procedure established the IASS model and generated all parameters required for subsequent scoring, including the mean (μ) and standard deviation (σ) of five selected behavioral parameters, the precomputed projection matrix P , and the weighting coefficients (

w_1 and w_2) of Eq. (2), which were subsequently used to calculate anxiety scores for new individuals.

Calculation of anxiety scores for new samples: To compute the AS for a new individual, its raw behavioral data vector $X = [x_1, x_2, x_3, x_4, x_5]$ was processed using the established sex-specific model in three sequential steps:

Standardization: The raw data vector X were standardized using the model's precomputed mean (μ) and standard deviation (σ) vectors to produce the standardized vector $Z = [z_1, z_2, z_3, z_4, z_5]$:

$$Z_j = (x_j - \mu_j) / \sigma_j, \quad j = 1, \dots, 5 \quad (4)$$

Projection: The standardized vector Z was multiplied by the precomputed projection matrix P (Eq. 1) to obtain the scores of the first two retained PCs:

$$[PC_1 \quad PC_2] = Z \times P = [z_1, z_2, z_3, z_4, z_5] \times \begin{bmatrix} P_{11} & P_{12} \\ P_{21} & P_{22} \\ P_{31} & P_{32} \\ P_{41} & P_{42} \\ P_{51} & P_{52} \end{bmatrix} \quad (5)$$

Score Calculation: The AS was calculated by applying the variance-weighted sum defined in Eq. (2): $AS = (PC_1 \times w_1) + (PC_2 \times w_2)$.

Calculation template and reproducibility: To facilitate reproducibility and application, we provided an automated Excel template in the Supplementary Materials (Anx_score_template.xlsx), containing the complete workflow from raw behavioral parameters to final anxiety scores (ASs) with all calculation formulas embedded and visible to users. The template supports both batch calculation of ASs and customization of PCA-derived model parameters.

Agreement analysis between IASS and NTT

Pairwise comparisons between the ASs of IASS and the traditional NTT (based on behavioral parameters and trajectories) were performed in a systematically sampled cohort. Systematic sampling was performed on 120 female/male zebrafish rank-ordered by descending

computational ASSs. 14 individuals were selected at defined rank positions (1, 5, 15, 25, ..., 115, 120) to span the full phenotypic spectrum from extreme high-anxiety to low-anxiety phenotypes.

For each of the 91 pairwise combinations ($n=14$, $C(14,2)=91$), we evaluated congruence between two methods. For traditional NTT, two blinded raters independently conducted pairwise comparisons of eight behavioral parameters and swimming trajectories between specimens, assessing relative anxiety intensity based on established ethological metrics (Cachat et al., 2010). For each pair, the raters designated the less anxious individual (A or B), or “ND” if a reliable judgment could not be made. Inter-rater reliability was assessed as the proportion of pairwise comparisons receiving identical classifications from both raters. Any discrepancies were then resolved by discussion to reach a consensus final designation.

Agreement Rate was defined as the proportion of all pairwise comparisons in which computational scores and consensus NTT evaluations concurred on anxiety-level hierarchy. Pairwise comparisons categorized as “ND” by blinded raters were conservatively retained in the denominator and treated as non-concordant cases.

$$\text{Agreement Rate (\%)} = \text{Number of concordant pairs} / \text{Total pairs} \times 100\% \quad (6)$$

Binomial 95% confidence intervals (CI) for Agreement Rates were calculated using the modified Wald method.

Caffeine-induced anxiety test

Twelve female zebrafish were randomly selected and exposed to a Caffeine (95.2%, CAS:58–08–2, TMRM, China) solution at a concentration of 100 mg/L for 20 minutes. NTTs were conducted both before and after caffeine exposure. Data were analyzed using the IASS and the traditional NTT statistical method for evaluation and comparison.

WGCNA-based correlational analysis between brain transcriptomic profiles and IASS



Zebrafish with stable anxiety phenotypes ($n=12$) were screened using IASS and marked with their ASs. Whole-brain transcriptomic profiling via RNA Sequencing (RNA-seq) was conducted on the individuals. The resulting gene expression data, combined with the quantitative IASS scores, were analyzed using Weighted Gene Co-expression Network Analysis (WGCNA). The most significant anxiety-related modules were then functionally annotated through KEGG pathway enrichment analysis.

Stable anxiety phenotype samples screening

Using the established IASS, zebrafish were assessed twice with a two-week interval to select individuals exhibiting stable anxiety phenotypes (Supplementary Figure S1A). For females, stable high anxiety-like behavior (f_HAB) was defined by $AS \leq -0.5$ in both assessments, whereas stable low anxiety-like behavior (f_LAB) required $AS > 0$ in both assessments. For males, the corresponding thresholds were $AS \leq -0.6$ for m_HAB and $AS > -0.15$ for m_LAB.

RNA-seq

Six stable f_HAB and six stable f_LAB female zebrafish were selected. Brain tissues were rapidly dissected on ice and subsequently subjected to RNA-seq, which was commercially performed by BGI Genomics (China).

WGCNA

The obtained RNA-seq data were analyzed using the WGCNA (v.1.48) and KEGG pathway enrichment analysis on BGI's Dr. Tom platform (<https://biosys.bgi.com/>). The Pearson correlation coefficient (r) and its significance (P , uncorrected for multiple comparisons) were calculated between modules and ASs. The significant associated modules were filtered based on the criteria ($|r| > 0.6$, $P < 0.05$).

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis was conducted on the genes within the significantly associated modules, applying a significance threshold of $P < 0.05$ (uncorrected for multiple

comparisons). The correlation analysis of co-expressed genes within the selected enriched pathways and ASs was performed using Origin 2019b software. Highly correlated genes were filtered according to the criteria ($|r| > 0.8$, $P < 0.05$).

RT-qPCR

Total RNA was extracted from individual zebrafish brains using the RNeasy Mini Kit (Qiagen, Germany). cDNA synthesis was performed with the RevertAid RT Kit (Thermo Scientific, USA). Quantitative PCR amplification used ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) with gene-specific primers (sequences in Supplementary Table S3). The housekeeping gene *rpl7* served for mRNA expression normalization (Lang et al., 2016). Relative expression of target genes was quantified via the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical analysis

Behavioral data were analyzed statistically and graphically using GraphPad Prism 9.5 software. Normality of all datasets was assessed using the Shapiro-Wilk test. For unpaired behavioral comparisons, the Mann-Whitney U test was used, and raw P values were adjusted using the Benjamini-Hochberg (BH) false discovery rate (FDR) correction procedure with significance defined as FDR $Q < 0.05$. For pre- and post-caffeine comparisons, all NTT behavioral parameters and the ASs were analyzed using the Wilcoxon signed-rank test; the raw P values from the behavioral parameters were adjusted using the BH procedure (FDR $Q < 0.05$), and the ASs were evaluated at the nominal significance level ($P < 0.05$).

PCA of the behavioral data was performed using the PCA plugin in Origin 2019b software for data analysis and graphing. Data processing of new screened samples was conducted using Microsoft Excel 2016 for matrix calculations and group determinations.

RESULTS

Heterogeneity in baseline anxiety profiles of zebrafish

Baseline anxiety profiles were assessed in 240 genetically homogeneous, experimentally naïve zebrafish using the NTT (Figure 1A), yielding significant insights into their anxiety levels. Behavioral analysis demonstrated notable inter-individual heterogeneity across eight quantified parameters (Supplementary Table S4), with coefficient of variation (*CV*) exceeding 100% for some measures. Extreme dispersion was observed in top-zone entries (Num_top: *CV*=127.5%), freezing duration (Dur_fre: *CV*=128.8%), and time spent in top (Time_top: *CV*=172.0%), demonstrating fundamental variability in stress responses. A total of 36.7% of the zebrafish exhibited complete top-zone avoidance, indicating a high level of anxiety (Levin et al., 2007). In contrast, extreme explorers entered the top zone up to 48 times, demonstrating pronounced inter-individual variability in anxiety responses under standardized conditions (Figure 1B).

Sex significantly modulated these baseline profiles (Figure 1C), with females exhibiting higher anxiety metrics: prolonged latency to enter the top ($Q<0.05$; Figure 1D), fewer top-zone entries ($Q<0.05$; Figure 1E), shorter distance traveled in the top ($Q<0.05$; Figure 1G) and increased freezing duration ($Q<0.01$; Figure 1I). Locomotor parameters also showed sexual dimorphism—females displayed shorter total distance traveled ($Q<0.0001$; Figure 1H), lower average movement velocity ($Q<0.0001$; Figure 1K). Male zebrafish exhibited a greater degree of exploratory behavior compared to females, characterized by a higher proportion of entries into the upper space (83 males vs. 69 females) and a greater number of upper space entries, with males reaching a maximum of 48 times and females a maximum of 38 times.

The analysis revealed pronounced sexual dimorphism in anxiety profiles. Consequently, we developed a sex-specific scoring system.

Development of the female IASS

We analyzed 120 naïve female zebrafish by recording 7 behavioral parameters during their initial NTT. Using PCA, we systematically evaluated multiple parameter combinations and

developed a model based on an optimal set of five parameters. The first two PCs (cumulative variance>80%) were used to construct a scoring system via weighted summation, thereby enabling the assessment of anxiety levels in individual female zebrafish. The development of the female IASS is presented in three sequential stages: behavioral parameter screening, construction of the final PCA-based scoring model using the selected behavioral parameters, and application of the finalized model to independent samples.

Behavioral parameters screening for female IASS: We initially performed PCA on 7 observational behavioral parameters from the NTT ($n=120$, females). The parameters included Latency, Num_top, Time_top, Dis_top, Dis_total, Dur_fre, Dur_bur (Supplementary Table S1). V_mov, a calculated metric, was excluded from this analysis. Results demonstrated that the first two PCs with eigenvalues>1 collectively accounted for over 85% of the total variance (Figure 2A). Three-dimensional PCA visualization revealed clear spatial segregation between pre_High_Anxiety_Beh (Num_top=0) and pre_Low_Anxiety_Beh (Num_top>0) cohorts (Figure 2B), demonstrating PCA's discriminative capacity for anxiety phenotypes. However, Three-dimensional loading plot analysis (Figure 2C) revealed tight spatial clustering of specific parameters (such as, Dis_top and Time_top) in vector space, visually indicating strong inter-parameter associations. Subsequent correlation analysis confirmed these observations (Figure 2D): Dis_top and Time_top exhibited near-perfect collinearity ($r=0.99$, $P<0.0001$), while Dis_total correlated strongly with Dur_bur ($r=0.89$, $P<0.0001$), and Num_top showed high concordance with both Time_top ($r=0.88$, $P<0.0001$) and Dis_top ($r=0.88$, $P<0.0001$). This robust multicollinearity necessitated parameter refinement to eliminate redundancy and enhance scoring system sensitivity.

We systematically evaluated seven parameter combinations (7P, 6P-1, 6P-2, 5P-1, 5P-2, 4P-1, 4P-2; Supplementary Table S2) using two criteria: cumulative variance explained by the first two PCs (PC1 and PC2) and linear fit accuracy R^2 (Figure 2E-K). All 7 parameter

combinations demonstrated adequate factorability (with KMO>0.6 and significant Bartlett's test of sphericity $P<0.001$; Supplementary Table S5–female). The 5P–2 parameter combination (Latency, Num_top, Time_top, Dis_total, Dur_fre) demonstrated optimal performance for anxiety quantification (Figure 2L), achieving 87.08% cumulative explained variance (Figure 2M) and a near-perfect linear fit ($R^2=0.9956$; Figure 2I). Therefore, the five behavioral parameters—Latency, Num_top, Time_top, Dis_total, and Dur_fre—were selected as the final behavioral feature set for subsequent model construction.

After the optimal behavioral parameter set had been identified, PCA was repeated using only the screened five behavioral parameters to establish the final female IASS model. PCA performed on the five parameters from 120 female zebrafish showed that samples with similar levels of anxiety were clustered together, while the 95% Confidence Ellipsoid of the pre_High_Anxiety_Beh group (Num_top=0) approximated a plane (Figure 2N), with samples displaying linear characteristics in Two-dimensional projections (Figure 2O). Individuals with lower anxiety levels were situated farther away from the line formed by the high-anxiety cluster. Directional analysis linked anxiety levels to parameter loadings (Figure 2O): proximity to point A (BA direction) correlated with prolonged freezing (Dur_fre) and reduced movement (Dis_total), indicative of high anxiety. While proximity to point D (CD direction) associated with shorter latency (Latency) and increased top exploration (Time_top, Dis_top), signifying low anxiety. Thus, for samples exhibiting significant differences, we can preliminarily assess the anxiety levels of various zebrafish based on their positional relationships on biplot.

These results confirmed that the retained five behavioral parameters adequately captured the major variation associated with anxiety-like behavior and therefore provided the basis for constructing the final scoring model.

construction of the final IASS model: The IASS was constructed by performing PCA in Origin (v.2019b) on the screened 5P–2 behavioral parameter set (Latency, Num_top, Time_top,

Dis_total, Dur_fre) of 120 female zebrafish. The first two principal components (PC1 and PC2) had eigenvalues of 3.28 and 1.08, both exceeding 1.0, and together explained 87.08% of the total variance, thereby satisfying the predefined retention criteria.

After PCA, all parameters required for the female IASS model were fixed. These included: the mean (μ) and standard deviation (σ) vectors (Supplementary Table S6A); the matrix L for the retained PCs (Supplementary Table S6B); the eigenvalues λ and their square roots for the retained PCs (Supplementary Table S6C), and the percentages of variance explained by PC1 (65.57%) and PC2 (21.50%).

We further calculated the projection matrix P using Eq. (1) (Supplementary Table S6D) and the variance weights using Eq. (3), yielding $w_1=75.31\%$ and $w_2=24.69\%$. Accordingly, the anxiety score for female zebrafish was defined as:

$$AS_{\text{female}} = PC_1 \times 75.31\% + PC_2 \times 24.69\% \quad (7)$$

Together, these fixed parameters constitute the complete female scoring model established under the present experimental conditions. The model allows direct calculation of anxiety scores for new individuals generated under the same NTT protocol, strain background, tracking system, and laboratory settings.

Application of the female IASS to new samples: For a new sample, five behavioral parameters (vector X)—Latency, Num_top, Time_top, Dis_total, and Dur_fre—were first standardized according to Eq. (4) to obtain the normalized vector Z . This vector was then projected onto the retained PC space via the projection matrix P (Eq. (5)), yielding the PC scores (PC_1 and PC_2). Finally, the individual AS_{female} was computed using Eq. (7). To illustrate this procedure, a representative calculation example is provided below:

For a representative female zebrafish (Latency = 42 s, Num_top = 24, Time_top = 25.13 s, Dis_total = 21.83 m, Dur_fre = 1.8 s), the raw behavioral vector is defined as:

$$X=[42, 24, 25.13, 21.83, 1.8];$$

After standardization according to Eq. (4), the normalized feature vector is obtained as:

$$Z=[-1.5148, 1.4850, 0.4035, 0.5906, -0.8772];$$

Projection onto the retained principal components according to Eq. (5) yields:

$$[PC_1 \quad PC_2] = [-1.5148 \ 1.4850 \ 0.4035 \ 0.5906 \ -0.8772] \times \begin{bmatrix} -0.2423 & -0.3072 \\ 0.2749 & 0.3078 \\ 0.2575 & 0.3440 \\ 0.2078 & -0.6366 \\ -0.2475 & 0.4662 \end{bmatrix}$$

$$[PC_1 \quad PC_2] = [1.2190 \quad 0.2763],$$

where $PC_1=1.2190$ and $PC_2=0.2763$;

Substitution into Eq. (7) yields:

$$AS_{\text{female}} = (1.2190 \times 0.7531) + (0.2763 \times 0.2469) = 0.9862.$$

Thus, the final anxiety score of this representative individual is 0.9862.

An automated calculation template is provided in the Supplementary Materials (“Anx_score_template.xlsx”). This pipeline provides a standardized and reproducible framework for calculating individual anxiety scores under the same experimental conditions.

Development of the male IASS

The same analytical procedure was applied to a cohort of 120 male zebrafish, establishing a parallel, sex-specific IASS. PCA identified an optimal 5-parameter set (5P-1: Latency, Num_top, Dis_top, Dis_total, Dur_fre; Figure 3A) for males, which yielded the highest cumulative values for PC1 and PC2, with an R^2 value of 0.9934 (Figure 3B–D). Importantly, Dis_top was selected for males, whereas Time_top was selected for females, highlighting the presence of sex-specific behavioral indicators.

The IASS for male zebrafish was established using the same methodology, based on the 5P-1 behavioral parameter set (Figure 3E, F). PC1 and PC2 together accounted for 81.15% of the total variance and were retained for score calculation. After PCA, all relevant model parameters

and results are detailed in Supplementary Table S7 A–D. The definitive formula for calculating the AS for any new male sample was defined as:

$$AS_{\text{male}} = PC_1 \times 71.73\% + PC_2 \times 28.27\% \quad (8)$$

As with the female model, anxiety scores for new male subjects can be directly calculated by standardizing the five behavioral parameters, projecting them onto the retained PC space, and applying Eq. (8). An automated Excel template of IASS for male zebrafish is also provided in the Supplementary Materials (“Anx_score_template.xlsx”).

Agreement analysis between IASS and NTT

We evaluated the agreement of the established IASS with the NTT results by performing pairwise comparisons of all possible dyadic combinations ($C(14,2)=91$ pairs) within a cohort of systematically sampled female zebrafish ($n=14$, Supplementary Table S8A). The two blinded raters showed highly consistent pairwise NTT-based classifications (inter-rater agreement=96.7%). Inter-method agreement analysis revealed concordant classifications in 89 of 91 pairwise comparisons (Agreement Rate=97.8%, 95% CI: 91.9–99.9%), whereas the remaining two pairs could not be unambiguously classified by the NTT-based assessment. Representative examples illustrating the concordance between IASS-derived scores and NTT-based rankings are shown in Figure 4A. Furthermore, female zebrafish stratified into f_LAB ($AS>0$) and f_HAB ($AS\leq-0.5$) subgroups based on IASS scores exhibited statistically robust intergroup differences across eight behavioral parameters (V_{mov} : $Q<0.001$; others: $Q<0.0001$; Figure 4B). These findings indicate that IASS-based subgrouping captures meaningful behavioral heterogeneity while remaining highly concordant with conventional NTT assessment.

Similarly, a separate agreement analysis in the male cohort produced consistent findings. The two blinded raters showed highly consistent pairwise NTT-based classifications (97.8% raw agreement). Concordant classifications were observed in 88 of the 91 pairwise



comparisons (Agreement Rate=96.7%, 95% CI: 90.4–99.3%) between ASs and NTT (Supplementary Table S8B; Supplementary Figure S2A). Subgroup differentiation by IASS (m_LAB vs. m_HAB) showed significant differences across eight parameters (V_mov and Dur_bur: $Q < 0.01$; Dis_total: $Q < 0.001$; others: $Q < 0.0001$; Supplementary Figure S2B). These findings from both sexes demonstrate a high degree of concordance between the IASS and conventional NTT-based assessment.

Responsiveness of IASS to caffeine-induced anxiety

Since studies have established that short-term caffeine exposure induces anxiety behavior in zebrafish (Alia & Maureen, 2019; Carvalho et al., 2019; Maximino et al., 2011), we employed this paradigm to evaluate the responsiveness and sensitivity of the IASS (experimental workflow: Figure 5A). Female zebrafish ($n=12$) were randomly selected and subjected to a 20 min acute caffeine challenge (100 mg/L), with behavioral changes comparatively analyzed between traditional NTT and IASS.

The NTT results (Figure 5B) revealed that, compared to pre-exposure levels, caffeine exposure significantly increased Latency, while decreasing Num_top, Time_top, and Dis_top ($Q < 0.05$). The Dur_fre increased after caffeine exposure, but was not significant ($Q = 0.064$). These behavioral changes collectively indicate elevated anxiety levels, aligning with established findings on caffeine (Egan et al., 2009; Li et al., 2024). Correspondingly, the anxiety assessment performed with the established IASS revealed a significant post-exposure decrease in scores ($P < 0.01$; Figure 5C), denoting heightened anxiety severity. This finding was highly consistent with the results obtained from conventional NTT analysis.

Moreover, the IASS successfully identified heterogeneous drug responses among individuals, a capability lacking in conventional NTT analysis. A representative case was sample n6, which was initially high-anxiety but exhibited reduced anxiety level after caffeine exposure (n6 to y6; Figure 5C, D)—a pattern that would likely be overlooked in population-



averaged analyses. These findings demonstrate that the IASS is sensitive to pharmacologically induced behavioral changes and can reveal inter-individual heterogeneity that may not be readily captured by conventional group-level analyses.

WGCNA-based correlational analysis between brain transcriptomic profiles and IASS

To investigate the transcriptomic correlates of individual anxiety scores, we leveraged WGCNA to identify gene co-expression modules associated with the ASs and examined whether these modules were enriched in pathways previously implicated in anxiety-related processes (Figure 6A). 12 female zebrafish with stable anxiety phenotypes (6 f_HAB and 6 f_LAB) were screened for whole-brain RNA-seq analysis, with their corresponding ASs presented in Figure 6B. A gene co-expression network was successfully constructed (soft threshold $\beta=7$, scale-free topology fit $R^2=0.9$; Figure 6C), yielding 16 distinct co-expression modules (Figure 6D). Two gene co-expression modules—blue2 ($r=-0.75$) and aquamarine2 ($r=0.65$)—showed nominal associations with ASs (Figure 6E, $P<0.05$).

KEGG pathway enrichment analysis were performed on the two modules. Results showed that the blue2 module exhibited an enrichment in several anxiety-related pathways, including Dopaminergic Synapses, Amphetamine Addiction, Cocaine Addiction, and Long-term Potentiation ($P<0.05$; Figure 6F). Similarly, the aquamarine2 module displayed significant enrichment in Circadian Rhythm, ErbB Signaling Pathway, Dopaminergic Synapses and Glutamatergic Synapses ($P<0.05$; Figure 6G). The gene modules highly correlated with individual ASs were significantly enriched in anxiety-related pathways, suggesting that individual differences in IASS scores are associated with anxiety-related molecular features. RT-qPCR analysis of representative genes (including *th* and five circadian rhythm-related genes) showed expression changes in agreement with the RNA-seq data (Figure 6H), thereby supporting the reliability of the RNA-seq findings.



Furthermore, we identified 20 genes from key anxiety-related pathways (e.g., dopaminergic synapse, circadian rhythm) that showed strong correlations with individual anxiety scores ($|r| > 0.8$, $P < 0.05$; Figure 6I). To assess the translational relevance of these hub genes, we identified their human orthologs through zebrafish genome annotations and then assessed their overlap with a reference anxiety disorder gene set. This list was compiled from a GeneCards database search for “anxiety disorder” (accessed on January 15, 2026), which retrieved 751 protein-coding genes relevant to human anxiety (Genecards, 2026). Five of the 20 candidate genes (e.g., *th*, *perl1a*, *perl1b*, *per2*, and *csnk1da*) had human orthologs that were present in this anxiety disorder gene set and these overlaps provide supportive cross-species contextual evidence for the potential biological relevance of the IASS.

In summary, the above results indicated that the anxiety-related gene set associated with IASS scores shows overlap with the anxiety risk genes or targets reported in existing literature (De Bundel et al., 2013; Spencer et al., 2013; Ventresca et al., 2023; Zafar et al., 2022). Together with the pathway enrichment and RT-qPCR results, these findings suggest that the behavioral variation captured by the IASS is associated with anxiety-related transcriptional signatures.

DISCUSSION

This study successfully developed a sex-specific IASS for quantifying anxiety-like behavioral variation among individual zebrafish. Comprehensive evaluation showed that the IASS exhibited high concordance with the traditional NTT, sensitively detected anxiety dynamics induced by pharmacological challenge, and effectively identified heterogeneous drug responses.

The development of the IASS is inherently grounded in the NTT, which served as the behavioral foundation for all subsequent modeling. The NTT is one of the most widely used and well-standardized paradigms for assessing anxiety-like behavior in zebrafish and is

particularly suitable for constructing standardized individual-level scoring systems. The assay generates multiple continuous and quantifiable behavioral variables related to vertical exploration, locomotion, and defensive freezing within a relatively short testing period, while maintaining high experimental reproducibility and throughput. In addition, the innate bottom-dwelling tendency of zebrafish in a novel environment provides a robust ethological basis for anxiety-related behavioral assessment (Haghani et al., 2019; Kysil et al., 2017). These characteristics make the NTT especially compatible with multivariate behavioral integration and standardized computational modeling.

The construction of a sex-specific model was a critical step, based on our initial characterization of baseline anxiety in 240 zebrafish. All zebrafish shared identical or closely matched genetic backgrounds to minimize genetic contributions to individual behavioral variation (Buenhombre et al., 2021). This initial characterization revealed pronounced sexual dimorphism. Males exhibited more active exploration and lower anxiety-like behavior, whereas females displayed higher vigilance and a stronger freezing response in novel environments, consistent with established literature (Bruna et al., 2021; Fontana et al., 2020). These sex-dependent behavioral differences may be associated with multiple biological mechanisms, including estrogen-associated regulation (Tao et al., 2025), mutations in brain aromatase that synthesizes estrogen (Malleret et al., 2025), as well as sex differences in noradrenergic receptor distribution (Ampatzis & Dermon, 2016) and brain metabolic dimorphism (Xiang et al., 2023). Importantly, previous studies have further demonstrated that sex differences in zebrafish extend beyond baseline anxiety phenotypes to differential responses to pharmacological intervention (Li et al., 2024) and environmental stressor responses (Atheena & Binu, 2023). Therefore, these sex-dependent phenotypic differences highlight the necessity of sex-specific analytical approaches and scoring models for accurate behavioral phenotyping and therapeutic evaluation (Sarapultsev et al., 2025).



Accordingly, we further applied PCA to screen and integrate the most representative sex-specific behavioral parameters and construct a composite scoring model. Rather than relying on single behavioral endpoints, the IASS captures latent anxiety-related dimensions emerging from coordinated behavioral patterns across individuals. Ultimately, the female model retained Latency, Num_top, Time_top, Dis_total, and Dur_fre, whereas the male model retained Latency, Num_top, Dis_top, Dis_total, and Dur_fre. These parameters cover vertical exploration, freezing, and locomotion, thereby integrating defensive and exploratory components (Golushko et al., 2025). Latency and Num_top, retained in both sex-specific models, primarily reflect vertical exploratory behavior. Prolonged Latency and fewer Num_top indicate enhanced risk avoidance and elevated anxiety (Cachat et al., 2010). Dur_fre reflects a passive defensive response to threat, and its increase indicates behavioral inhibition and heightened vigilance (Egan et al., 2009). Dis_total was incorporated into both models but should be interpreted with other behavioral parameters. Anxiety may manifest as freezing (reduced locomotion) or increased activity (Wendt et al., 2017), with the latter resembling anxioescapic in rodents (Shanazz et al., 2022) and fight-flight responses in humans (Roelofs, 2017). Time_top was specifically retained in the female model and Dis_top was specifically retained in the male model, indicating that Time_top demonstrated greater discriminative power for anxiety levels in females, whereas Dis_top did so in males. These results underscore the value of PCA-based feature selection, as unbiased dimensionality reduction and parameter integration enabled the identification of the most informative anxiety-like behavioral indicators for each sex.

Based on these PCA-derived models with sex-specific parameters, all computational parameters—including variable means and standard deviations, the matrix L , and variance contribution weights—were subsequently fixed to establish a complete sex-specific computational standard. In application, raw data from new individuals are standardized using



their respective sex-specific parameters. Their PC scores are computed via the corresponding projection matrix and synthesized into a score. This standardized pipeline ensures that every score is generated under an identical covariance structure and weighting system, allowing for reliable, comparable assessment of anxiety levels across individuals and experimental batches.

Although PCA has previously been used in behavioral neuroscience to dissect complex behavioral constructs (Audira et al., 2020; Chen et al., 2017; Clément et al., 2007; Feyissa et al., 2017; Re et al., 2010), the present study further demonstrates that PCA can be applied to extract latent anxiety-related dimensions from multidimensional behavioral patterns and establish a standardized framework for individual-level anxiety quantification. Its implementation as the core engine of a standardized scoring system for individual-level anxiety assessment has rarely been reported. The robustness of this PCA-based approach is further supported by its proven efficacy in generating composite metrics across diverse scientific fields (Stephanie et al., 2017; Xu et al., 2020), ensuring that the derived IASS score is both statistically rigorous and biologically interpretable.

Our findings provide strong support for the reliability of the IASS under the present experimental conditions. The agreement rate between the IASS and traditional NTT-based classification exceeded 96%. In the caffeine-induced anxiety test, the IASS not only sensitively tracked dynamic shifts in individual anxiety levels but also identified subpopulations with heterogeneous responses to the drug. These observations offer preliminary clues for the potential use of IASS in detecting individual differences in drug responsiveness, although further validation in larger cohorts is required.

More importantly, we provided transcriptomic evidence supporting the biological relevance of the IASS. By correlating individual ASs with brain transcriptomic profiles, we identified gene co-expression modules significantly associated with IASS scores that were enriched in pathways previously implicated in anxiety-related processes, including dopaminergic synapses,



glutamatergic synapses, and circadian rhythm. Key hub genes screened subsequently showed strong correlations with ASs, and their human orthologs have been implicated in anxiety disorders. This suggests that the behavioral variations captured by the IASS are associated with conserved molecular features related to anxiety.

The primary advantage of the IASS lies in its capacity to move from population-averaged analysis to individual quantification. This shift addresses two fundamental limitations of conventional group-level analyses: firstly, the inability to achieve direct comparisons of anxiety levels at the individual level; and secondly, the tendency of multi-parameter between-group mean comparisons to mask biologically meaningful individual heterogeneity. Our assessment revealed that approximately 15.6% males and 16.7% females maintained a stable high-anxiety phenotype under baseline conditions (Supplementary Figure S1B). When analyzed via group-mean methods, this substantial subpopulation can substantially influence group averages and potentially obscure treatment-related effects. This limitation was illustrated by the heterogeneous drug responses observed in our caffeine challenge experiment. Therefore, the ability of the IASS to stratify individuals may facilitate a more refined assessment of behavioral heterogeneity. Furthermore, by establishing a one-to-one mapping between individual ASs and multi-omics profiles, the IASS may facilitate detailed investigations of molecular correlates and biomarker identification.

This study has certain limitations. First, although the IASS demonstrated robust performance under the present conditions, its applicability to other zebrafish strains and laboratory settings remains to be further evaluated. The current model was established under a specific NTT protocol, strain background, tracking system, and laboratory setting and may therefore require recalibration and external validation before being applied to other experimental conditions. Second, the RNA-seq analysis (WGCNA) was limited by a small sample size ($n=6$ per group), which reduces statistical power and elevates the risk of both false positive and false negative



results. In addition, module-trait correlation analysis, gene-level screening within enriched pathways, and KEGG pathway enrichment analysis were performed using *P* values ($P < 0.05$) without multiple-comparison correction, and these findings should therefore be interpreted with caution. Third, the current IASS is derived exclusively from NTT behavioral parameters and does not yet incorporate behavioral features from other anxiety-related paradigms, personality traits, or stress coping styles.

Future directions will focus on validating the system across different strains and ages, extending the framework to additional anxiety-related behavioral paradigms and personality-associated behavioral dimensions, and incorporating biochemical indicators to establish a more robust and comprehensive assessment system.

CONCLUSION

In conclusion, we developed a sex-specific, data-driven IASS that enables quantitative assessment of anxiety-like behavioral variation at the individual level in zebrafish. The IASS showed a high degree of concordance with conventional NTT-based assessment and sensitively detected pharmacologically induced behavioral changes while revealing substantial inter-individual heterogeneity that may be obscured by population-averaged analyses. By linking individual behavioral scores with brain transcriptomic profiles, our findings further suggest that the behavioral variation captured by the IASS is associated with distinct molecular correlates relevant to anxiety. Although further evaluation in independent cohorts and experimental settings is required, the IASS provides a practical framework for individual-level behavioral phenotyping and provides a foundation for future studies integrating behavioral phenotypes with multi-omics data in zebrafish anxiety research.

DATA AVAILABILITY

Data will be made available on request.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization: X.G., Y.Z., S.W. Methodology: X.G., D.L., W.Y.L., Y.S.L., Z.P.S., and H.L.

Investigation: X.G., D.L., Y.S.L. Visualization: X.G., W.Y.L., and Z.P.S. Supervision: X.G., and H.L.

Writing—original draft: X.G. Writing—review and editing: Z.P.S., S.W., and Y.Z. All authors read and approved the final version of the manuscript.

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FIGURES

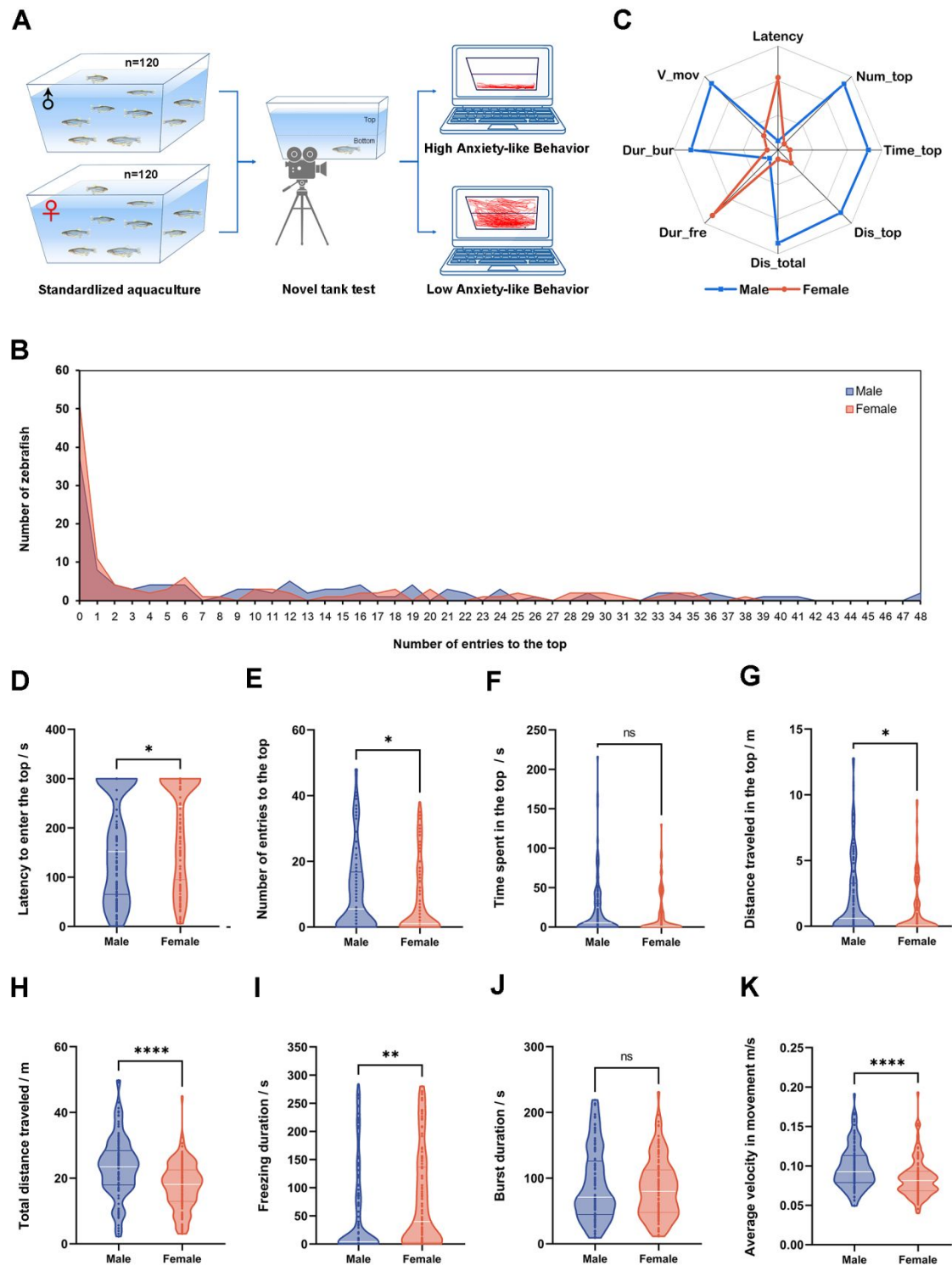


Figure 1. Baseline anxiety profiles reveal individual and sex differences in zebrafish

A: Schematic of the NTT procedure ($n=240$, 120 males and 120 females). B: Population

distribution of the number of top zone entries. C: Sex-specific patterns across eight key NTT behavioral parameters. D–K: Violin plots showing the distribution of each behavioral parameter compared between sexes, with median and quartiles indicated. Raw P values from Mann-Whitney U tests were adjusted using the Benjamini-Hochberg procedure; ns: Not significant; *: $Q < 0.05$; **: $Q < 0.01$; ****: $Q < 0.0001$.

Note: NTT, Novel Tank Test.

uncorrected proof



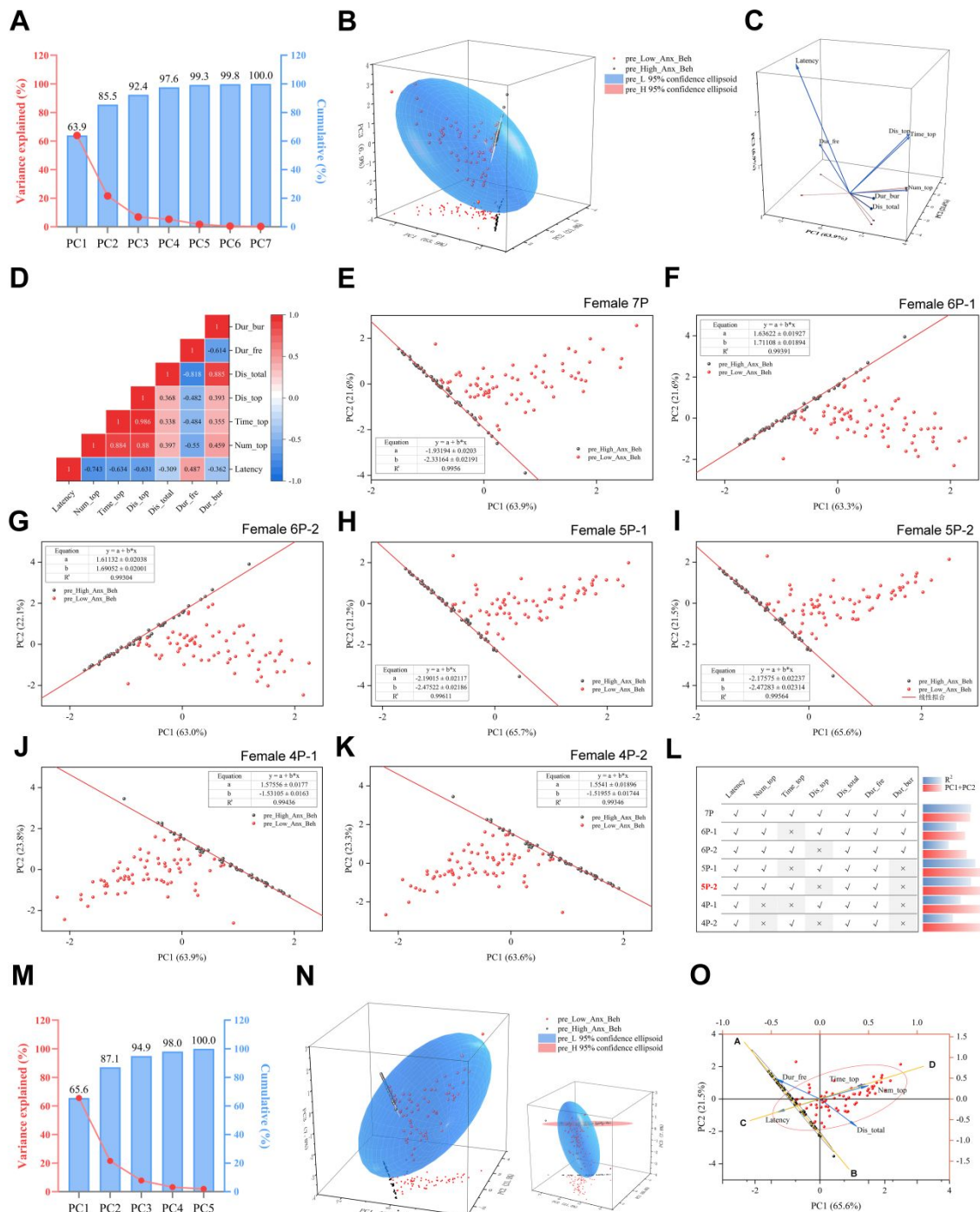


Figure 2. Identification of key parameters for the female zebrafish anxiety model

A: Scree plot from the PCA of seven initial behavioral parameters. B: 3D score plot showing pre-grouped samples based on initial behavior: pre-high anxiety-like behavior (Num_top=0) and pre-low anxiety-like behavior (Num_top>0). C: 3D loading plot corresponding to B. D:

Pearson correlation heatmap of the behavioral parameters. E–L: Evaluation of seven different parameter combinations for model construction; the 5P–2 set was selected for optimal PCA performance. M–O: Final PCA results based on the selected 5P–2 parameter set.

Note: All combinations passed KMO and Bartlett’s tests (Supplementary Table S5 female).

The selected 5P–2 set comprises: Latency (Latency to enter the top), Num_top (Number of entries to the top), Time_top (Time spent in the top), Dis_total (Total distance traveled), and Dur_fre (Freezing duration). PCA, Principal Component Analysis.

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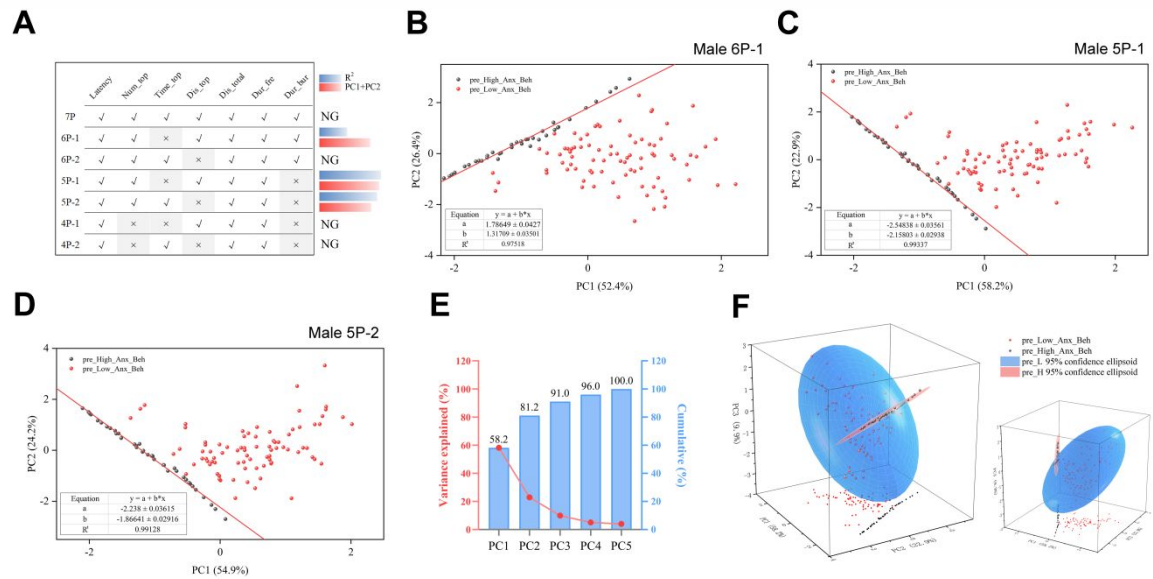


Figure 3. Identification of key parameters for the male zebrafish anxiety model

A–D: Evaluation of seven parameter combinations for model construction; the 5P–1 set was selected for optimal PCA performance. E, F: Final PCA results based on the selected 5P–1 parameter set.

Note: Three of the seven combinations met the assumptions for PCA ($KMO > 0.5$ and significant Bartlett's test, see Supplementary Table S5). The selected 5P–1 set comprises: Latency (Latency to enter the top), Num_top (Number of entries to the top), Dis_top (Distance traveled in the top), Dis_total (Total distance traveled), and Dur_fre (Freezing duration). PCA, Principal Component Analysis.

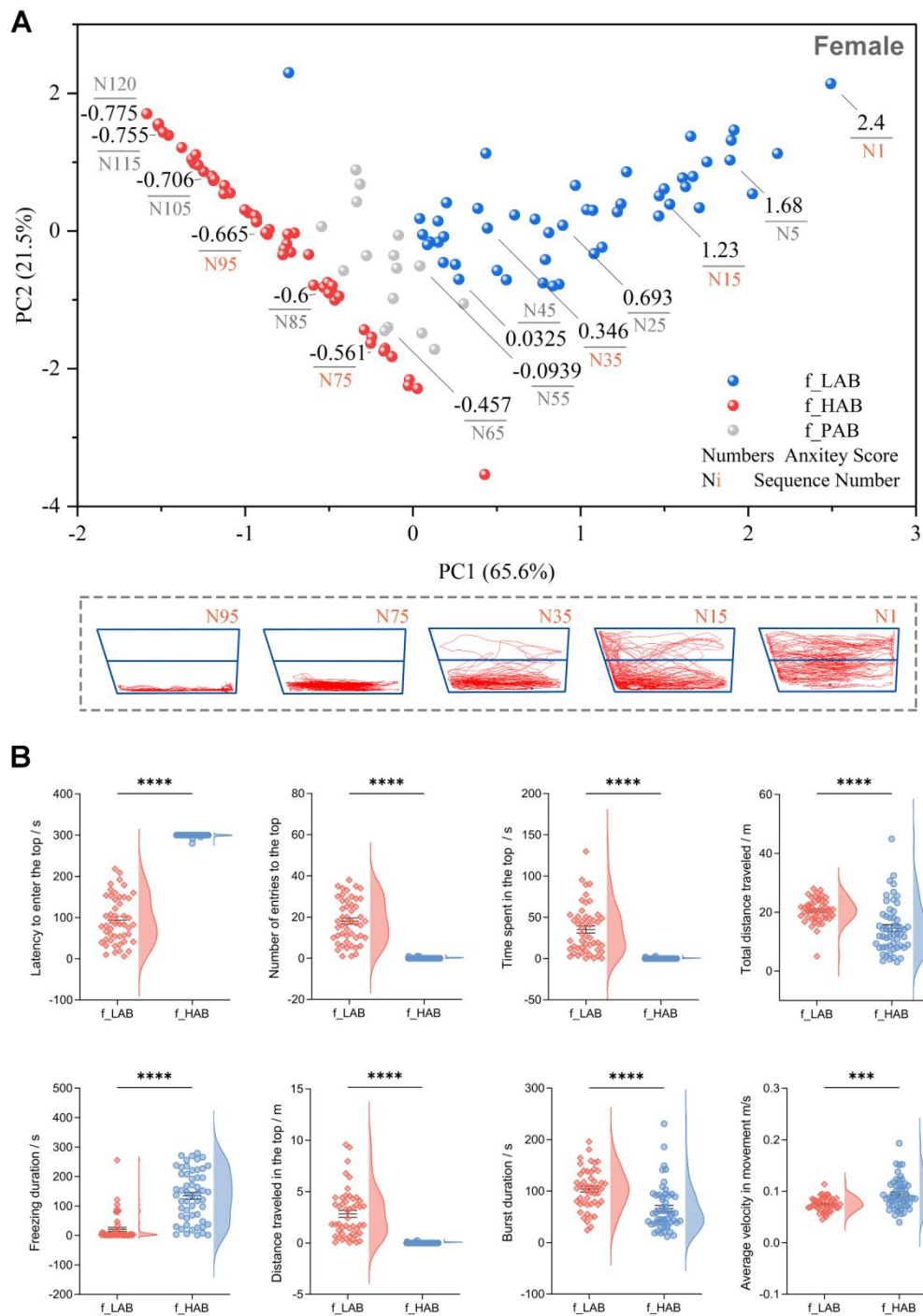


Figure 4. High consistency between the IASS and traditional NTT assessment in female zebrafish

A: IASS scores reflect anxiety levels evident in NTT swimming trajectories: lower IASS scores (higher anxiety) correlate with restricted movement and reduced top exploration, while higher

scores (lower anxiety) correlate with extensive movement and increased top entries. B: Significant behavioral divergence between IASS-stratified subgroups in females (f_LAB, $n=49$; f_HAB, $n=54$; Mann-Whitney U test, with raw P values adjusted using the Benjamini-Hochberg procedure; ***: $Q<0.001$; ****: $Q<0.0001$).

Note: IASS, Individual Anxiety Scoring System; NTT, Novel Tank Test.

uncorrected proof



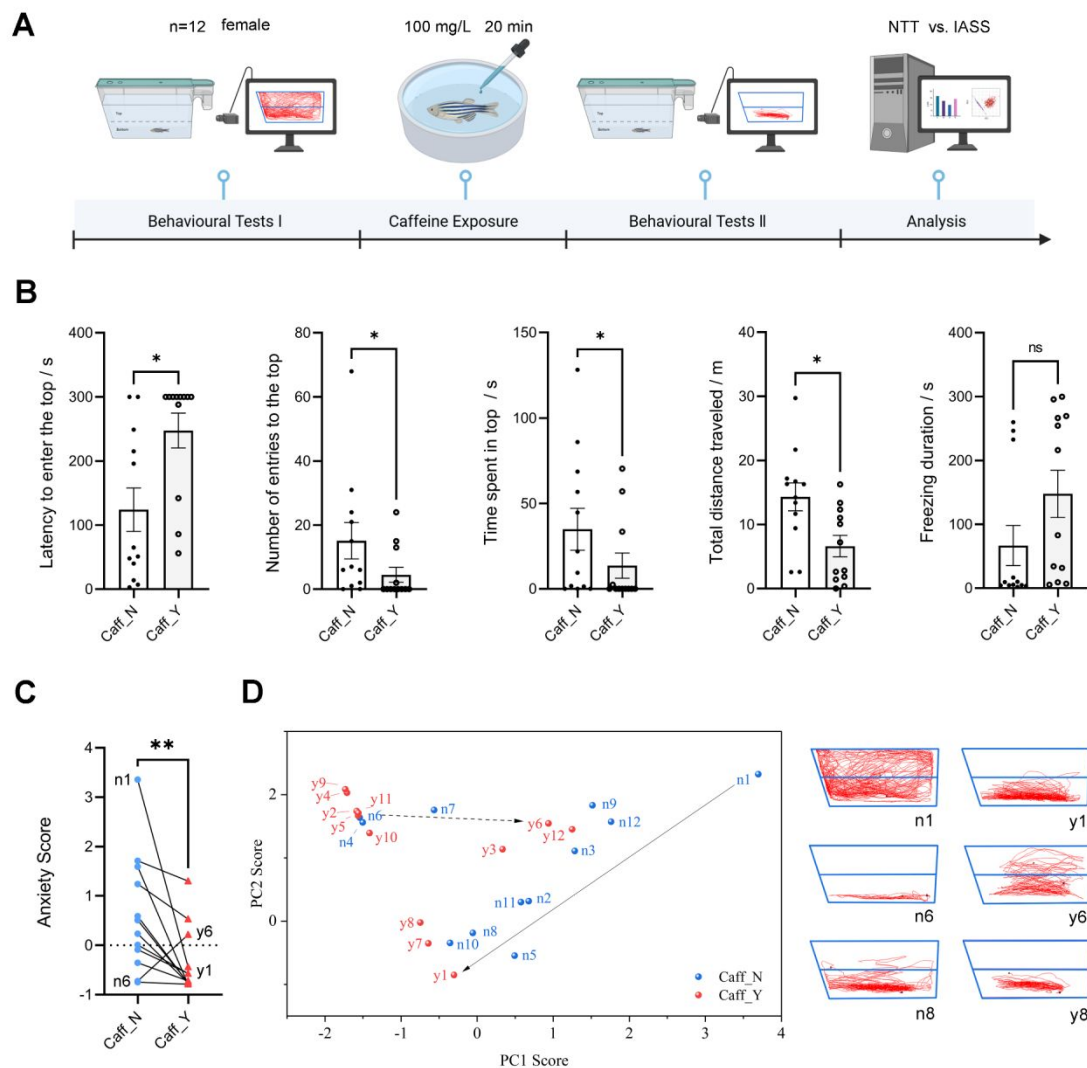


Figure 5. The IASS quantifies caffeine-induced anxiety and heterogeneous responses

A: Schematic of the caffeine exposure and testing workflow (created with BioRender.com). B: Increased anxiety levels following caffeine exposure, as measured by traditional NTT metrics (Data are presented as mean±SEM; $n=12$ females; Wilcoxon signed-rank test, with raw P values adjusted using the Benjamini-Hochberg procedure; ns: Not significant, *: $Q < 0.05$). C: Corresponding increase in anxiety quantified by the IASS (Wilcoxon signed-rank test, **: $P < 0.01$). D: Visualizations of 12 samples pre and post-exposure by IASS, with identification of a heterogeneous responder. Representative samples' anxiety scores were consistent with NTT trajectories.

Note: IASS, Individual Anxiety Scoring System; NTT, Novel Tank Test.

uncorrected proof



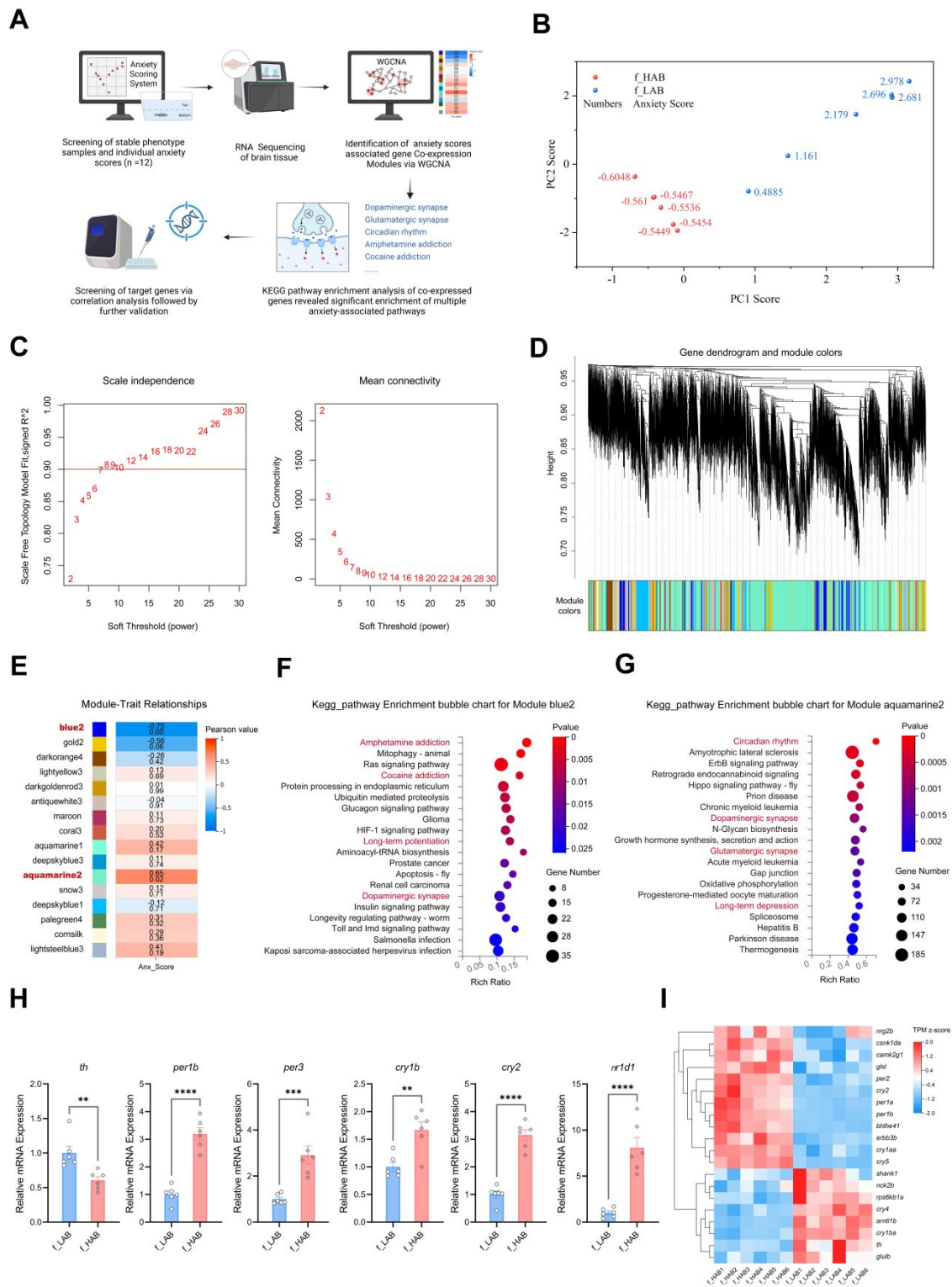


Figure 6. Biological validity of IASS through molecular enrichment in anxiety pathways

A: Schematic of the experimental workflow (created with BioRender.com). B: IASS scores of the 12 female zebrafish with stable phenotypes (6 f_HAB and 6 f_LAB) selected for RNA-seq

analysis. C: Scale-free topology analysis for choosing the soft-thresholding power ($\beta=7$, scale-free fit $R^2=0.9$). D: Gene dendrogram and module assignment, resulting in 16 co-expression modules. E: Module-trait correlation heatmap. Two modules show significant correlation with anxiety scores: blue2 ($r=-0.75$) and aquamarine2 ($r=0.65$). F-G: KEGG pathway enrichment analysis of the blue2 (F) and aquamarine2 (G) modules. Significantly enriched anxiety-related pathways ($P<0.05$) are labeled. H: RT-qPCR confirmation of transcriptional differences between anxiety phenotypes (Student's t test, **: $P<0.01$, ***: $P<0.001$, ****: $P<0.0001$). I: RNA-seq heatmap of the 20 genes from anxiety-related pathways that show high correlation with individual anxiety scores (Pearson $|r|>0.8$, $P<0.05$).

Note: IASS, Individual Anxiety Scoring System; WGCNA, Weighted Gene Co-expression Network Analysis.



Supplementary Materials for

**Individual anxiety assessment in zebrafish: development and validation of a sex-specific
Individual Anxiety Scoring System (IASS)**

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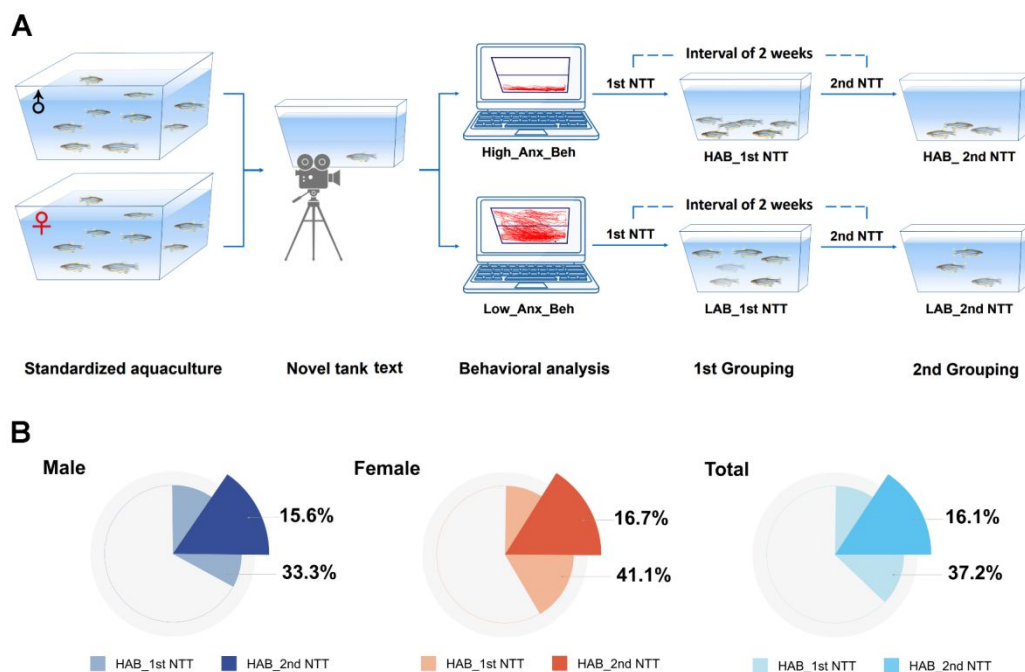


Figure S1. Individual differences in stable anxiety-like behavior in zebrafish

A: Flowchart for screening stable anxiety phenotypes via two-time NTTs. B: Proportion of zebrafish exhibiting stable high anxiety-like behavior phenotypes ($n=180$, male: female=1:1).

Note: NTT, Novel Tank Test; LAB / Low_Anx_Beh, low anxiety-like behavior; HAB / High_Anx_Beh, high anxiety-like behavior.

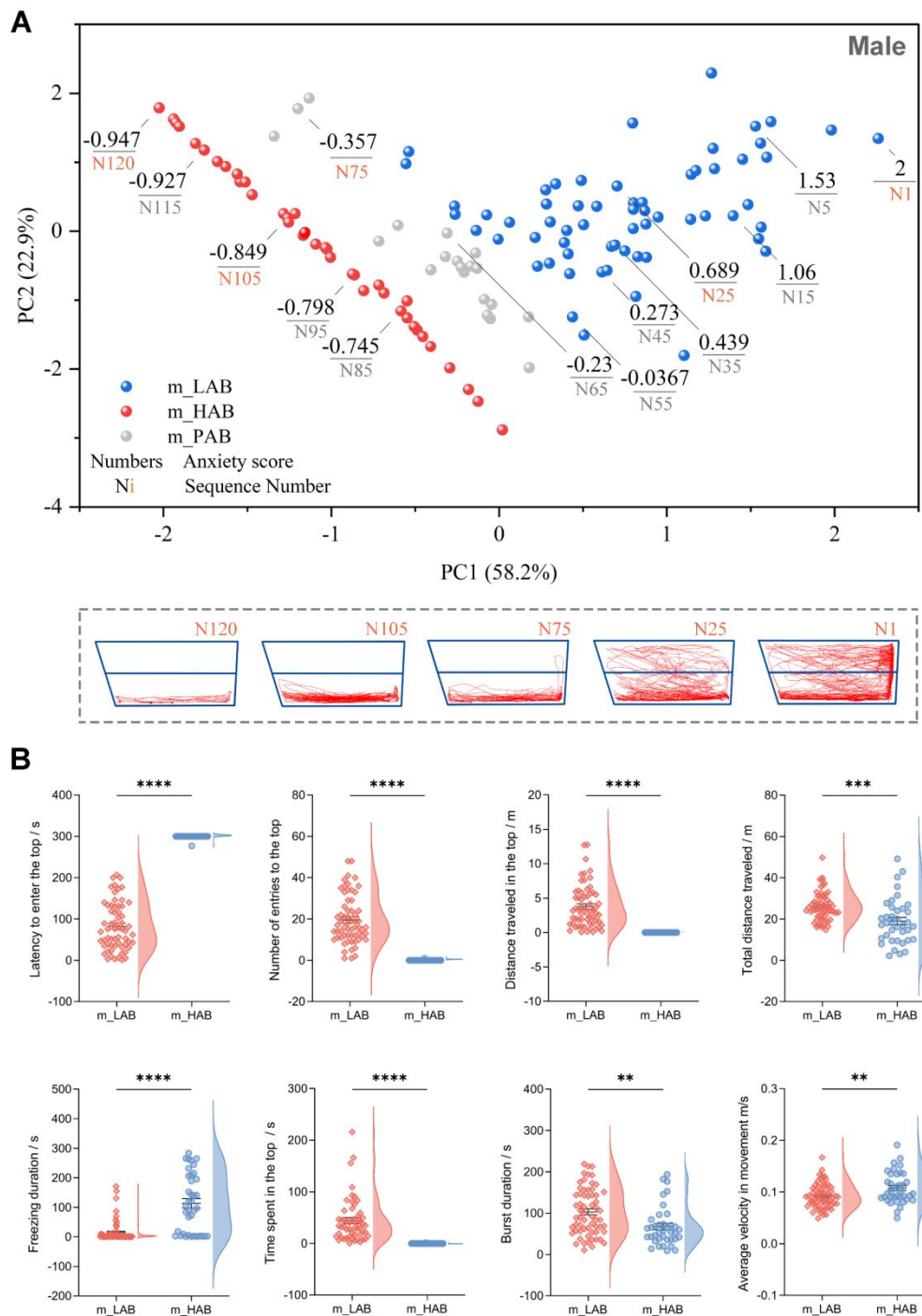


Figure S2. High consistency between the IASS and traditional NTT assessment in male zebrafish

A: IASS scores reflect anxiety levels evident in NTT swimming trajectories: lower IASS scores (higher anxiety) correlate with restricted movement and reduced top exploration, while higher

scores (lower anxiety) correlate with extensive movement and increased top entries. B: Significant behavioral divergence between IASS-stratified subgroups in males (m_LAB, $n=62$; m_HAB, $n=38$; Mann-Whitney U test, with raw P values adjusted using the Benjamini-Hochberg procedure; **: $Q<0.01$, ***: $Q<0.001$, ****: $Q<0.0001$).

Note: IASS, Individual Anxiety Scoring System; NTT, Novel Tank Test.

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Table S1. Abbreviations and definitions of terminology in NTT

Behavioral parameters	Abbreviation	Definition
Latency to enter the top (s)	Latency	Time spent for zebrafish crossed the midline from the bottom to the top for the first time.
Number of entries to the top	Num_top	Cumulative times of zebrafish from the bottom area to the top area.
Time spent in the top (s)	Time_top	Total time spent by the zebrafish in the top area of the novel tank.
Distance traveled in the top (m)	Dis_top	Total distance traveled by the zebrafish in the top.
Total distance traveled (m)	Dis_total	Total distance traveled by the zebrafish in the entire tank.
Burst duration (s)	Dur_bur	The duration when the zebrafish were in the highly active statement (Quantization mode of the ViewPoint ZebraLab system: sensitivity=15, burst threshold>200 pixels/frame).
Freezing duration (s)	Dur_fre	The duration when the zebrafish remains stationary. (Quantization mode of the ViewPoint ZebraLab system: sensitivity=15, freezing threshold=0 pixels/frame).
Average velocity in movement (m/ s)	V_mov	Mean value of velocity in movement (Total distance divided by the non_freezing duration).



Table S2. Parameter combination list for PCA

Combinations	7P	6P-1	6P-2	5P-1	5P-2	4P-1	4P-2
Parameters	Latency	Latency	Latency	Latency	Latency	Latency	Latency
	Num_top	Num_top	Num_top	Num_top	Num_top	/	/
	Time_top	/	Time_top	/	Time_top	/	Time_top
	Dis_top	Dis_top	/	Dis_top	/	Dis_top	/
	Dis_total	Dis_total	Dis_total	Dis_total	Dis_total	Dis_total	Dis_total
	Dur_fre	Dur_fre	Dur_fre	Dur_fre	Dur_fre	Dur_fre	Dur_fre
	Dur_bur	Dur_bur	Dur_bur	/	/	/	/



Table S3. Primer sequences for RT-qPCR

Symbol	Gene name	Forward primer (5'–3')	Reverse primer (5'–3')	Accession number
<i>rpl7</i>	ribosomal protein L7	CAGAGGTATCAATGGTGTGTCAGCCC	TTCGGAGCATGTTGATGGAGGC	NM_213644.2
<i>th</i>	tyrosine hydroxylase	GACCCAGCCGAAAACCAAAG	GGGTCAGTAAATCCTGGGTGA	NM_131149.1
<i>per1b</i>	period circadian clock 1b	GGTCCTCCTTCATCAACCCC	TCGTTCAAGTGGAGAGGTTCTG	NM_212439.3
<i>per3</i>	period circadian clock 3	TTCACAGCACTCATCACACTGA	GTACTCGCTGTTTGCTTGACT	NM_131584.2
<i>cry1b</i>	cryptochrome circadian regulator 1b	CCCTTGGAATGCCCCAGAAA	CAGTTGCAAGGAGCCCAAG	NM_131790.5
<i>cry2</i>	cryptochrome circadian regulator 2	CCGACCATCACTCAAGAGCA	ATTTGCTACCCAGGCCTTCC	NM_131786.2
<i>nr1d1</i>	nuclear receptor subfamily 1, group d, member 1	GACACGAACAACACAGGTGG	TGATCCGAAGCAGGGTTGTG	NM_205729.2



Table S4. Baseline anxiety levels in zebrafish and sex differences

	Male (n=120)	Female (n=120)	<i>Q</i> value	Total (n=240)	<i>CV</i> %
Latency / s	165.24±9.75	201.86±9.59	0.0261	183.55±6.94	58.59
Num_top	10.64±1.13	7.79±0.99	0.0485	9.22±0.76	127.51
Time_top / s	20.96±3.23	14.92±2.3	0.0954	17.94±1.99	172.01
Dis_top / m	2.03±0.26	1.21±0.18	0.0261	1.62±0.16	154.83
Dis_total / m	23.29±0.84	17.63±0.65	<0.0001	20.46±0.56	42.34
Dur_fre / s	53.44±7.39	76.79±7.77	0.0027	65.12±5.41	128.80
Dur_bur / s	89.03±4.94	84.63±4.06	0.8883	86.83±3.20	57.14
V_mov / m·s ⁻¹	0.098±0.002	0.084±0.002	<0.0001	0.09±0.002	28.82

Note: Results are presented as mean±SEM. Sex differences were assessed by the Mann-Whitney *U* test, with raw *P* values adjusted by the Benjamini-Hochberg procedure (*Q* value). The coefficient of variation (*CV*%) was calculated for each parameter across all zebrafish (*n*=240).



Table S5. KMO and Bartlett's test of sphericity of different parameter combinations

Female		7P	6P-1	6P-2	5P-1	5P-2	4P-1	4P-2
KMO		0.694	0.618	0.622	0.700	0.699	0.631	0.632
	Approximate Chi-Square	1117.889	688.977	692.747	445.342	448.491	231.979	232.797
Bartlett's test of sphericity	Degrees of freedom	21	15	15	10	10	6	6
	Significance	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Male		7P	6P-1	6P-2	5P-1	5P-2	4P-1	4P-2
KMO		0.535	0.601	0.578	0.684	0.666	0.593	0.555
	Approximate Chi-Square	629.416	515.270	476.485	291.468	252.089	164.960	158.029
Bartlett's test of sphericity	Degrees of freedom	21	15	15	10	10	6	6
	Significance	0.000	0.000	0.000	0.000	0.000	0.000	0.000



Table S6A. Means and standard deviations (SD) of screened behavioral parameters in female zebrafish

	f_Latency	f_Num_top	f_Time_top	f_Dis_total	f_Dur_fre
Mean (μ)	201.86	7.79	14.92	17.63	76.79
SD (σ)	105.53	10.91	25.31	7.11	85.49

Note: f_Latency, f_Time_top and f_Dur_fre are measured in seconds (s); f_Dis_total is measured in meters (m).

Table S6B. Matrix L of the retained PCs for the female zebrafish scoring model

	PC1	PC2
f_Latency	-0.4388	-0.3185
f_Num_top	0.4977	0.3191
f_Time_top	0.4663	0.3567
f_Dis_total	0.3762	-0.6601
f_Dur_fre	-0.4481	0.4834

Table S6C. Eigenvalues and their square roots from PCA modeling in female zebrafish

	PC1	PC2
Eigenvalue (λ)	3.2786	1.0752
Square root of eigenvalue	1.8107	1.0369

Table S6D. Projection Matrix P of PCA modeling in female zebrafish

	PC1	PC2
f_Latency	-0.2423	-0.3072
f_Num_top	0.2749	0.3078
f_Time_top	0.2575	0.3440
f_Dis_total	0.2078	-0.6366
f_Dur_fre	-0.2475	0.4662

Table S7A. Means and standard deviations (SD) of screened behavioral parameters in male zebrafish

	m_Latency	m_Num_top	m_Dis_top	m_Dis_total	m_Dur_fre
Mean (μ)	165.24	10.64	2.03	23.29	53.44
SD (σ)	107.30	12.46	2.86	9.19	81.26

Note: m_Latency and m_Dur_fre are measured in seconds (s); m_Dis_top and m_Dis_total are measured in meters (m).

Table S7B. Matrix L of the retained PCs for the male zebrafish scoring model

	PC1	PC2
m_Latency	-0.43476	-0.32583
m_Num_top	0.50643	0.27733
m_Dis_top	0.45124	0.39793
m_Dis_total	0.37097	-0.66443
m_Dur_fre	-0.46181	0.46595

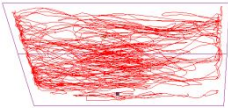
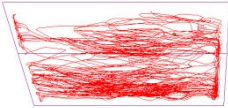
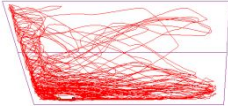
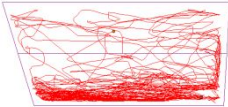
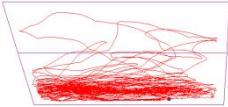
Table S7C. Eigenvalues and their square roots from PCA modeling in male zebrafish

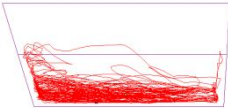
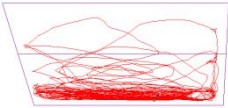
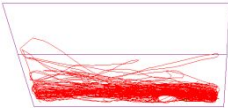
	PC1	PC2
Eigenvalue (λ)	2.91039	1.14727
Square root of eigenvalue	1.71	1.07

Table S7D. Projection Matrix P of PCA modeling in male zebrafish

	PC1	PC2
m_Latency	-0.2548	-0.3042
m_Num_top	0.2969	0.2589
m_Dis_top	0.2645	0.3715
m_Dis_total	0.2175	-0.6203
m_Dur_fre	-0.2707	0.4350

Table S8A. Eight NTT behavioral parameters, trajectory patterns, and anxiety scores in 14 systematically sampled female zebrafish

Sort Number	Latency	Num_top	Time_top	Dis_top	Dis_total	Dur_fre	Dur_bur	V_mov	Trajectories	Anxiety Score
1	39	34	129.77	9.58	20.24	3.37	109.20	0.068		2.40
5	99	29	89.60	7.96	23.51	11.17	108.53	0.081		1.68
15	57	23	53.10	4.29	22.60	0.77	139.40	0.076		1.23
25	153	16	41.93	3.42	20.34	0.70	98.30	0.068		0.69
35	60	6	13.33	0.92	16.43	2.33	47.60	0.055		0.35

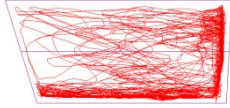
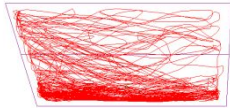
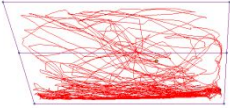
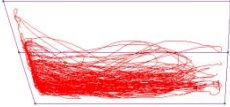
45	154	7	3.60	0.31	20.62	3.73	79.83	0.070		0.03
55	201	6	8.73	0.66	20.44	54.10	90.93	0.083		-0.09
65	284	3	0.50	0.09	24.86	43.80	124.00	0.097		-0.46
75	300	0	0	0	25.83	21.23	108.37	0.093		-0.56
85	300	0	0	0	19.30	68.70	103.07	0.083		-0.60
95	300	0	0	0	14.58	147.27	56.70	0.095		-0.67

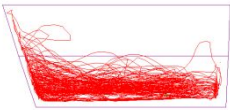
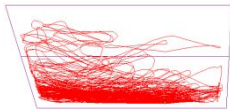
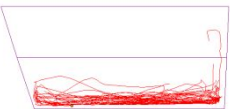


105	300	0	0	0	8.58	196.47	43.37	0.083		-0.71
115	300	0	0	0	5.10	255.63	18.47	0.115		-0.75
120	300	0	0	0	3.08	279.93	13.67	0.154		-0.77



Table S8B. Eight NTT behavioral parameters, trajectory patterns, and anxiety scores in 14 systematically sampled male zebrafish

Sort Number	Latency	Num_top	Time_top	Dis_top	Dis_total	Dur_fre	Dur_bur	V_mov	Trajectories	Anxiety Score
1	76	48	93.16	10.68	31.10	1.27	172.53	0.104		2.00
5	9	41	76.27	5.77	19.79	2.17	29.63	0.066		1.53
15	107	29	46.23	6.59	32.65	0.50	175.37	0.109		1.06
25	71	21	45.73	3.60	19.65	1.578 8	44.33	0.066		0.69
35	140	29	13.70	1.21	27.25	0.93	84.07	0.091		0.44

45	62	15	6.70	0.91	31.46	1.27	127.47	0.105		0.27
55	200	12	14.20	2.03	36.47	0.53	213.57	0.122		-0.04
65	68	1	0.03	0.05	25.16	105.20	94.77	0.129		-0.23
75	85	1	0.33	0.08	8.80	244.63	37.30	0.159		-0.36
85	300	0	0	0	23.49	1.70	43.60	0.079		-0.75
95	300	0	0	0	21.02	67.90	60.03	0.091		-0.80



105	300	0	0	0	14.69	141.63	36.40	0.093		-0.85
115	300	0	0	0	8.27	245.63	35.57	0.152		-0.93
120	300	0	0	0	2.22812	283.566	9.1	0.136		-0.95



Supplementary File 1. Anx_score_template.xlsx - Template for calculating individual anxiety scores.

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斑马鱼个体焦虑评估：性别特异性个体焦虑评分系统（IASS）的建立与验证

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摘要

焦虑障碍是全球范围内重要的公共卫生问题之一，造成了巨大的社会负担。临床前动物模型，如斑马鱼新水箱试验（Novel Tank Test, NTT），在焦虑相关研究中发挥着重要作用。然而传统的行为学分析方法主要基于多个行为参数组间均值比较，难以反映焦虑样行为的个体异质性。为突破这一限制，本研究基于 240 尾斑马鱼基线焦虑样行为特征及性别差异分析，构建了一种性别特异性的个体焦虑评分系统（Individual Anxiety Scoring System, IASS）。该系统通过主成分分析（Principal Component Analysis, PCA）筛选 NTT 中的性别特异性关键行为参数，并将其整合为单一的量化焦虑评分。结果表明，IASS 与传统 NTT 行为评价结果的一致率在雌、雄斑马鱼中均超过 96%。在咖啡因暴露实验中，IASS 能够有效量化暴露前后个体焦虑水平的变化，并识别药物反应的异质性个体。进一步结合雌性个体脑转录组数据与 IASS 评分，利用加权基因共表达网络分析（Weighted Gene Co-expression Network Analysis, WGCNA）鉴定出与焦虑评分显著相关的基因共表达模块。模块内基因在已知焦虑相关信号通路中富集，为 IASS 评分与焦虑相关转录特征之间的关联提供了探索性证据。综上，本研究建立了一种用于个体水平焦虑评估的 IASS 框架，实现了行为表型与分子特征的整合分析，为揭示传统群体水平分析难以捕获的个体差异提供了新的研究策略。

关键词：个体焦虑评分系统（IASS）；斑马鱼；新水箱试验（NTT）；主成分分析（PCA）；个体异质性；加权基因共表达网络分析（WGCNA）

