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Comparative assessment of behavioural alterations induced by common laboratory solvents in zebrafish (*Danio rerio*) larvae

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Abstract

Organic solvents are routinely used to dissolve poorly water-soluble chemicals in zebrafish-based assays. However, their intrinsic biological activity may confound behavioural endpoints and compromise data interpretation. This study systematically evaluated the effects of seven commonly used laboratory solvents, dimethyl sulfoxide (DMSO), ethanol (EtOH), methanol (MeOH), acetone (Ace), acetonitrile (ACN), isopropanol (IPA), and ethyl acetate (EtOAc), at four concentrations (1, 0.5, 0.1, 0.05%; *v*/*v*), on locomotor behaviour of zebrafish *Danio rerio* larvae at 120 hours post fertilization. Larval activity was quantified using automated video tracking under alternating light-dark conditions, assessing distance moved, velocity, acceleration, mobility states, and turning behaviour. Behavioural responses were strongly solvent- and concentration-dependent. EtOH, MeOH, Ace, and IPA induced hyperactivity at ≥ 0.5%, while DMSO showed biphasic effects, stimulating activity at 0.5% but suppressing it at 1%. ACN demonstrated a significant inhibitory response even at 0.5%, while EtOAc was tolerated only at ≤ 0.1%. Principal component analysis (PCA) and hierarchical clustering on principal components (HCPC) further revealed coherent behavioural signatures shaped not only by solvent identity and concentration but also strongly by light–dark transitions, underscoring the critical importance of consistent illumination conditions in zebrafish behavioural assays. Overall, ≤ 0.1% of Ace and EtOAc, and ≤ 0.05% of DMSO, EtOH, MeOH, ACN, and IPA did not significantly alter locomotion, suggesting their suitability for zebrafish behavioural assays. These findings establish reference points for solvent selection and concentration thresholds in zebrafish behavioural assays under the specific experimental conditions tested, thereby supporting reproducibility and reliability in scientific research.

Keywords: Zebrafish, Organic solvents, Locomotor activity, Bioassays, PCA

1. Introduction

Many environmental contaminants, pharmaceuticals and natural compounds exhibit limited water solubility, which requires the use of organic solvents or solubilizing agents prior to dilution in aqueous exposure media [1,2]. Although commonly considered chemically inert, these solvents may alter organismal physiology or behaviour, making it difficult to interpretsublethal toxicity endpoints [3].

Among *in vivo* models, zebrafish *Danio rerio* has emerged as an important vertebrate model in ecotoxicology, developmental biology, neurotoxicology, and drug screening. This is primarily due to its rapid *ex utero* development, high fecundity, transparency of embryos, and a well-annotated genome that exhibits significant genetic homology with humans [4,5]. In zebrafish studies, behaviour has been one of the most significant parameters because it reflects nervous system function and can reveal subtle neurotoxic effects before morphological damage occurs [6]. By 72–120 hours post fertilization (hpf), major subdivisions of the nervous system are established, the swim bladder is inflated, and larvae exhibit robust swimming behaviour, making locomotor behaviour a reliable indicator of integrated neural and muscle function [7]. Moreover, behavioural assays conducted during the larval stage are high-throughput, cost-effective, and associated with fewer ethical constraints compared to adult vertebrate models. Therefore, establishing solvent-specific concentration thresholds during the larval stage (i.e. 120 hours post fertilization; hpf), when behavioral phenotyping is most commonly performed, is essential for ensuring reproducibility and reliable interpretation of behavioral endpoints.

Since bioactive compounds differ greatly in polarity, hydrophobicity, and volatility, their solubilization requires solvents with distinct chemical properties. Dimethyl sulfoxide (DMSO) and ethanol (EtOH) are among the most versatile and widely used solvents in zebrafish assays, as they effectively dissolve both polar and non-polar compounds [8–11]. Methanol (MeOH) and acetone (Ace) are more suitable for small polar molecules [12], while acetonitrile (ACN), although less commonly used due to its higher toxicity, is often chosen for compounds that require stronger polar aprotic solvents [13]. Isopropanol (IPA), a polar protic solvent and secondary alcohol, dissolves a wide range of non-polar compounds [14]. Ethyl acetate (EtOAc), a moderately polar ester, is frequently used due to its ability to dissolve a broad range of non-polar and slightly polar compounds. However, despite their usefulness in chemical applications, these solvents have limited biological compatibility. Higher concentrations may disrupt physiological processes *in vivo* and should therefore be used with caution [15].

Despite the widespread use of solvents in zebrafish assays, most available studies focus on single solvents (most commonly DMSO and ethanol), rather than providing systematic, cross-solvent comparisons of neurobehavioural effects [8,16–18]. Interpretation of solvent-induced behavioural effects is further complicated by methodological variability among studies. Important factors include exposure duration [16,19], illumination conditions [16,20], behavioural stimuli [21–29], and developmental stage [16]. These discrepancies can markedly influence behavioural outcomes, limiting reproducibility and comparability. As a result, distinguishing genuine solvent-induced effects from methodological artefacts becomes challenging, highlighting the need for their evaluation under standardised conditions.

While some studies suggested that concentrations of certain solvents up to 1% (*v*/*v*) do not markedly interfere with zebrafish survival or development [8,13], regulatory guidelines such as the OECD Test Guideline 236 [30] recommend a substantially lower threshold of 0.01% (*v*/*v*). However, these thresholds clearly cannot be applied uniformly to all solvents, given their distinct toxicity profiles. Moreover, it should be noted that the absence of mortality or abnormalities does not necessarily imply the absence of biological effects, as even subtle behavioural or neurophysiological changes may remain undetected yet still compromise assay validity [3,31–33].

In the present study, we systematically evaluated the effects of seven widely used solvents, DMSO, EtOH, MeOH, Ace, ACN, IPA, and EtOAc, on locomotor activity of 120 hpf zebrafish larvae. In addition to conventional behavioural endpoints, we incorporated multivariate analysis, including principal component analysis (PCA) and hierarchical clustering on principal components (HCPC), to identify integrated behavioural patterns and characterize solvent-dependent profiles across light and dark phases. By combining univariate and multivariate approaches, this study provides a comprehensive evaluation of solvent-induced effects and offers practical guidance for selecting suitable solvents and concentrations for zebrafish behavioural assays. Overall, this study defines solvent- and concentration-dependent behavioural thresholds under standardized experimental conditions. These findings support the selection of appropriate carriers and concentrations in zebrafish-based assays and improve assay robustness, reproducibility, and reliability of sublethal assessments

2. Materials and methods

2.1 Chemicals

Dimethyl sulfoxide (99.9%; CAS No. 67-68-5) and ethanol (absolute anhydrous; CAS No. 64-17-5) were obtained from Carlo Erba Reagents, France. Ethyl acetate (99.9%; CAS No. 141-78-6) and methanol (≥ 99.9%; CAS No. 67-17-5) were from VWR, France. Remaining solvents: acetone (≥ 99.8%; CAS No. 67-64-1), acetonitrile (≥ 99.9%; CAS No. 75-05-8), and isopropanol (≥ 99.9%; CAS No. 67-63-0) were acquired from Honeywell, Germany.

To prepare E3 medium, sodium chloride (CAS No. 7647-14-5), potassium chloride (CAS No. 7447-40-7), and magnesium sulfate heptahydrate (CAS No. 10034-99-8) were purchased from Gram-mol (Croatia), and calcium chloride dihydrate (CAS No. 10035-04-8) from Merck, Germany.

2.2 Experimental setup

Adult wild-type WIK zebrafish (*Danio rerio*), obtained from the European Zebrafish Resource Center at the Karlsruhe Institute of Technology, were maintained in a ZebTEC rack system with Active Blue technology (Tecniplast S.p.A.) under a controlled photoperiod of 14 h light and 10 h dark. Water quality parameters were daily monitored and maintained within the following - ranges: temperature 26.72 ± 0.33 °C, conductivity 496.13 ± 3.08 µS/cm, pH 8.11 ± 0.04 and dissolved oxygen ≥ 95% saturation. Fish were fed two times daily with dry food (Zebrafish diet standard 05.08, Mucedola, Italy) and frozen mosquito larvae (PETRA-AQUA; Red mosquito larvae Frozen, Czech Republic). Spawning was conducted three times per week

using different parental pairs to prevent repeated use of the same breeders. One day prior to the experiment, adult males and females were transferred to an iSpawn-S benchtop breeding system (Tecniplast S.p.A.) at a 2:1 male-to-female ratio and separated by a divider. Spawning was initiated the following day by removing the divider and lifting the spawning platform. Fertilized eggs were collected within 15 min using an 800 μm mesh and gently rinsed to remove debris prior to further handling.

Fertilized embryos at 4 to 64-cell stage [34] were individually placed in 96-well plates (polystyrene and non-pyrogenic non-tissue culture treated, flat bottom with low evaporation lid; Falcon, Corning), with one embryo per well containing 300 μL of test solution. To further minimize evaporation and concentration fluctuations during the exposure period, plates were additionally sealed with Parafilm. Embryos were continuously exposed from the embryonic stage until 120 hpf to one of seven organic solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, or methanol – MeOH), each tested at four concentrations (1, 0.5, 0.1, and 0.05%, *v/v*), prepared by dilution in E3 medium. E3 medium without solvent served as the control. Exposure was conducted under static conditions. Solvent density was not considered when calculating volume-based percentages. Therefore, in this study, all results are expressed as nominal percentages (%).

The experiment was performed in three independent replicates, each conducted by a different operator. For each replicate, 40 embryos were tested per solvent concentration and control group. This resulted in 120 specimens being tested at each concentration, including controls. Embryos were randomly distributed across multiple 96-well plates, with solvents, concentrations, and control treatments interspersed within and across plates to minimize plate-position effects. Plates with embryos were placed in an incubator (Phoenix Instrument TININ35, Naperville, IL, USA) and maintained for 120 h at 27.50 ± 0.50 °C.

2.3 Analysis of larval locomotor activity

Behavioural recordings were performed at 120 hpf using a DanioVision observation chamber (Noldus Information Technology, The Netherlands). All behavioural assessments were conducted during the light phase of the incubator photoperiod, between 08:30 a.m. and 13:00 p.m., to minimize circadian variability. Prior to recording, experimental settings were adjusted in EthoVision XT 17 software (Noldus Information Technology, The Netherlands), and the well size was calibrated according to the manufacturer's instructions (top well diameter: 6.85 mm). Larvae were allowed to acclimate for 10 min under dark conditions before the start of behavioural recording to reduce handling-induced stress [11]. Light and dark periods were alternated throughout the 20-minute measurement protocol, which consisted of two identical cycles of 5 minutes of light exposure followed by 5 minutes of darkness. During the light period, illumination was set to 100% intensity, corresponding to 5,450 lux as specified by the manufacturer. The integrated DanioVision temperature control unit was used to keep the temperature at 28 °C. Obtained tracks were analysed using EthoVision XT 17 software and only live larvae without visible developmental abnormalities, including normally inflated swim bladders, were selected for further analysis. An input filter of 0.2 cm (minimum distance moved) was used to remove system noise. Analysed behavioural parameters included total

distance moved (mm), velocity (mm/s), acceleration (mm/s^2), mobility (s), and turning behaviour quantified as turning angle (degrees). All locomotion data are expressed per segment of testing, from which total activity was calculated for each larva in both light and dark periods. The mobility state was categorised based on the percentage of body position/pixel change detected between consecutive video frames by the EthoVision XT software as follows: highly mobile (> 10%), mobile (4–10%), and immobile (< 4%). These thresholds reflect movement-derived pixel change rather than absolute locomotor velocity. Lower thresholds were used because 5 dpf zebrafish larvae exhibit subtle movements and short locomotor bursts. Significantly higher thresholds would mainly detect extreme burst activity or tracking artifacts while underestimating typical spontaneous swimming behaviour.

2.4 Statistical analysis

Behavioural data exported from the EthoVision XT 17 software were analysed using the GraphPad Prism 8.0 software (San Diego, CA, USA). No systematic operator effects were observed; therefore, data from all replicates were pooled for analysis. Results are presented as means ± standard error (SEM). Data normality was assessed using the D'Agostino-Pearson omnibus normality test. Depending on data distribution, statistical analyses were performed using either one-way ANOVA followed by Tukey's multiple comparisons test or non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. Significance was set at $p \leq 0.05$. Exact *p*-values from pairwise comparisons between solvents for each measured parameter are provided within the Supplementary material, along with the corresponding Kruskal-Wallis test statistics (H), degrees of freedom (df), and overall *p*-values for each analysis (Tables S1–S3), while the main text and figures present significance relative to the control group. For multivariate analysis principal component analysis (PCA) was performed using the singular value decomposition method with scaling of variables to unit variance. Active variables for PCA were the measured behavioural endpoints and phase (light/dark periods), solvents, and concentrations served as supplementary variables. Agglomerative hierarchical clustering on principal components was performed using Ward's method on Euclidean distances and optimal number of clusters determined by absolute loss in within-cluster inertia. PCA was performed using R v4.5.2 (R: A language and environment for statistical computing. R Core Team (2025), URL: https://www.R-project.org/).

3. Results and discussion

3.1 Locomotor activity parameters

Behavioural profiling of zebrafish larvae exposed to seven commonly used solvents (DMSO, EtOH, MeOH, Ace, ACN, IPA, and EtOAc) revealed distinct concentration- and solvent-dependent alterations in locomotor activity under alternating light and dark conditions. Larvae are inherently more active in the dark compared to light conditions [35], and this contrast offers a robust framework for identifying stress-related behavioural modulation. The degree and direction of behavioural effects differed markedly across solvents, reflecting their unique chemical properties, biological compatibility, and neuroactive potential.

To characterize these effects comprehensively, locomotor behaviour was quantified using complementary endpoints, distance moved (mm; Figure 1), mean velocity (mm/s; Figure 2), and acceleration (mm/s^2; Figure S1), which provided an index of overall locomotor output, while cumulative duration of mobility states (s; Figure 3) showed how activity was distributed over time. Turning behaviour, measured as turn angle (deg; Figure 4), offered an additional insight into motor control and directional stability.

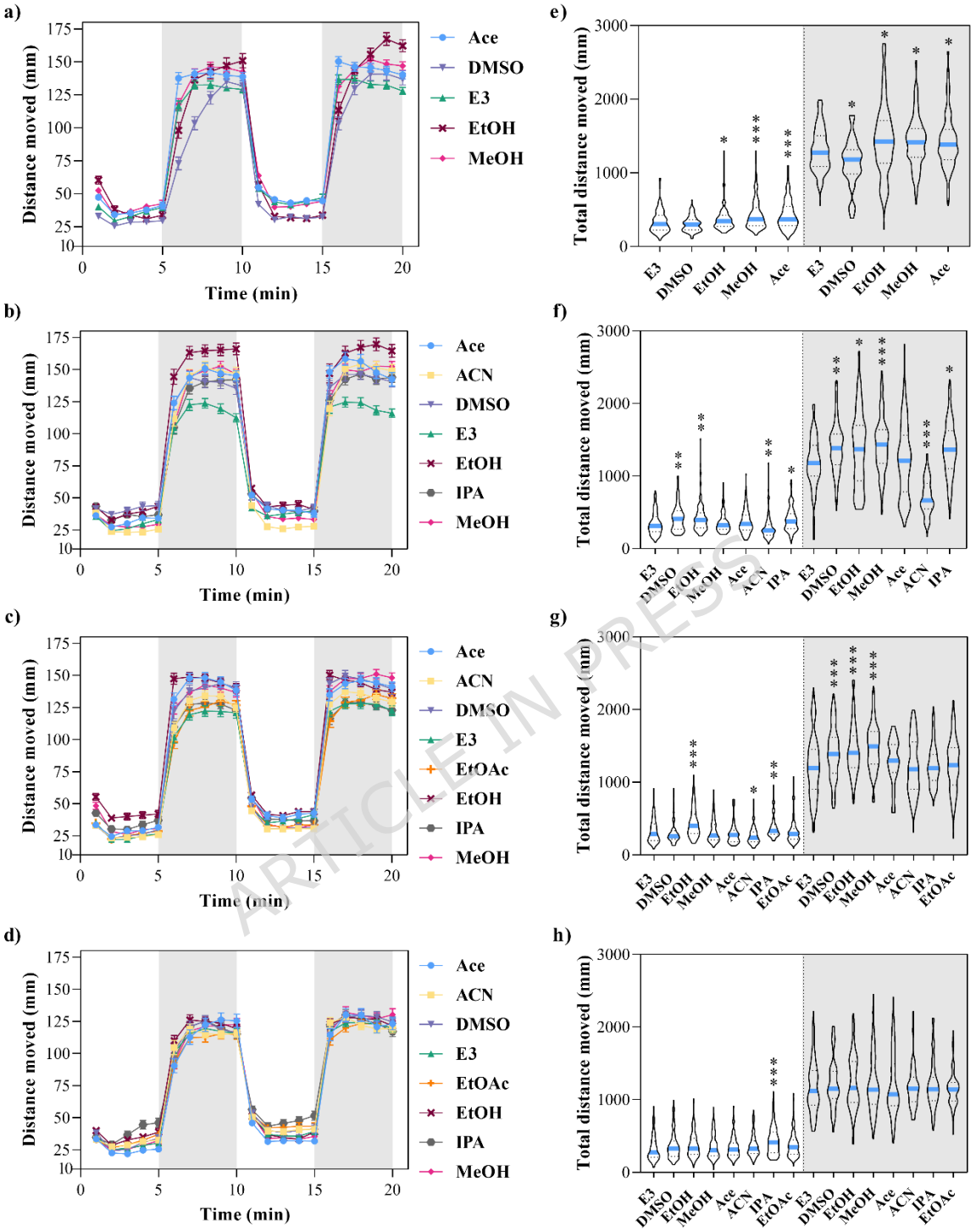

Figure 1. Effect of different solvent (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH) concentrations: 1% (a, e), 0.5% (b, f), 0.1% (c, g), and 0.05% (d, h), on zebrafish larval locomotor activity. Activity was recorded for 20 minutes under alternating 5-minute light (white background) and dark (gray background) periods. Control larvae were kept in E3

medium. The left panels (a-d) show the total distance moved by each experimental group, presented by 1-minute time bins. Results are presented as mean ± SE. The right panels (e-h) show the total distance moved during light (white background) and dark (gray background) phases. Violin plots represent the distribution of movement data, with the blue line representing the median and dotted lines indicating the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

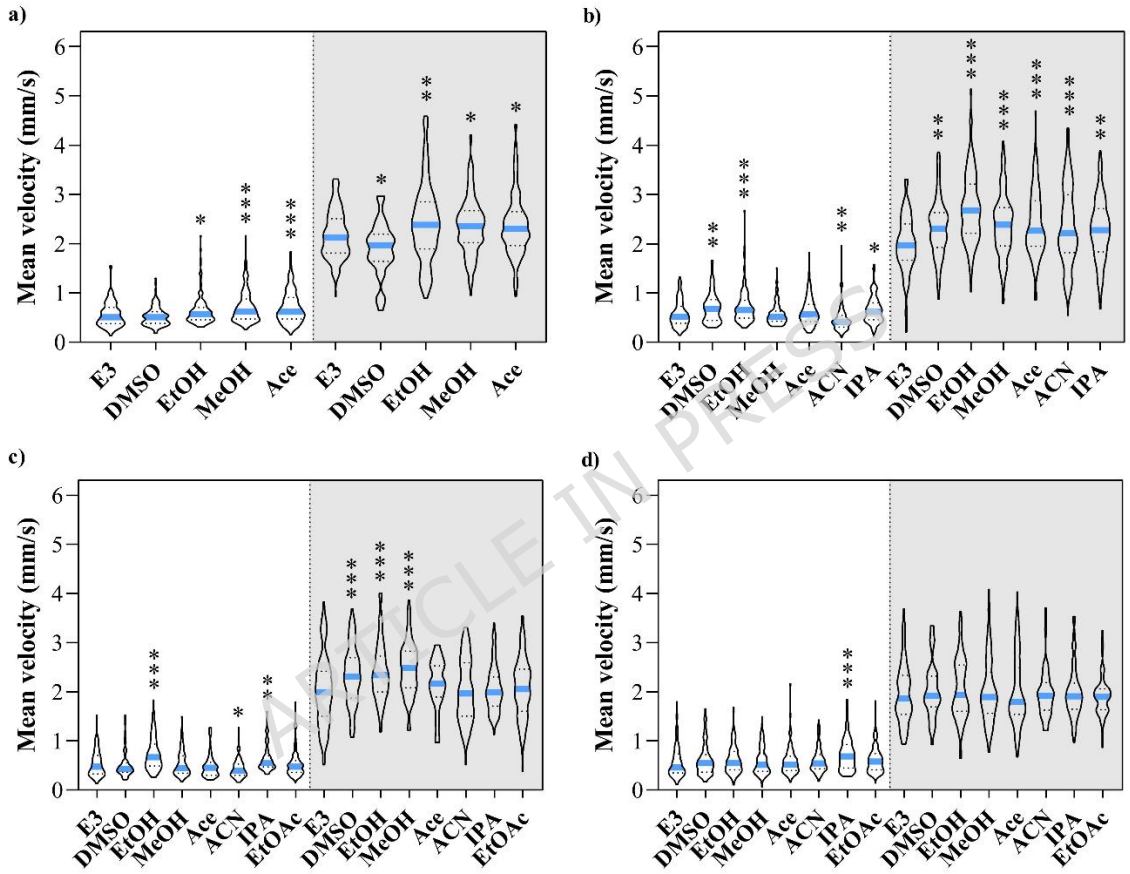

Figure 2. Effect of different solvent (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH) concentrations: a) 1%, b) 0.5%, c) 0.1%, and d) 0.05%, on zebrafish larval velocity. Swimming velocity (mm/s) was presented as mean values for the light (white background) and dark (gray background) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were kept in E3 medium. Violin plots represent the distribution of mean velocity. The horizontal blue line inside the violins represents the median, while dotted lines indicate the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

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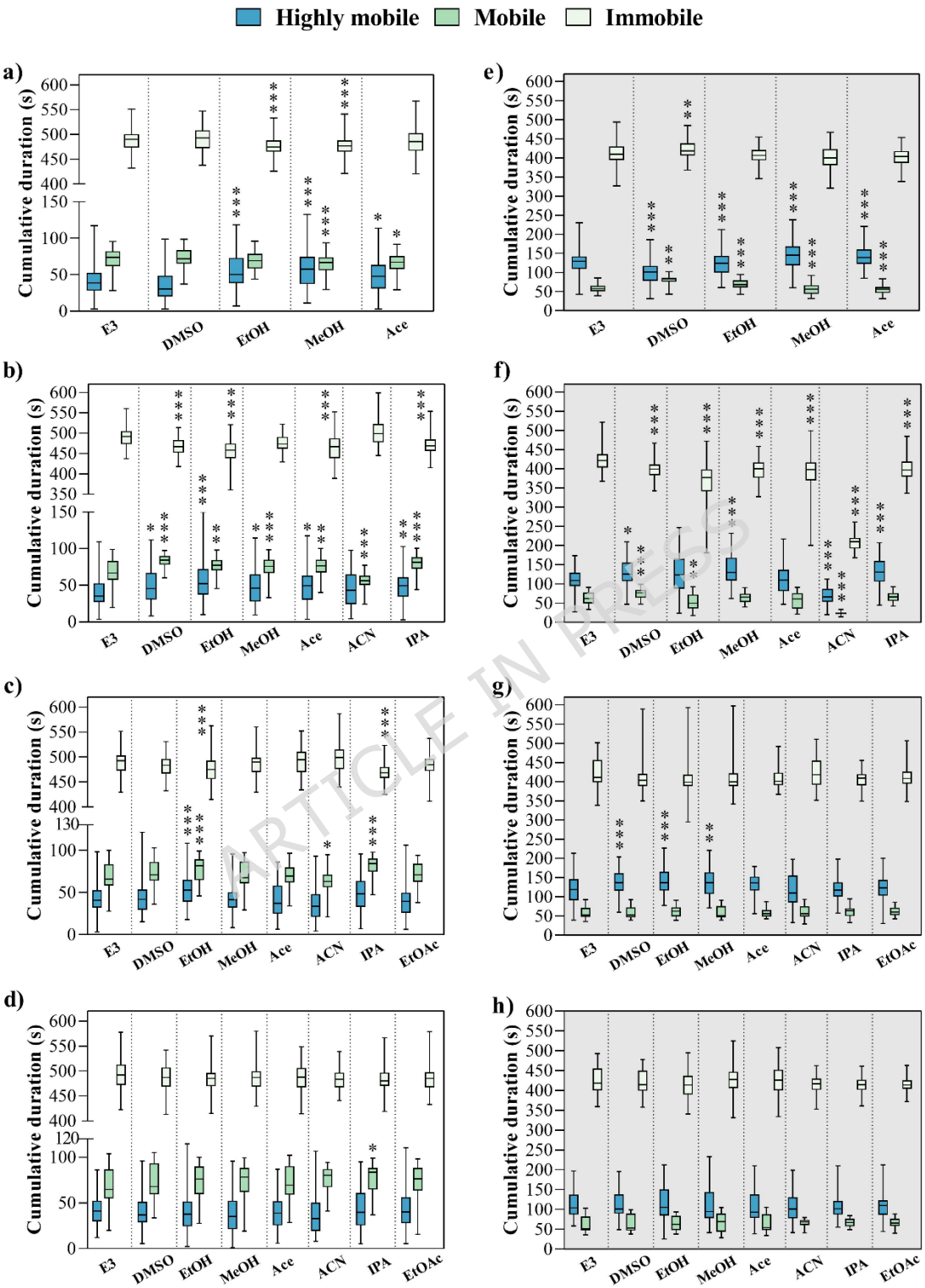


234

235 **Figure 3.** Effect of different solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide
236 – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH)
237 concentrations: 1% (a, e), 0.5% (b, f), 0.1% (c, g), and 0.05% (d, h), on zebrafish larval mobility

states (highly mobile, mobile, and immobile). The cumulative duration of each state (s) is shown separately for the light (white background; a-d) and dark (gray background; e-h) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were maintained in E3 medium. Box plots show the distribution of cumulative duration for each mobility state. Each box represents the interquartile range (between the first (Q1) and third (Q3) quartiles), the horizontal line inside the box denotes the median, and the whiskers extend to the minimum and maximum values. Significant differences compared to the control group are indicated by asterisks: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

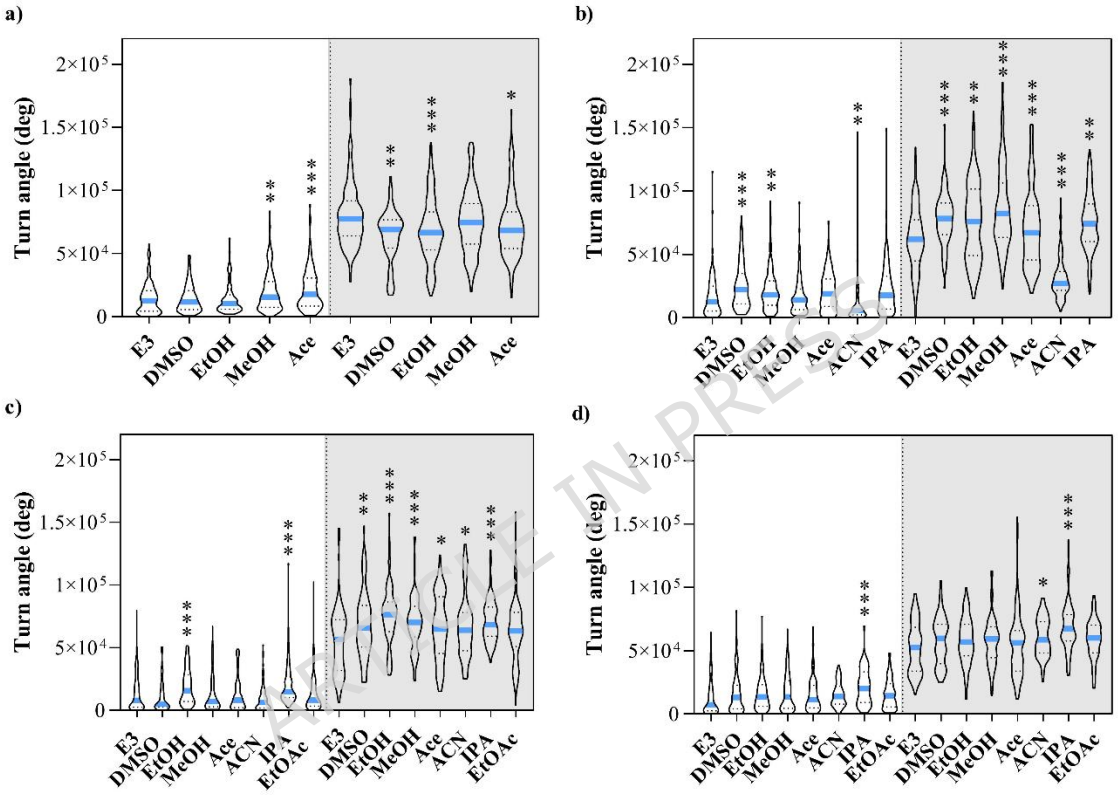


Figure 4. Effects of solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, or methanol – MeOH) at different concentrations: a) 1%, b) 0.5%, c) 0.1%, and d) 0.05%, on zebrafish larval turning behaviour. Absolute turn angle (in degrees) was presented as total values for the light (white background) and dark (gray background) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were kept in E3 medium. Violin plots represent the distribution of turning angle data. The horizontal blue line inside the violins represents the median, while dotted lines indicate the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

DMSO showed biphasic effects on larval locomotor behaviour. At 1%, it significantly reduced movement and velocity during the dark phase (13.1%; $p = 0.0201$) and lowered the proportion

of highly mobile larvae (19.2%; $p = 0.0002$), while slightly increasing immobility (3.6%; $p = 0.0046$), indicating a mild sedative or depressant effect. In contrast, 0.5% DMSO enhanced activity in both light and dark phases, increasing distance moved by 23.7% and 17.6%, respectively (Figure 1f). The increased activity observed at 0.5% DMSO was accompanied by a higher proportion of highly mobile state and increased turning under light conditions (Figures 3b and 4b), suggesting an excitatory and exploratory response. Lower concentrations (0.1% and 0.05%) generally did not differ from controls, aside from a moderate activity increase at 0.1% during darkness, reflected across velocity (16%; $p = 0.0008$) (Figure 2c) and turning metrics (23.3%; $p = 0.0022$) (Figure 4c). These concentration- and light-dependent responses are consistent with the heterogeneous findings reported in the literature. Chen et al. [32] observed increased activity between 0.01–1%, whereas Christou et al. [16] and Licitra et al. [36] found no significant behavioural effects up to 1% or 0.2%, respectively. Such discrepancies likely arise from differences in exposure duration, developmental stage, and testing protocols. For instance, in this study, larvae were exposed for 120 h and recorded for 20 min under alternating 5-min light/dark cycles, whereas Licitra et al. [36] exposed zebrafish for 96 h under longer light phases.

At 1%, EtOH significantly increased distance moved and velocity, together with a pronounced rise in highly mobile behaviour and reduced immobility under both illumination conditions. Despite this overall stimulation, turning behaviour decreased during the dark phase (14.8%; $p = 0.0004$) (Figure 4a), indicating more stereotyped and less exploratory swimming. A similar stimulatory response occurred at 0.5%, where distance moved increased by 23.5% and 15.3% in the light and dark, respectively (Figure 1f), accompanied by more time spent in the highly mobile state (Figures 3b and 3f). Even at 0.1%, EtOH enhanced activity during darkness, reflected in elevated distance moved, velocity, and turning behaviour (Figures 1g, 2c, and 4c, respectively), suggesting increased exploratory behaviour during the naturally more active phase. These findings correspond to ethanol's well-documented excitatory effects in zebrafish, where concentrations between 0.1% and 1% enhance locomotor performance through mild stimulation of the central nervous system [12,16,32]. The combination of increased continuity of swimming and reduced immobility likely reflects lowered anxiety-like inhibition and enhanced arousal under both light and dark conditions [35]. This behavioural pattern represents the excitatory phase of ethanol's biphasic behavioural profile, showing stimulation at low-to-moderate concentrations (up to ~2%) and sedation at higher doses (≥ 4%) [20,32]. At the neurophysiological level, ethanol may modulate locomotor behaviour via oxidative stress and neuroinflammatory signaling, elevating reactive oxygen species (ROS) and pro-inflammatory cytokines in glial cells, which can alter neurotransmission and neuronal excitability [37]. It was also shown that ethanol acts as $GABA_A$-type receptors agonist and that ethanol exposure affects the GABAergic system and the expression of GABA subunits [38].

MeOH similarly induced strong, dose-dependent locomotor stimulation. At 1%, both distance and velocity increased under both illumination conditions (Figures 1e and 2a), and larvae spent considerably more time in the highly mobile state (up to 39.7% higher compared to control at the light; $p \leq 0.0001$) with reduced immobility (Figure 3a). Increased turning behaviour (40% compared to the control; at light; $p < 0.05$) supported this hyperactive profile (Figure 4a). At 0.5% and 0.1%, stimulatory effects persisted, particularly in darkness (Figures 1f-g, 4b-c).

Studies by Hallare et al. [12] and Lockwood et al. [39] reported no significant changes in the swimming performance of zebrafish larvae up to 1.5%, while Christou et al. [16] observed only a slight, non-significant increase at 1%. The more pronounced stimulatory response observed in our study may be attributed to differences in assay design, which may enhance sensitivity to subtle neuroactive effects. This is in line with the slight excitatory trends reported by Hamilton et al. [17] in adult zebrafish. In addition, it was shown that zebrafish embryos exposure to 2% methanol caused no differences in retinal morphology or alterations in photoreceptor functions [40,41]. Ace significantly increased larval locomotor activity at 1%, increasing distance moved by 27% ($p \leq 0.0001$) in light and 9% ($p = 0.0434$) in darkness (Figure 1e). This stimulatory response was accompanied by longer durations of highly mobile and mobile behaviour and reduced immobility, indicating a pronounced activation of motor output. At 0.5%, Ace did not affect the total distance moved (Figure 1f), although mean velocity increased by 21.2% in the dark (Figure 2b). Mobility state analysis revealed an inconsistent pattern at 0.5%, with simultaneous increases in highly mobile, mobile, and immobile states under light, and elevated immobility in the dark. This pattern indicates a variable and uncoordinated behavioural response, suggesting mild stress or disruption of normal activity regulation. Lower concentrations (0.1% and 0.05%) did not produce significant changes. Behavioural data for Ace in zebrafish larvae are scarce. Audira et al. [14] demonstrated that exposure of adult zebrafish to 0.1% Ace did not cause a negative effect on behaviour. Acetone's high volatility, but also quick metabolism and restricted bioaccumulation in aquatic organisms, are the reasons for its minimal developmental and behavioural toxicity, as shown in this and earlier investigations [12]. Additionally, compared to alcohol-based solvents, acetone does not strongly interact with biological membranes or neuronal receptors, reducing the likelihood of direct neuroactive effects.

ACN produced a significant inhibitory effect on zebrafish locomotor activity. At 0.5%, it markedly reduced the distance moved under both light (16.8%; $p = 0.0027$) and dark (40.4%; $p \leq 0.0001$) conditions (Figure 1f). Cumulative duration analysis revealed significant reductions in the proportions of highly mobile, mobile, and immobile larvae during the dark phase, indicating suppression of motor activity rather than a shift between mobility states. This decline was accompanied by decreased acceleration (Figure S1b) and reduced turn angle (Figure 4b) under both illumination conditions ($p < 0.05$), further supporting a strong inhibitory effect on swimming dynamics and motor coordination. At 0.1%, distance moved decreased by 20% under light ($p < 0.05$) (Figure 1g), while turn angle increased significantly during the dark phase (Figure 4c), indicating alterations in directional control at lower concentrations. A similar turning pattern at 0.05% indicates that even low ACN concentrations may alter movement stability, whereas higher concentrations induce consistent and pronounced behavioural inhibition. These findings align with known mechanisms of aliphatic nitriles, which are metabolically converted to cyanide and can impair oxidative metabolism and neural function [42]. To our knowledge, no previous study has reported the effects of ACN on zebrafish behaviour.

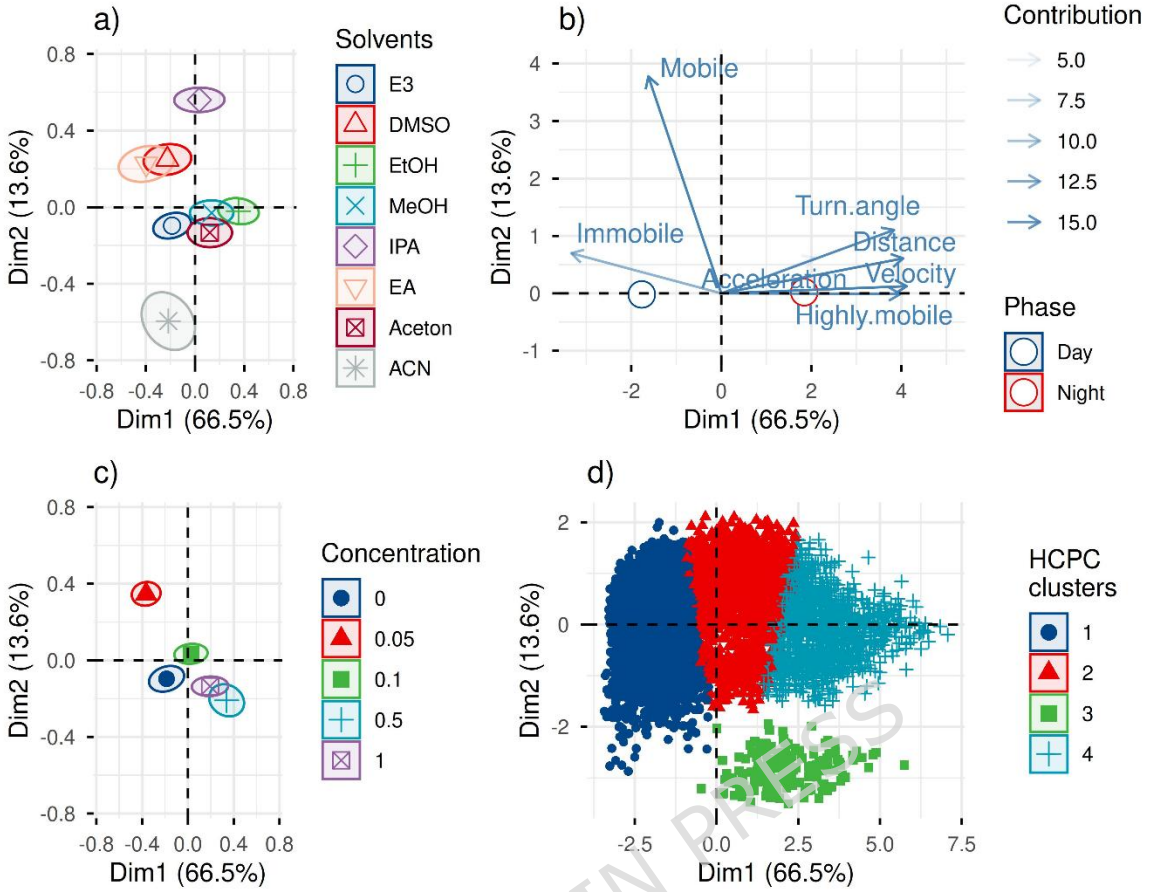
At 0.5%, IPA significantly increased movement (Figure 1f) and velocity (Figure 2b) during both illumination conditions, while 0.1% and 0.05% caused a similar but weaker increase, most evident in the light phase. Even at 0.05%, distance, velocity, and turn angle increase

significantly (Figures 1h, 2d, and 4d, respectively), indicating that IPA stimulates activity even at very low concentrations. These findings align with previous studies showing that short-chain alcohols induce moderate stimulatory effects on zebrafish behaviour. According to Audira et al. [14], exposure to 0.1% IPA in adult zebrafish induces a temporary excitatory or anxiolytic-like response, characterized by increased swimming and reduced freezing behaviour. Similar increases in locomotion were observed at comparable concentrations without developmental abnormalities, supporting the assumption that IPA primarily acts as a behavioural stimulant rather than a neurotoxicant. Although IPA is less used than DMSO or MeOH in zebrafish assays, it can serve as a solvent for hydrophobic compounds. However, its potential biological effects, including toxicity or behavioural alterations, should be carefully evaluated when applied *in vivo*.

The lipophilic and volatile nature of EtOAc allows it to rapidly pass through biological membranes and disturb cellular homeostasis during early development, which likely explains the toxicity observed at 0.5% and 1%. Once absorbed, EtOAc is hydrolysed to ethanol and acetic acid, which can temporarily change the pH and metabolic balance and impact the viability of embryos [43]. Consequently, only the two lowest concentrations (0.1% and 0.05%) were included in behavioural testing, neither of which produced significant changes across measured endpoints. Given that no previous studies have investigated the behavioural effects of EtOAc in zebrafish, either in larvae or adults, these findings provide the first evidence that low subtoxic concentrations of EtOAc do not elicit detectable neurobehavioural alterations in zebrafish.

3.2 Integrated behavioural profiling

Multivariate analysis confirmed associations observed univariately, showing that all included variables had a significant effect. Over 80% of the total variance was explained by the first two principal components (PC), with PC1 dominating the most (Figure 5). Acceleration and immobile state neither significantly contributed to the first two components nor were qualitatively represented by them. Distance moved, velocity, turn angle, and the duration of the highly mobile state positively correlated with PC1, while the mobile state was primarily associated with PC2 (Figure 5b). The most pronounced separation in the PCA corresponded to the light-dark periods (Figure 5b), whose influence on behavioural variation spanned orders of magnitude beyond that of solvent type or concentration. This emphasizes the critical importance of experimental design, as behavioural datasets generated under various illumination conditions are not directly comparable. Variability driven by day–night phases can easily obscure solvent-induced effects, making interpretation and cross-study comparisons more difficult. Considering solvents, separation by the first dimension mostly affected alcohol-based solvents (EtOH and MeOH, but not IPA) and acetone, which, although distinct from each other, grouped and were most similar to the control (E3 medium). In contrast, the separation of the remaining solvents was predominantly driven by the second dimension (DMSO, EA, ACN, and IPA) (Figure 5a). However, these patterns are affected by concentration, as high concentrations (≥ 0.5%) cluster in the lower right quadrant opposite to the lowest solvent concentration used and all being distinct to the control (Figure 5c).



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387 **Figure 5.** Results of principal component analysis of behavioural endpoints in zebrafish larvae
388 are presented on the factor map. Supplementary variables are presented by barycenters and
389 confidence ellipses for: a) solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide –
390 DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, methanol – MeOH, and
391 E3 medium as control group), b) phase (light and dark periods as day vs. night) with active
392 variables included in blue and their transparency set by contribution, c) concentration (*v/v*) of
393 solvents with 0% denoting control group in E3 medium. Individuals (6388 larvae) are plotted
394 only in d) with colors denoting the cluster assigned based on hierarchical clustering on principal
395 components (HCPC).

396

397 Hierarchical clustering on principal components showed 4 distinct clusters present in the data
398 with the 3rd cluster having complete separation from all others (Figure 5d). The 1st cluster is
399 heavily dominated by daytime behaviour 95% of data) and shows a relatively uniform
400 representation of solvents and concentrations (Table 1). This suggests homogeneous patterns
401 in daytime locomotor activity, regardless of solvent treatment, to be consistent with the
402 inherently lower variability of larval behaviour. In contrast, the other 3 clusters predominantly
403 consisted of night-phase data, confirming that behavioural differentiation is substantially
404 greater in darkness. Clusters 3 and 4 were almost exclusively composed of night data, whereas
405 cluster 2 contained a small but notable proportion of daytime data (approx. 20%). Although all
406 night-phase clusters shared elevated activity relative to daytime, important distinctions
407 emerged between them. Cluster 2 had a relatively higher contribution from alcohol-based

solvents (EtOH, MeOH) compared to Ace, ACN and EA which was inverse in cluster 4. More importantly, cluster 2 had higher proportions of lower concentrations, while higher concentrations dominated cluster 4. This pattern aligns well with the activity-enhancing or inhibitory responses observed at higher solvent concentrations, reflecting the stronger perturbation of locomotor patterns documented in the individual endpoint analyses. This in turn, made cluster 3 the most different and smallest cluster that accounted for only 3% of all data (192/6388) exclusively made of night period data, 0.5% concentrations of EtOH, Ace, and ACN which were characterised by the lowest locomotor activities in many endpoints (Table 1). Overall, these findings indicate specific patterns in behavioural data are present and results are significantly affected by conditions and methodological protocol employed. That is, certain combinations of solvent, concentration, and environmental conditions may produce particular patterns which is in line with a study of solvents on behavioural data of adult zebrafish sub-chronically exposed to 0.1% concentrations [14]. In that study various other behavioural tests were used such as novel tank, mirror biting, predator avoidance, social interaction and shoaling, which through PCA and HCPC analyses showed that each common solvent induced unique behavioural alteration patterns. Across clusters, all active variables had significant correlations, positive or negative, except for mobile state and both acceleration and turn angle. This suggests that mobility state transitions and fine motor control are partly independent dimensions of the behavioural response. Details of variable correlations overall and within clusters are available in the Supplementary material (Figure S2).

Table 1. Description of clusters based on hierarchical clustering on principal components (HCPC) by supplementary variables (solvent, concentration, and phase) and active variables (measured behavioural endpoints) from 6388 zebrafish larvae placed in microtiter plates observed by an automated tracking device. Supplementary variables are described by their counts and proportions and active variables by summary indices.

Variable	HCPC Cluster			
	1	2	3	4
Solvents				
E3	533 (17.3%)	336 (18.3%)	0 (0.0%)	194 (15.2%)
DMSO	372 (12.1%)	220 (12.0%)	0 (0.0%)	122 (9.6%)
EtOH	512 (16.6%)	295 (16.1%)	44 (22.9%)	295 (23.1%)
MeOH	460 (14.9%)	250 (13.6%)	0 (0.0%)	254 (19.9%)
Ace	424 (13.8%)	230 (12.5%)	42 (21.9%)	194 (15.2%)
ACN	280 (9.1%)	127 (6.9%)	106 (55.2%)	53 (4.2%)
IPA	257 (8.3%)	228 (12.4%)	0 (0.0%)	103 (8.1%)
EtOAc	245(7.9%)	152 (8.3%)	0 (0.0%)	60 (4.7%)
Concentration % (*v/v*)				
0.05%	729 (23.6%)	537 (29.2%)	0 (0.0%)	171 (13.4%)
0.1%	657 (21.3%)	372 (20.2%)	0 (0.0%)	339 (26.6%)
0.5%	609 (19.8%)	302 (16.4%)	192 (100.0%)	269 (21.1%)
1%	555 (18.0%)	291 (15.8%)	0 (0.0%)	302 (23.7%)
Phase				
Day	2936 (95.2%)	323 (17.6%)	0 (0.0%)	2 (0.2%)
Night	147 (4.8%)	1515 (82.4%)	192 (100.0%)	1273 (99.8%)
Measured endpoints	**Summary** mean (SD; standard deviation) min (minimum) ≤ med (median) ≤ max (maximum) IQR (interquartile range) (CV; coefficient of variability)			
Distance (mm)	338.1 (137.6) 38.0 ≤ 310.6 ≤ 849.2 176.9 (0.4)	1038.2 (234.1) 343.0 ≤ 1061.6 ≤ 1581.7 328.1 (0.2)	728.8 (236.5) 164.9 ≤ 682.1 ≤ 1541.1 326.7 (0.3)	1643.7 (284.5) 1048.8 ≤ 1589.5 ≤ 2816.9 356.5 (0.2)
Velocity (mm/s)	0.6 (0.2) 0.1 ≤ 0.5 ≤ 1.4 0.3 (0.4)	1.7 (0.4) 0.6 ≤ 1.8 ≤ 2.6 0.5 (0.2)	2.4 (0.8) 0.6 ≤ 2.3 ≤ 5.1 1.1 (0.3)	2.7 (0.5) 1.7 ≤ 2.7 ≤ 4.7 0.6 (0.2)
Acceleration (mm/s^2)	1235.7 (532.1) 132.6 ≤ 1194.6 ≤ 3088.2 780.8 (0.4)	1698.8 (405.2) 370.3 ≤ 1651.5 ≤ 3260.9 551.1 (0.2)	1483.8 (375.2) 594.4 ≤ 1430.5 ≤ 2832.4 492.0 (0.3)	1739.0 (334.9) 931.3 ≤ 1709.1 ≤ 3152.2 447.7 (0.2)
Highly mobile (s)	42.8 (20.5) 1.2 ≤ 40.9 ≤ 132.7 27.9 (0.5)	98.4 (20.6) 39.0 ≤ 99.9 ≤ 154.2 30.3 (0.2)	72.5 (21.2) 19.2 ≤ 70.9 ≤ 139.3 30.6 (0.3)	159.1 (24.5) 107.6 ≤ 155.2 ≤ 246.7 36.6 (0.2)
Mobile (s)	72.1 (15.9) 15.8 ≤ 72.2 ≤ 109.6 25.1 (0.2)	73.6 (15.4) 29.4 ≤ 77.2 ≤ 104.9 22.4 (0.2)	26.4 (6.2) 14.1 ≤ 25.4 ≤ 46.5 8.6 (0.2)	55.6 (11.8) 31.3 ≤ 54.2 ≤ 88.8 18.3 (0.2)
Immobile (s)	484.4 (23.5) 412.7 ≤ 483.1 ≤ 579.7 30.4 (0.04)	427.3 (20.6) 384.6 ≤ 424.7 ≤ 506.1 31.0 (0.04)	201.1 (19.6) 143.3 ≤ 200.5 ≤ 261.2 24.4 (0.1)	384.9 (19.7) 313.9 ≤ 387.8 ≤ 449.0 24.7 (0.1)
Turn angle (deg)	14559.6 (11672.1) 83.5 ≤ 11813.3 ≤ 76504.4 16744.3 (0.8)	56106.4 (16603.4) 12606.5 ≤ 56589.7 ≤ 149514 25108.1 (0.3)	37164.9 (16149.3) 4964.5 ≤ 35395.9 ≤ 94483.4 21319.5 (0.4)	87114.8 (23626.1) 39760 ≤ 84717.6 ≤ 188526 29405.9 (0.3)

4. Conclusions

This study provides a comprehensive assessment of the behavioural effects of seven commonly used solvents in zebrafish larvae, demonstrating solvent- and concentration-dependent alterations across several locomotor endpoints. While several solvents (EtOH, MeOH, Ace, and IPA) caused stimulatory effects at moderate concentrations, ACN produced significant behavioural suppression, and DMSO showed biphasic responses. Multivariate analyses further revealed coherent behavioural patterns that closely matched univariate findings, highlighting that solvent effects cannot be interpreted independently of experimental conditions, especially light–dark transitions. The pronounced distinction between daytime and nighttime behaviours emphasizes how crucial it is to use a consistent assay design when evaluating neurobehavioural toxicity. Taken together, these integrated behavioural assessments provide concentration limits ($\leq 0.05\%$ for DMSO, EtOH, MeOH, ACN, and IPA, and $\leq 0.1\%$ for Ace and EtOAc) that can be considered behaviourally inert under the specific experimental conditions used. In order to prevent confounding effects and ensure repeatability in behavioral research, the obtained results collectively emphasize the importance of careful solvent and concentration selection in zebrafish-based tests.

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Author contributions

Tamara Vujović: Conceptualization**,** Methodology, Data curation, Investigation, Writing - original draft; **Karolina Begić**: Data curation, Formal analysis, Visualization, Writing - original draft; **Krunoslav Bojanić:** Software**,** Visualization, Writing- Reviewing and Editing; **Rozelindra Čož-Rakovac:** Resources; **Sanja Babić Brčić:** Conceptualization, Methodology, Data curation, Visualization, Funding acquisition, Writing-Reviewing and Editing**.**

Data availability statement

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Ethics declarations

Animal housing and spawning were carried out in aquaria units approved by the Croatian Ministry of Agriculture (HR-POK-023). Adult zebrafish were used only for breeding purposes to obtain embryos. All experimental procedures in this study were performed on non-protected embryonic stages (up to 120 hpf), which do not require approval from animal welfare committees according to the Council Directive 2010/63/EU. The applicable institutional and national rules and regulations for the use and care of laboratory animals were followed in the execution of all procedures.

Declaration of competing interest

The authors have nothing to declare.

Figure and table captions

Figure 1. Effect of different solvent (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH) concentrations: 1% (a, e), 0.5% (b, f), 0.1% (c, g), and 0.05% (d, h), on zebrafish larval

locomotor activity. Activity was recorded for 20 minutes under alternating 5-minute light (white background) and dark (gray background) periods. Control larvae were kept in E3 medium. The left panels (a-d) show the total distance moved by each experimental group, presented by 1-minute time bins. Results are presented as mean ± SE. The right panels (e-h) show the total distance moved during light (white background) and dark (gray background) phases. Violin plots represent the distribution of movement data, with the blue line representing the median and dotted lines indicating the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Figure 2. Effect of different solvent (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH) concentrations: a) 1%, b) 0.5%, c) 0.1%, and d) 0.05%, on zebrafish larval velocity. Swimming velocity (mm/s) was presented as mean values for the light (white background) and dark (gray background) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were kept in E3 medium. Violin plots represent the distribution of mean velocity. The horizontal blue line inside the violins represents the median, while dotted lines indicate the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Figure 3. Effect of different solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, and methanol – MeOH) concentrations: 1% (a, e), 0.5% (b, f), 0.1% (c, g), and 0.05% (d, h), on zebrafish larval mobility states (highly mobile, mobile, and immobile). The cumulative duration of each state (s) is shown separately for the light (white background; a-d) and dark (gray background; e-h) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were maintained in E3 medium. Box plots show the distribution of cumulative duration for each mobility state. Each box represents the interquartile range (between the first (Q1) and third (Q3) quartiles), the horizontal line inside the box denotes the median, and the whiskers extend to the minimum and maximum values. Significant differences compared to the control group are indicated by asterisks: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Figure 4. Effects of solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, or methanol – MeOH) at different concentrations: a) 1%, b) 0.5%, c) 0.1%, and d) 0.05%, on zebrafish larval turning behaviour. Absolute turn angle (in degrees) was presented as total values for the light (white background) and dark (gray background) phases during a 20-minute session consisting of alternating 5-minute light and dark periods. Control larvae were kept in E3 medium. Violin plots represent the distribution of turning angle data. The horizontal blue line inside the violins represents the median, while dotted lines indicate the inter-quartile range. Significant difference between the treatment and the control group is marked with asterisks: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Figure 5. Results of principal component analysis of behavioural endpoints in zebrafish larvae are presented on the factor map. Supplementary variables are presented by barycenters and confidence ellipses for: a) solvents (acetone – Ace, acetonitrile – ACN, dimethyl sulfoxide – DMSO, ethyl acetate – EtOAc, ethanol – EtOH, isopropanol – IPA, methanol – MeOH, and

698 E3 medium as control group), b) phase (light and dark periods as day vs. night) with active
699 variables included in blue and their transparency set by contribution, c) concentration (*v/v*) of
700 solvents with 0% denoting control group in E3 medium. Individuals (6388 larvae) are plotted
701 only in d) with colors denoting cluster assigned based on hierarchical clustering on principal
702 components (HCPC).

703 **Table 1**. Description of clusters based on hierarchical clustering on principal components
704 (HCPC) by supplementary variables (solvent, concentration, and phase) and active variables
705 (measured behavioural endpoints) from 6388 zebrafish larvae placed in microtiter plates
706 observed by an automated tracking device. Supplementary variables are described by their
707 counts and proportions and active variables by summary indices.

708