UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

TEN YEARS OF UNPREDICTABLE CHRONIC STRESS RESEARCH IN ZEBRAFISH: A SYSTEMATIC REVIEW AND META-ANALYSIS

MATHEUS GALLAS LOPES

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Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de grau de Farmacêutico.

Orientadora: Profª. Drª. Ana Paula Herrmann

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APRESENTAÇÃO

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Ten years of unpredictable chronic stress research in zebrafish: a systematic review and meta-analysis

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ABSTRACT

The zebrafish (*Danio rerio*) is a model animal that is being increasingly used in neuroscience research. A decade ago, the first study on chronic unpredictable stress (UCS) in zebrafish was published, inspired by protocols established for rodents. Since then, several studies have been published by different groups, in some cases with conflicting results. We conducted a systematic review to identify studies evaluating the effects of UCS in zebrafish and meta-analytically synthesised the data of neurobehavioral outcomes and relevant biomarkers. Literature searches were performed in three databases (PubMed, Scopus and Web of Science) and a two-step screening process based on inclusion/exclusion criteria. The included studies underwent extraction of qualitative and quantitative data, as well as risk of bias assessment. Outcomes of included studies (n = 38) were grouped into anxiety/fear-related behaviour, locomotor function, social behaviour, cortisol levels, *bdnf*, or *crf* expression domains. UCS increased anxiety/fear-related behaviour and cortisol levels while decreased locomotor function, but no effects were found for social behaviour and expression of *bdnf* and *crf.* Despite including a significant number of studies, the high heterogeneity and the methodological and reporting problems evidenced in the risk of bias analysis make it difficult to assess the internal validity of most studies and the overall validity of the model. Our review thus evidences the need to conduct well-designed experiments to better evaluate the effects of UCS on the behaviour of zebrafish.

Keywords: Unpredictable chronic stress, *Danio rerio*, animal model, anxiety, locomotor function, social behaviour, cortisol, systematic review, meta-analysis, depression

CONTENTS

I. INTRODUCTION

The origins of the unpredictable chronic stress (UCS) protocol go back to the early 1980s, when researchers proposed the chronic administration of a variety of stressors to rodents as a way to induce behavioural alterations relevant to the study of depression (Katz & Hersh, 1981; Katz, Roth & Carroll, 1981; Katz, 1982; Willner *et al.*, 1987). Construct, face, and predictive validities of this model are supported by many studies that show that rodents exposed to the UCS protocol develop anhedonia-like behaviour, cognitive deficits, hormonal and neurochemical imbalances, weight loss, and many other changes that can be reversed by using antidepressant treatments (Willner, 1997). Given its translational potential, there has been an exponential growth in the implementation of this protocol across laboratories as it has become an important tool for the study of the neurobiological basis of depression and antidepressant action (Willner, 2017a; Nollet, 2021).

Whereas this intervention became popular, researchers started adapting the UCS protocol and reports of controversial data and reproducibility problems have also increased (Strekalova & Steinbusch, 2009; Willner, 2017b; Antoniuk *et al.*, 2019). The protocol has been largely criticized for its lack of reliability as many known elements such as the training level of experimenters, the duration of the protocol, and animal characteristics (species, strain, sex, and others) can introduce variability to the intervention and influence the results (Willner, 2017b). Apart from that, even with heterogeneous protocols, the UCS was able to replicate behavioural and physiological alterations within and between labs, adding to the internal and external validity of the model.

More than a decade ago, researchers made an effort to transpose this intervention for studies using zebrafish (*Danio rerio* Hamilton, 1822), an emerging

model animal in the field of neuroscience at the time (Piato *et al.*, 2011). Cross-species approaches are important tools to evaluate the validity of an intervention, and translating the UCS protocol to zebrafish can help reduce species-specific biases originating from studies conducted solely with rodents (Maximino *et al.*, 2015; Weber-Stadlbauer & Meyer, 2019). In zebrafish, this protocol is also able to induce anxiety-like behaviour and alterations in outcomes like locomotion, cognition, sociability, cortisol levels, and in the mechanisms of defence against oxidative damage (Piato *et al.*, 2011; Marcon *et al.*, 2016, 2018b; Bertelli *et al.*, 2021). But just as in the experiments carried out with rats and mice, the heterogeneity between protocols established in each laboratory has grown throughout the years as investigators needed to adapt the procedures to different facilities or the outcomes of interest sought in the studies. Such problems culminated in the publication of many discrepant results for key outcomes to understand the impacts of UCS, like social behaviour, which was shown to be altered in opposing directions depending on the duration of the protocol (Piato *et al.*, 2011), or not altered at all (Golla, Østby & Kermen, 2020; Bertelli *et al.*, 2021).

Aiming to estimate the overall validity and to summarise the evidence regarding the effects of UCS on behavioural and biochemical outcomes relevant to the study of psychiatric disorders, we conducted a systematic review and meta-analysis of the available scientific literature using zebrafish. We analysed the evolution of this intervention in the first ten years of its use, qualitatively describing the published studies, establishing the direction of the effect of chronic stress on neurobehavioural and neurochemical parameters, detecting patterns and effect moderators, and evaluating the impact of bias arising from methodological conduct, reporting quality, and selective publication.

II. METHODS

A protocol for conducting this review was registered on Open Science Framework prior to the screening of records and data collection. Preregistration is available at https://osf.io/9rvyn (Gallas-Lopes *et al.*, 2021). The reporting of this study complies with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page *et al.*, 2021).

(1) Search strategy

Searches were conducted in three bibliographic databases: PubMed, Scopus, and Web of Science. The search strategy was designed to include broad terms that describe the intervention (UCS protocol) and the desired population (zebrafish). The complete query for each database can be found at https://osf.io/9rvyn (Gallas-Lopes *et al.*, 2021). There were no language or date restrictions. The first search was performed on the 10th of July, 2021, with an update search carried out on the 26th of October, 2021. The bibliographic data acquired were imported to Rayyan software (Ouzzani *et al.*, 2016), where duplicates were detected and removed by one of the investigators (MGL). The reference lists of the included studies were also screened in order to detect additional relevant articles.

(2) Eligibility screening

After the removal of duplicates, the selection of eligible studies was conducted using Rayyann software in a two-step process based, initially, on title and abstract, followed by a full-text analysis. The screening of each record was performed by two independent investigators (MGL and LMB or RB) and disagreements were resolved

by a third investigator (APH). Peer-reviewed articles were eligible for inclusion if they had an appropriate control group and assessed the effects of unpredictable chronic stress in zebrafish (any strain or developmental stage) on any of the following domains of interest: morphometric measures, locomotor function, sensory function, learning and memory, social behaviour, reproductive behaviour, anxiety/fear-related behaviour, circadian cycle-related behaviour, and neurochemical or peripheral biomarkers (e.g., cortisol, cytokines, and oxidative stress).

In the first screening stage (title and abstract), studies were excluded based on the following reasons: (1) design: not an original primary study (e.g., review, commentary, conference proceedings, and corrections); (2) population: studies using other species than zebrafish (*Danio rerio*) or studies that did not use any animal; (3) intervention: non-interventional studies or studies using other interventions than unpredictable chronicle stress (e.g., acute stress (stressed only once) and repetitive or predictable stress (chronic stress using only a single stressor multiple times)). In the second stage (full-text screening), the remaining articles were assessed for exclusion based on the same reasons considered in the first stage plus the following additional reasons: (4) comparison: studies without an adequate control group; (5) outcome: studies that did not evaluate any of the target outcomes. All Rayyan files with investigators' decisions are available at the study repository in Open Science Framework (https://osf.io/j2zva/), section “Eligibility screening archives”.

(3) Data extraction

Data extraction from included studies was conducted by two independent investigators (MGL and LMB or RB) and disagreements were resolved by a third investigator (APH). Whenever available, the exact information and values were extracted directly from text

or tables. Otherwise, WebPlotDigitizer software (v4.5, Rohatgi, A., Pacifica, CA, USA, https://automeris.io/WebPlotDigitizer) was used to manually estimate numbers from the graphs. In cases of lacking or dubious information, investigators attempted to contact via e-mail the corresponding author of the study in two separate attempts, at least two weeks apart.

The following characteristics were extracted: (1) study characteristics: study title, digital object identifier (DOI), first and last authors, last author's institutional affiliation, and year of publication; (2) animal model characteristics: strain, sex, animal source (supplier of the animals used to develop the experiments), the total number of animals used, and the developmental stages during stress induction and outcome assessment; (3) UCS protocol characteristics: the number of different stressors, stress sessions per day, stress sessions in total, the duration of the stress protocol in days, and the time in days between the end of UCS protocol and outcome assessment; (4) test characteristics: experiment identification (to annotate whether the tests conducted within the same study used different sets of animals), the type of the test, test duration, habituation phase (whether the animals were subjected to an habituation phase in the experimental apparatus prior to the test), the category of measured variable, and the measured variable.

Outcome data were extracted for each of the variables within the domains of interest. The measure of central tendency and the number of animals (n) were extracted for the control and UCS groups along with the standard deviation (SD) or standard error (SEM) when the mean value was expressed, or the interquartile range (IQR) when data were expressed as the median value. Whenever sample size was reported as a range instead of the exact number of animals in each group, the lowest

value was extracted. If the study reported the SEM, SD was calculated by multiplying SEM by the square root of the sample size (SD = SEM $*$ $\sqrt{n}$).

(4) Bias assessment

In order to evaluate the quality of included studies, the risk of bias assessment was conducted by two independent investigators (MGL and LMB or RB) for each paper, and disagreements were resolved by a third investigator (APH). The analysis was conducted based on the SYRCLE's risk of bias tool for animal studies (Hooijmans *et al.*, 2014) with adaptations to better suit the model animal and the intervention of interest. The following items were evaluated for methodological quality: (1) description of random allocation of animals; (2) description of baseline characteristics; (3) description of random housing conditions during the experiments; (4) description of random selection for outcome assessment; (5) description of blinding methods for outcome assessment; (6) incomplete outcome data; (7) selective outcome reporting. Additionally, four other items were evaluated by the investigators to assess the overall reporting quality of the studies based on a set of reporting standards for rigorous study design (Landis *et al.*, 2012): (8.1) mention of any randomization process; (8.2) sample size estimation; (8.3) mention of inclusion/exclusion criteria; (8.4) mention of any process to ensure blinding during the experiments. For methodological quality, each item was scored with a "Yes" for low risk of bias, "No" for a high risk of bias or "Unclear" when it was not possible to estimate the risk of bias based on the information provided. Items regarding reporting quality were scored with only "Yes" or "No", meaning high or low risk of bias, respectively. A complete guide for assessing the risk of bias associated with each of the items in this review is available at https://osf.io/sdpwb. Risk of bias plots were created using *robvis* (McGuinness & Higgins, 2021).

Publication bias was investigated by generating funnel plots and performing Egger's regression test (Egger *et al.*, 1997). Analyses were only conducted when at least five studies were available within a given domain for funnel plots and at least ten studies for the regression test. A p-value < 0.1 was considered significant for the regression test.

(5) Meta-analysis

Studies were grouped based on the domains of interest (anxiety/fear-related behaviour, locomotor function, social behaviour, cortisol levels, *bdnf* expression, or *crf* expression), and a meta-analysis was performed for each group. When a study reported multiple outcomes for the same domain, only one outcome of interest was chosen for the meta-analysis based on a rank of frequency developed by one of the investigators (MGL). Tests and variables within each test were ranked prior to data extraction, and the most frequent in the rank was included in the meta-analysis. The ranking is available at https://osf.io/rvn8b. A minimum of five studies were required for each domain in order to conduct a meta-analysis, as established a priori in our protocol (Gallas-Lopes *et al.*, 2021).

The sample size of the control group was divided by the number of comparisons and rounded down whenever two or more experimental groups shared the same control (Vesterinen *et al.*, 2014). When outcomes were analysed across time, the last point was selected for analysis. When animals were subjected to experiments at different time points following the end of the UCS protocol, the outcomes assessed closest to the end of the protocol were chosen. Effect sizes were "flipped" (multiplied by minus one) when needed to adjust the direction of the effect for specific behavioural traits in order to properly interpret the effects of UCS. Studies that only reported

outcomes as the median value and interquartile range were excluded from the analyses along with studies with incomplete data (e.g., lacking sample sizes, SD, and SEM) when contact with the authors was unsuccessful.

Effects sizes were determined with standardised mean differences (SMD) using Hedge's G method. Analyses were conducted using JASP software version 0.16.3 (https://jasp-stats.org) with packages *metafor* (Viechtbauer, 2010) (https://cran.r-project.org/package=metafor) and *ggplot2* (Wilkinson, 2011) following Hedge's random effects model given the anticipated heterogeneity between studies. Values for SMD were reported with 95% confidence intervals. Heterogeneity between studies was estimated using both the I^2 and Chi^2 tests. Values of 25%, 50%, and 75% were considered as representing low, moderate, and high heterogeneity, respectively for the I^2, and a p-value ≤ 0.1 was considered significant for the Chi^2 (Higgins & Thompson, 2002). Furthermore, a subgroup meta-analysis was performed to evaluate if the duration of the UCS protocol was a potential source of heterogeneity. Studies were grouped into two categories: those with up to 7 days of UCS protocol and those with more than 7 days. Subgroup analysis was only performed when there were at least five unique studies for each subgroup.

(6) Sensitivity analysis

A sensitivity analysis was conducted in order to assess if any experimental or methodological difference between studies was distorting the main effect found in the meta-analysis. Analyses were conducted by excluding studies presenting a significant risk of bias, defined as either a high risk of bias in one of the main items evaluating methodological quality (items 1 to 7), or an unclear risk of bias in five or more of the

same items. A minimum of three comparisons were required for each domain in order to conduct a sensitivity analysis.

III. RESULTS

(1) Search results

From the search in the selected databases, 420 records were retrieved altogether. Following the removal of duplicates, 206 records were screened for eligibility based on title and abstract. After the first screening phase, 58 reports remained to be assessed based on full text, and 38 met the criteria and were included in the review (Fig. 1). Out of the reports included in the review, 34 were collected from the first database search on the 10th of July, 2021, and four additional reports were identified in the second search on the 26th of October, 2021. No extra studies were identified by reference list screening. Most of the records sought for inclusion in either stage of screening were excluded because they did not meet the criteria set for the intervention (*n* = 89), followed by the population of interest (*n* = 42), and the design of the study (*n* = 37). Two studies were excluded from the quantitative analyses because the minimum number of studies to perform a meta-analysis was not reached for the outcome reported (Zimmermann *et al.*, 2016; Marcon *et al.*, 2018b), and four studies were excluded because of missing information (Huang, Butler & Lubin, 2019; Zhang *et al.*, 2021; Kirsten *et al.*, 2021; Demin *et al.*, 2021).

(2) Study characteristics

As expected, the protocols implemented by each research group varied significantly. The duration of the stress protocol ranged between 3 and 77 days, with 15 studies (39.5%) implementing UCS for up to 7 days, and 27 (71%) for more than a week.

Protocols using 7 (*n* = 13, 34.2%) or 14 days (*n* = 12, 31.6%) of UCS were the most common. It is important to mention that some studies (*n* = 5, 13.2%) used UCS protocols of more than 15 days to explore the more severe or long-term impacts of UCS in zebrafish. The protocols were conducted using frequently a group of up to 10 different stressors to account for unpredictability. Outcome assessment usually took place within the 24 hours following the last stress session (*n* = 31, 81.6%), with only a few studies evaluating the effects of UCS after a longer washout period (*n* = 10, 26.3%). The tests were mostly scheduled to occur at least a day from the last stressor to avoid the acute interference from the last stress session but also not too far off the end of the protocol to avoid losing the effects of UCS.

The majority of studies were conducted by exposing adult zebrafish to the protocol (*n* = 34, 89.4%), followed by fish in the larval (*n* = 3, 7.9%), and juvenile life stages (*n* = 1, 2.6%). Of the publications implementing the UCS protocol in early developmental stages, one of them evaluated behavioural data of the exposed animals when animals were still larvae. The remaining were designed to assess the long-lasting effects of the stress and, in this case, animals were tested more than 75 days after the protocol ended, when they were considered adults. Experiments were conducted generally with a pool of both male and female zebrafish (*n* = 21, 55.2%). In only two studies both male and female zebrafish were used and sex was analysed as a biological variable, whereas in four papers animals of only one sex were selected (*n* = 2 for male and *n* = 2 for female fish). The sex of the animals was not specified in 11 studies (28.9%). A description of the studies included in the review can be found in Table 1, and the detailed extracted information is available at https://osf.io/2jzw9.

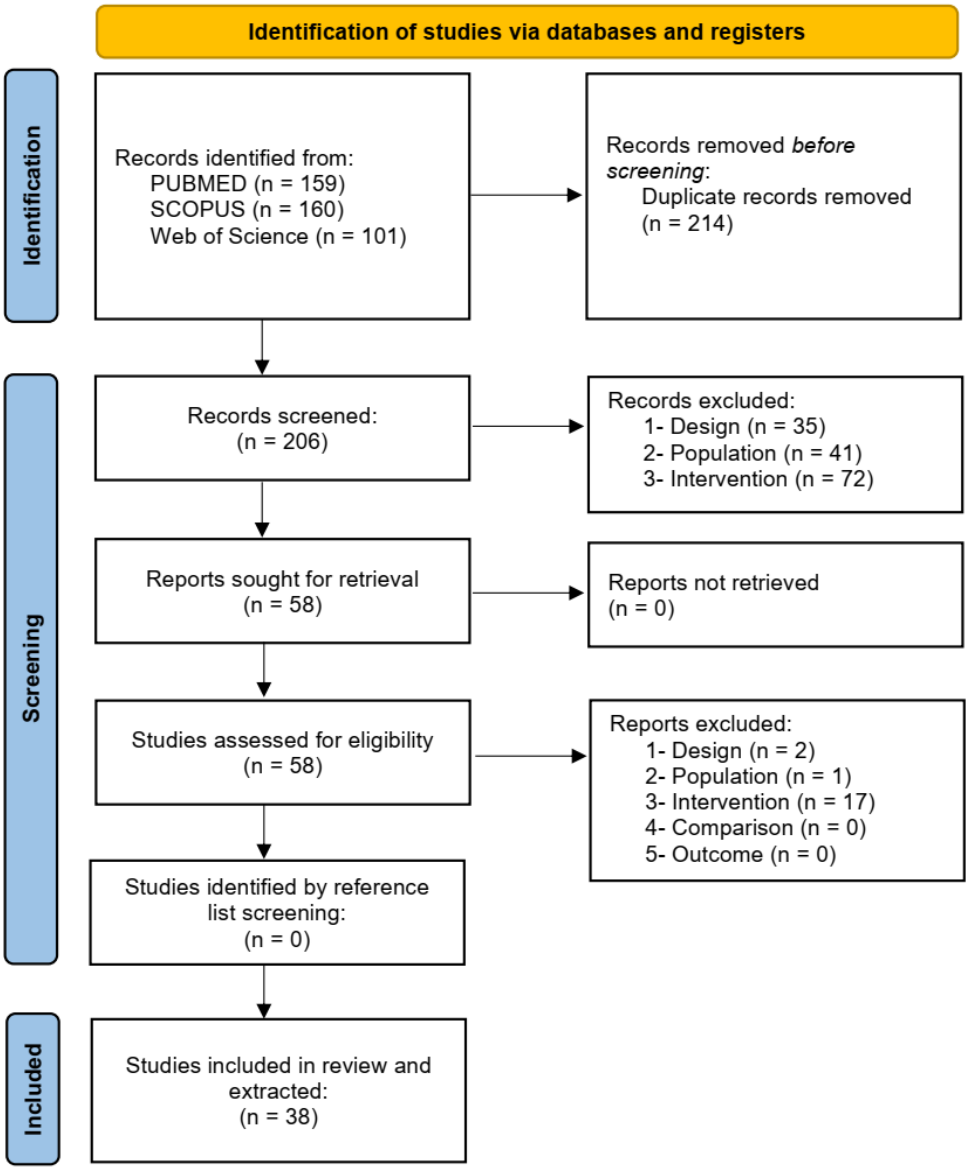

Fig 1. Flowchart diagram of the collection of studies and selection process.

(3) Bias assessment

The overall risk of bias associated with the items evaluated for methodological quality was considered unclear (Fig. 2). In more than 89% of the studies included, the information given was insufficient to rule out biases arising from the allocation of animals to the experimental groups or baseline characteristics. Although being an important methodological conduct, random housing allocation was not reported in any

publication. Bias related to blind assessment of outcomes was considered unclear in 14 studies (36.8%) and one study was deemed as having a high risk of bias for this item. Outcome data was incomplete in two studies (5.3%), and it was unclear whether data was complete in 63.2% of the assessed papers. For six studies (15.8%), cross-checking the information for outcomes measured between the methodology and the results was not possible and selective reporting was considered unclear.

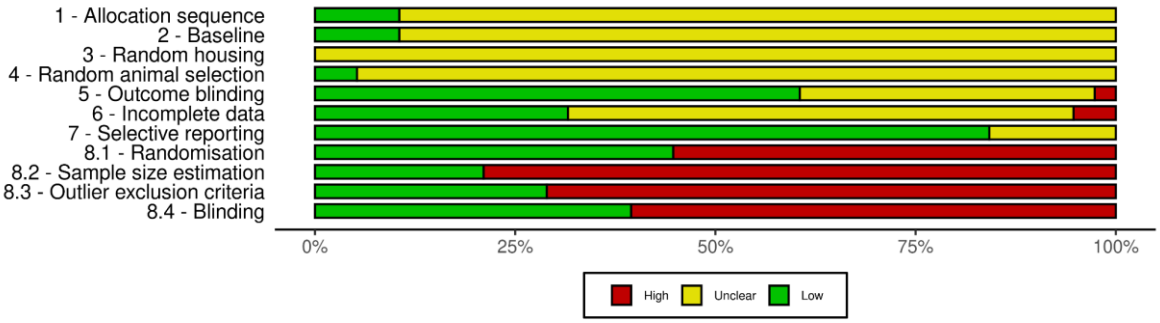

Fig. 2. Risk of bias assessment of included studies. The risk of bias assessment was performed by two independent investigators based on the SYRCLE's risk of bias assessment tool. Items 1 to 7 account for methodological quality and were scored as presenting a high, unclear or low risk of bias. Items 8.1 to 8.4 evaluate the reporting quality of the studies and were scored as presenting a high or low risk of bias. Classification is given as the percentage of assessed studies (*n* = 38) presenting each score.

As for the reporting quality, more than 50% of the studies failed to report any information on the items assessed. Researchers failed to describe if any randomization method was used in 21 studies (55.3%). Sample size estimation procedures were not informed in 30 papers (78.9%). Reporting quality was also considered unsatisfactory when evaluating the report of inclusion/exclusion criteria and blinding, since there were no reports of these items in 27 (71.1%) and 23 (60.5%) of the studies, respectively. Out of 418 scores given in the risk of bias assessment, there were 51 (12.2%) inconsistencies between investigators. Individualised scores for each study included are available at https://osf.io/zw6qg.

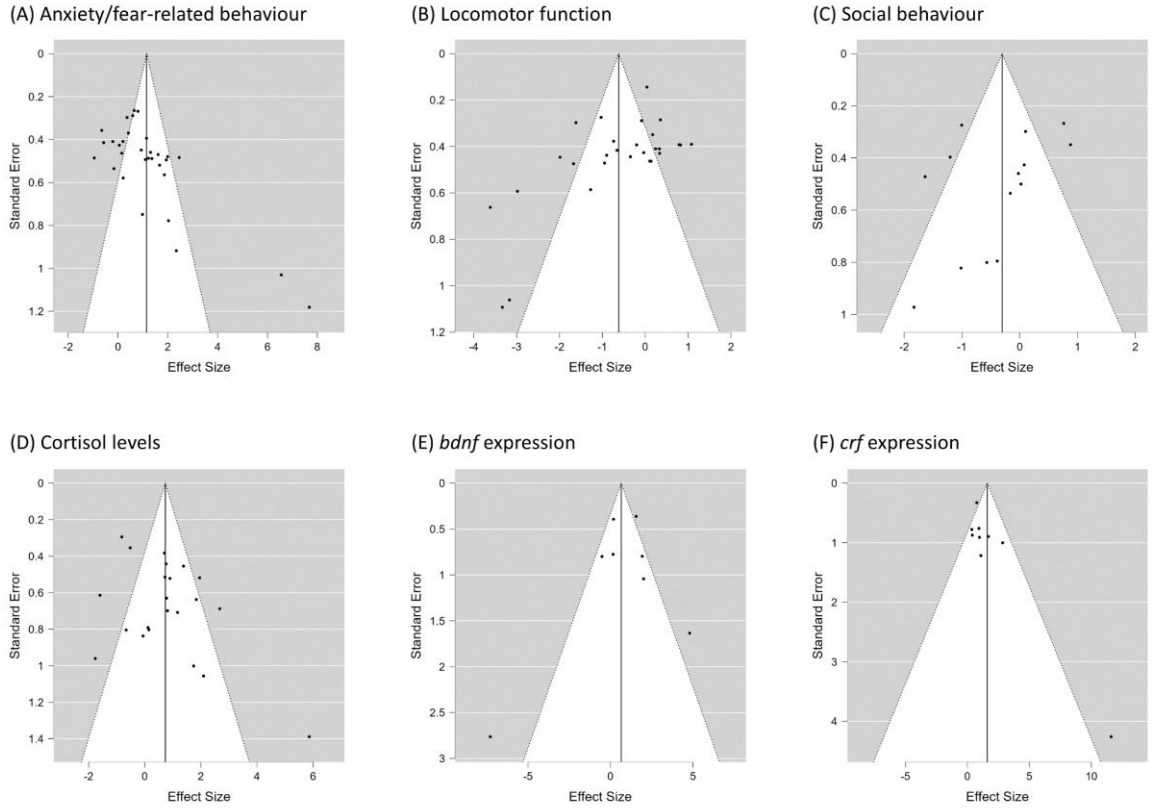

Fig 3. Funnel plots including studies analysed within each domain of interest: (A) anxiety/fear-related behaviour, (B) locomotor function, (C) social behaviour, (D) cortisol levels, (E) *bdnf* expression, and (F) *crf* expression. Each point represents a single comparison. The vertical line represents the overall effect size and the triangular region represents the 95% confidence interval.

Visual inspection of funnel plots demonstrated a substantial asymmetrical distribution of the studies within some domains of interest (Fig. 3). The scattered plot does not show the expected funnel-shaped distribution of experiments for anxiety/fear-related behaviour (Fig. 3A), locomotor function (Fig. 3B), and social behaviour (Fig. 3C). This could be attributed to sample heterogeneity, as the protocols, tests, and measured variables differ significantly among selected studies. On the other hand, funnel plots for cortisol levels (Fig. 3D), *bdnf* expression (Fig. 3E), and *crf* expression (Fig. 3F) show a relatively symmetrical distribution, with the limitation that the latter two are based on a small number of studies.

Egger's regression test indicated publication bias for all domains tested (Table 2): anxiety/fear-related behaviour ($p < 0.001$), locomotor function ($p < 0.001$), social behaviour ($p = 0.077$), and cortisol levels ($p = 0.086$). Both tests suggest a possible overestimation of the effects of UCS based on published data. Unfortunately, as mentioned above, studies reporting *bdnf* and *crf* expression were only a few, which hindered the inference of publication bias based on the regression test, as its statistical power depends on the number of experiments included in the analysis.

Table 2. Regression test for Funnel plot asymmetry ("Egger's test"). A *p*-value < 0.1 was considered significant for publication bias.

Domain	***z***	***p*-value**
Anxiety/fear-related behaviour	5.440	< 0.001
Locomotor function	-4.036	< 0.001
Social Behaviour	-1.771	0.077
Cortisol levels	1.717	0.086

(4) Anxiety/fear-related behaviour

The meta-analysis comprised 31 comparisons out of 23 independent studies. A total of 377 animals were used as controls and 439 composed the stressed groups. The most frequently used test to assess anxiety/fear-related behaviour in the included studies was the novel tank (31), followed by the open field (3), light/dark (1), and stress-induced analgesia tests (1).

The overall analysis revealed that stressed animals have higher levels of anxiety/fear-related behaviour when compared to control animals (SMD 1.15 [0.52, 1.78], $p < 0.001$, Fig. 4A). The estimated heterogeneity was high, with an $I^2 = 93.91\%$ and a $Chi^2 = 169.092$ ($df = 30$, $p < 0.001$). Subgroup analysis revealed that for

experiments with stress duration of up to 7 days there was no statistically significant effect on anxiety/fear-related behaviour (SMD 0.37 [-0.22, 0.97], p = 0.218, Fig. 4B). The heterogeneity was also high for this subgroup, with an I^2 = 82.69%, and a Chi^2 = 51.242 (df = 10, p < 0.001). For experiments with a UCS regimen of more than 7 days, it is possible to observe a significant effect of the stress on increasing anxiety-like behaviour (SMD 1.61 [0.75, 2.48], p < 0.001, Fig. 4B). The heterogeneity remained high when analysing this subgroup, resulting in an I^2 = 94.49%, and a Chi^2 = 100.689 (df = 19, p < 0.001).

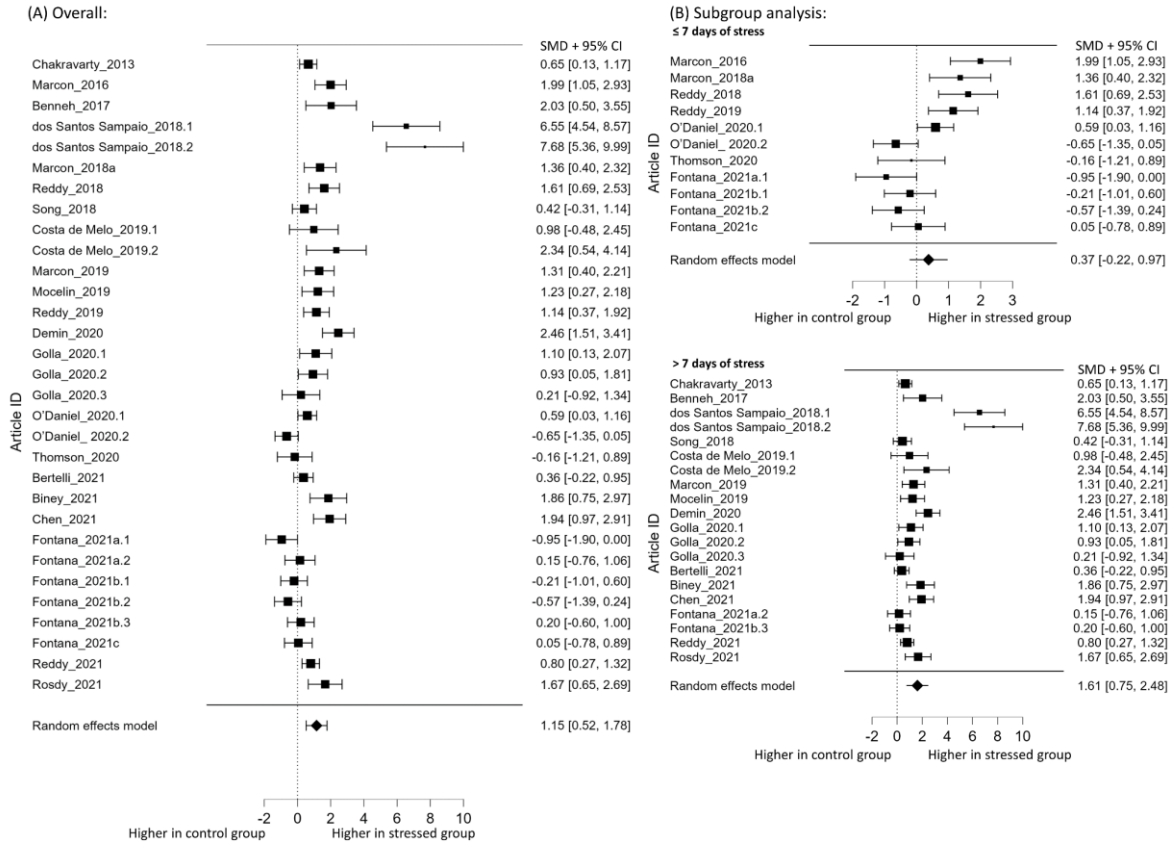

Fig 4. The effect of unpredictable chronic stress UCS protocol on anxiety/fear-related behaviour of zebrafish. (A) Overall effects of UCS on anxiety/fear-related behaviour in included studies. (B) Subgroup analyses based on the duration of the stress protocol (either ≤ 7 days or > 7 days of stress). Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

(5) Locomotor function

The meta-analysis comprised 28 comparisons out of 21 independent studies. A total of 454 animals were used as controls and 510 composed the stressed groups. The most frequently used test to assess locomotor function in the included studies was the novel tank test (21), followed by the open field (4), mirror-induced aggression (2), and stress-induced analgesia tests (1).

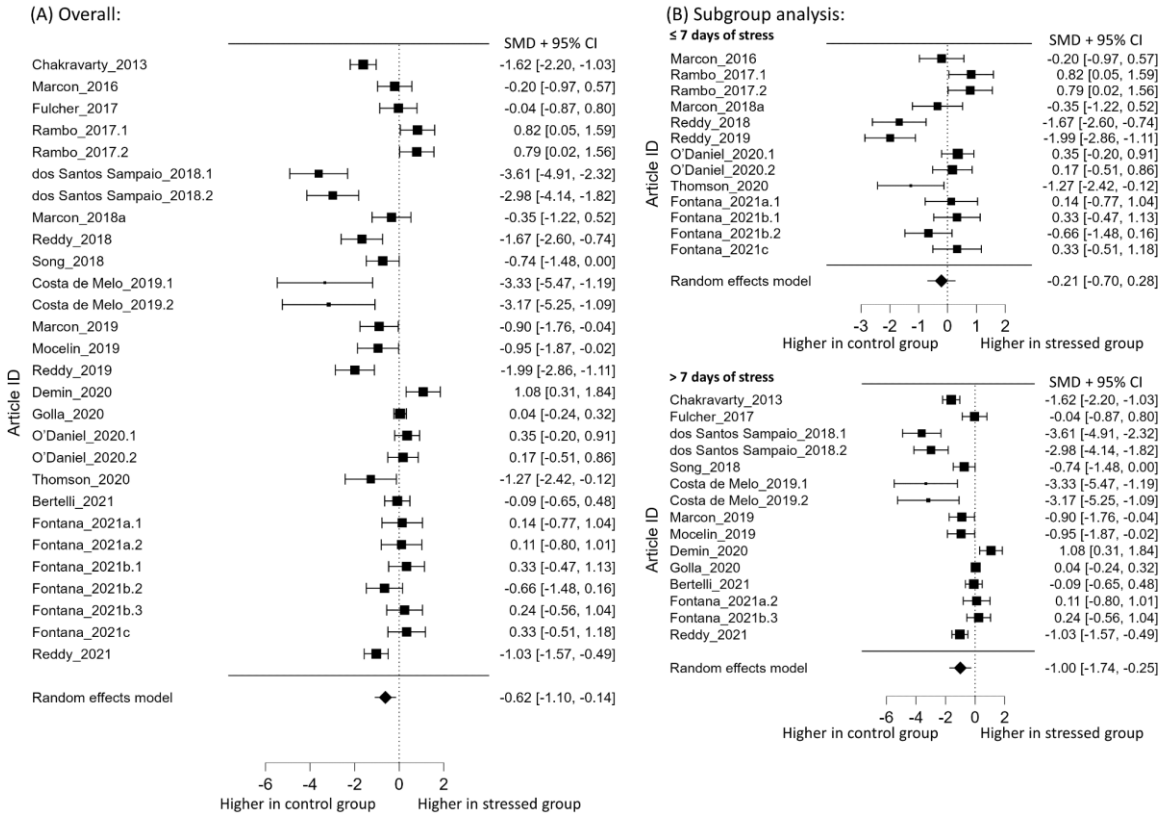

Fig 5. The effect of unpredictable chronic stress UCS protocol on the locomotor function of zebrafish. (A) Overall effects of UCS on the locomotor function in included studies. (B) Subgroup analyses based on the duration of the stress protocol (either ≤ 7 days or > 7 days of stress). Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

The overall analysis showed that stressed animals show lower levels of mobility when compared to control animals (SMD -0.62 [-1.10, -0.14], $p = 0.012$, Fig. 5A). The estimated heterogeneity was considered high, with an $I^2 = 91.30\%$ and a $Chi^2 = 168.198$ ($df = 27$, $p < 0.001$). When analysing separately experiments conducted with

a UCS protocol of up to 7 days, there was no statistically significant effect of the stress on locomotor function (SMD -0.21 [-0.70, 0.28], p = 0.4, Fig. 5B). The heterogeneity was also high for this subgroup, with an I^2 = 79.4%, and a Chi^2 = 51.207 (df = 12, p < 0.001). As for experiments conducted with a UCS regimen of more than 7 days, it is possible to observe a significant difference in locomotor function between stressed and unstressed groups, evidencing higher mobility for unstressed animals (SMD -1.00 [-1.74, -0.25], p = 0.009, Fig. 5B). The heterogeneity remained high when analysing this subgroup, resulting in an I^2 = 93.63%, and a Chi^2 = 110.784 (df = 14, p < 0.001).

(6) Social behaviour

The meta-analysis comprised 14 comparisons out of 11 independent studies. A total of 172 animals were used as controls and 190 composed the stressed groups. The most frequently used test to assess social behaviour in the included studies was the shoaling response test (8), followed by social interaction (4), and novel tank tests (2).

The overall analysis showed no significant effects of the UCS protocol on social behaviour (SMD -0.31 [-0.71, 0.10], p = 0.140, Fig. 6). The estimated heterogeneity was considered moderate, with an I^2 = 66.20% and a Chi^2 = 52.631 (df = 13, p < 0.001). There were no sufficient studies to perform a subgroup analysis.

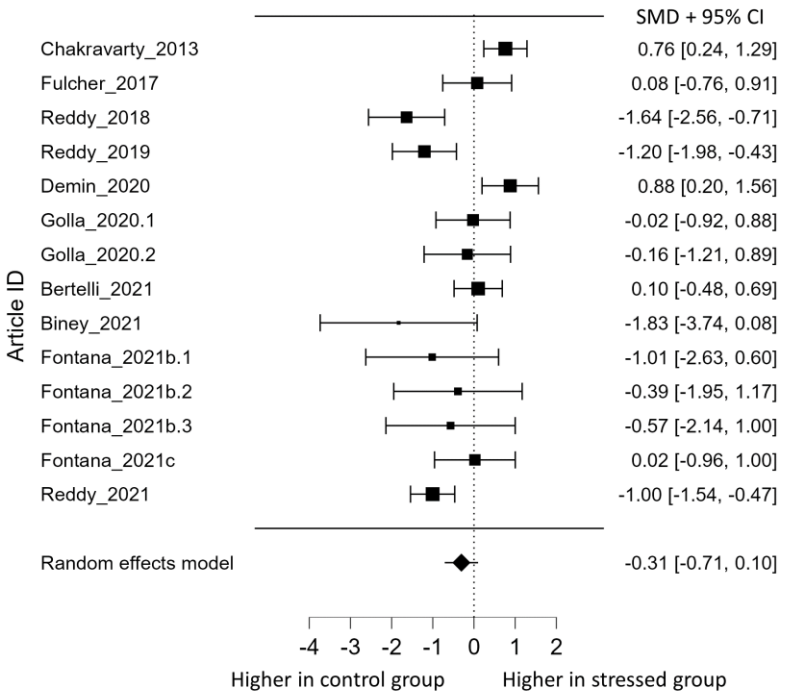

Fig 6. The effect of unpredictable chronic stress UCS protocol on the social behaviour of zebrafish. Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

(7) Cortisol levels

The meta-analysis comprised 22 comparisons out of 13 independent studies. A total of 150 animals were used as controls and 223 composed the stressed groups. Whole-body cortisol levels were measured in most studies (15), followed by trunk (5), and serum cortisol measurements (2).

The overall analysis showed that stressed animals have higher levels of cortisol when compared to control animals (SMD 0.73 [0.06, 1.40], p = 0.032, Fig. 7A). The estimated heterogeneity was considered high, with an I^2 = 86.68% and a Chi^2 = 95.623 (df = 21, p < 0.001). When analysing separately experiments conducted with a UCS regimen of up to 7 days, there was no statistically significant effect of the stress on cortisol levels (SMD 0.82 [-0.21, 1.85], p = 0.120, Fig. 7B). The heterogeneity was also high for this subgroup, with an I^2 = 91.05%, and a Chi^2 = 87.613 (df = 13, p < 0.001).

As for experiments conducted with a UCS protocol of more than 7 days, it is possible to observe a significant effect of the stress on increasing cortisol levels (SMD 0.69 [0.24, 1.13], p = 0.002, Fig. 7B). The heterogeneity significantly decreased when analysing this subgroup, resulting in an I^2 = 17.88%, and a Chi^2 = 5.381 (df = 7, p = 0.614).

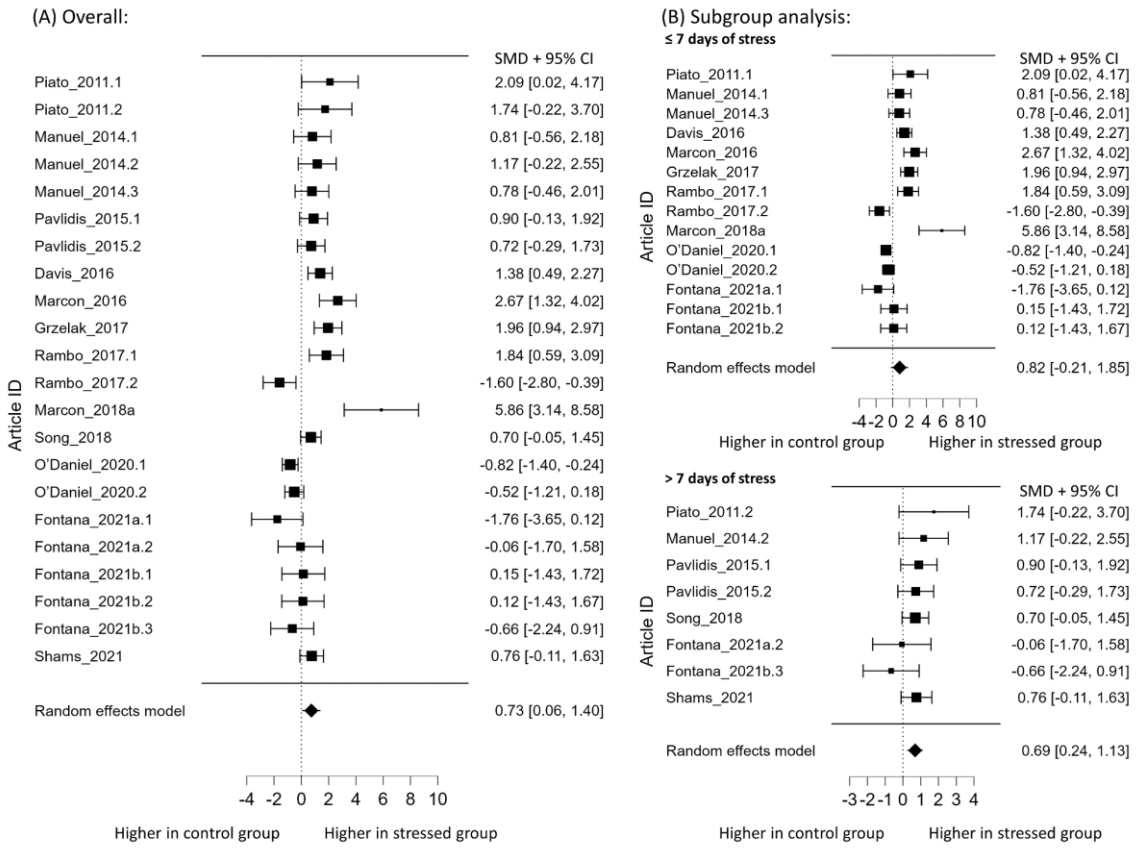

Fig 7. The effect of unpredictable chronic stress UCS protocol on cortisol levels in zebrafish. (A) Overall effects of UCS on the locomotor function in included studies. (B) Subgroup analyses based on the duration of the stress protocol (either ≤ 7 days or > 7 days of stress). Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

(8) *bdnf* and *crf* expression

The meta-analysis for *bdnf* expression comprised 8 comparisons out of 5 independent studies. A total of 45 animals were used as controls and 81 composed the stressed groups. The overall analysis showed no significant effects of the UCS protocol on the

expression of *bdnf* (SMD 0.65 [-1.74, 3.04], p = 0.592, Fig. 8A). The estimated heterogeneity was considered high, with an I^2 = 95.76% and a Chi^2 = 27.967 (*df* = 7, p < 0.001). There were no sufficient studies to perform a subgroup analysis. For the *crf* expression, the meta-analysis comprised 9 comparisons out of 5 independent studies. A total of 36 animals were used as controls and 73 composed the stressed groups. The overall analysis also showed no significant effects of the UCS protocol on the expression of *crf* (SMD 1.60 [-0.63, 3.82], p = 0.159, Fig. 8B). The estimated heterogeneity was considered high, with an I^2 = 94.13%. On the other hand, heterogeneity was found to not be significant with a Chi^2 = 11.865 (*df* = 8, p = 0.157). Again, there were no sufficient studies to perform a subgroup analysis.

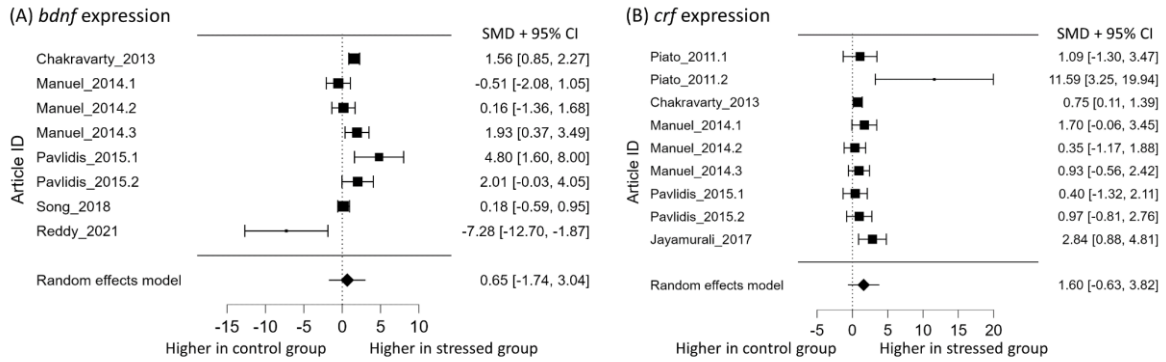

Fig 8. The effect of unpredictable chronic stress UCS protocol on the expression of *bdnf* and *crf* in zebrafish. (A) Meta-analysis of *bdnf* expression. (B) Meta-analysis of *crf* expression. Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

(9) Sensitivity analysis

The sensitivity analyses for studies presenting a significant risk of bias skewed the main effect of the domains tested (Fig 9). After excluding studies with a high risk of bias, no significant effects of UCS on anxiety/fear-related behaviour (SMD 1.07 [-0.13, 2.28], p = 0.081, Fig. 9A) and locomotor function (SMD -0.44 [-1.13, 0.25], p = 0.210, Fig. 9B) were observed. For social behaviour, the overall interpretation remained the

same, with no significant effects of the intervention on this behaviour (SMD 0.15 [-0.20, 0.49], p = 0.410, Fig. 9C). For cortisol levels, on the other hand, by excluding studies associated with a high risk of bias the direction of the effect was reversed, as the meta-analysis evidenced higher levels of cortisol in the control animals when compared to the stressed groups (SMD -0.61 [-0.99, -0.23], p = 0.002, Fig. 9D). As all studies included in the *bndf* and *crf* expression domains were considered as having a high risk of bias, conducting this sensitivity analysis was not possible for these outcomes.

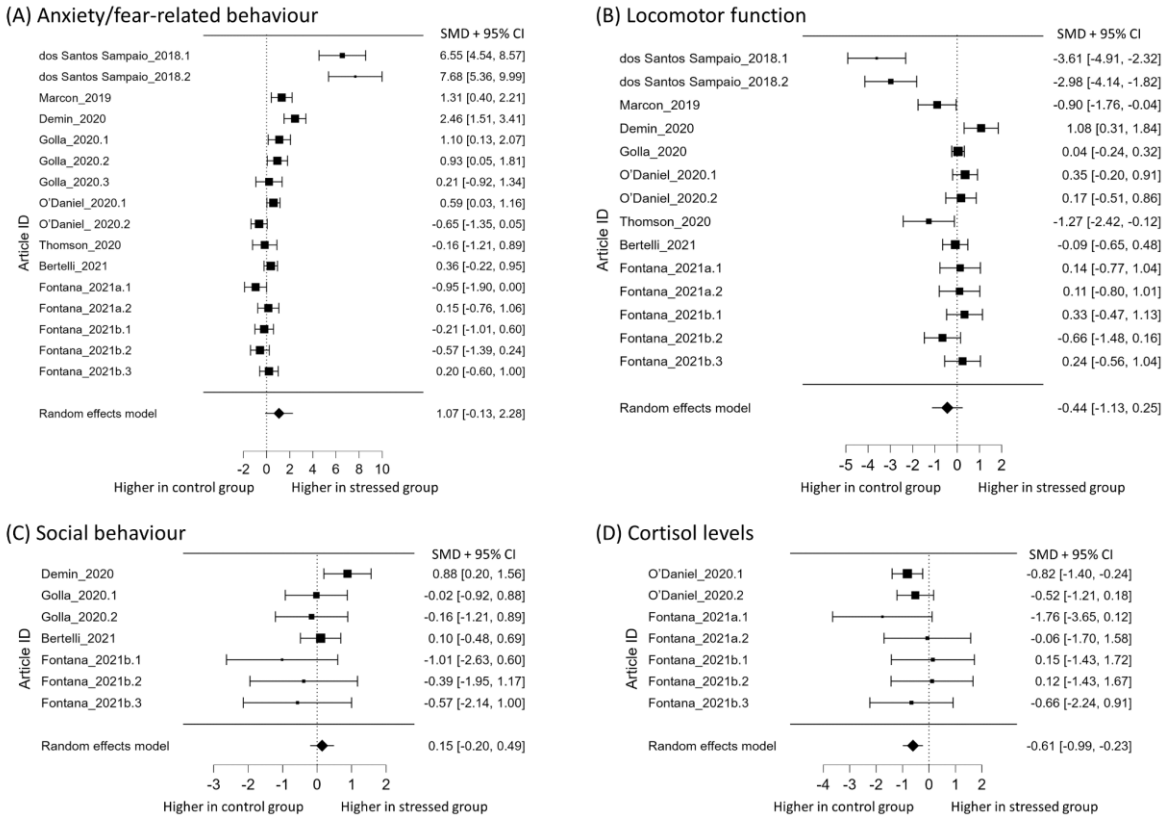

Fig 9. Sensitivity analyses for studies with a high risk of bias. The analyses were conducted by excluding studies presenting a significant risk of bias, defined as either a high risk of bias in one of the main items evaluating methodological quality in the risk of bias assessment (items 1 to 7), or an unclear risk of bias in five or more of the same items. Analyses were conducted for (A) anxiety/fear-related behaviour, (B) locomotor function, (C) social behaviour, and (D) cortisol levels. Data are presented as Hedges' G standardised mean differences and 95% confidence intervals.

Table 1. Qualitative description of studies reporting unpredictable chronic stress (UCS) protocols in research with zebrafish. The sex of the animals used was computed as: M, for male animals; F, for females; M:F, when male and female were included but tested and analyzed as a mixed group; M+F, when male and female fish were discriminated in the experiments; Unclear, for larvae and when the sex of the animals was not reported. Main findings were described as: ↑, higher when compared to the control group; ↓, lower when compared to the control group; =, no difference when compared to the control group.

Reference	Duration of stress protocol (days)	Number of different stressors	Interval between stress protocol and outcome assessment (days)	Developmental stage during stress/outcome assessment	Sex	Main findings
Piato *et al.*, 2011	7, 14	10	1	Adult	M	**Anxiety/fear-related behaviour** ↓ Height in the tank **Cortisol** ↑ Whole-body cortisol **Locomotor function** ↓ Locomotion (14 days) **Neurochemical outcomes** ↓ *gr* expression ↑ *crf* expression **Social behaviour** ↑ Shoal cohesion (7 days) ↓ Shoal cohesion (14 days)

Chakravarty *et al.*, 2013	15	10	1	Adult	M:F	**Anxiety/fear-related behaviour** ↑ Latency to upper zone ↓ Entries in the upper zone ↑ Freezing bouts ↑ Freezing duration ↓ Latency to dark compartment **Locomotor function** ↓ Crossings **Neurochemical outcomes** ↑ *crf* expression ↑ *ppp3r1a* expression ↑ *bdnf* expression **Social behaviour** ↓ Latency to together
Manuel *et al.*, 2014	7, 14	9	1	Adult	M:F	**Cortisol** ↑ Whole-body cortisol (14 days, 7 nights of UCS) **Learning and memory** ↓ Latency to black compartment day 2 (14 days of UCS) ↓ Latency to black compartment day 3 (14 days, 7 nights of UCS) **Neurochemical outcomes**

						↑ *cart* expression (7 days of UCS) ↑ *htr1ab* expression (7 days of UCS) = *crf-bp* expression = *crf* expression ↑ *bndf* expression (7 nights of UCS) ↑ *grβ* expression (7 nights of UCS) = *cnr1* expression ↑ *mr* expression (7 nights of UCS) ↑ *gra* expression (7 nights of UCS) = *mr/gra* ratio ↑ *grβ/gra* ratio (7 nights of UCS)
Pavlidis, Theodoridi & Tsalafouta, 2015	11	7-12	1	Adult	M:F	**Cortisol** ↑ Trunk cortisol concentration (Higher grade stressors) **Neurochemical outcomes** = *crf* mRNA relative levels ↑ *pomc* mRNA relative levels (Higher grade stressors) ↑ *gr* mRNA relative levels (Higher grade stressors) ↑ *mr* mRNA relative levels (Higher grade stressors) = *mc2r* mRNA relative levels ↑ *prl* mRNA relative levels (Higher grade stressors)

						= *avt* mRNA relative levels ↑ *hypocretin/orexin* mRNA relative levels (Higher grade stressors) ↑ *bdnf* mRNA relative levels ↑ *c-FOS* mRNA relative levels
Davis *et al.*, 2016	5	5	Unclear	Adult	Unclear	**Cortisol** ↑ Serum cortisol **Leukogram** ↓ Lymphocytes ↑ Monocytes = Neutrophils = Eosinophils
Marcon *et al.*, 2016	7	7	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↓ Entries in the upper zone **Cortisol** ↑ Whole-body cortisol **Locomotor function** = Total distance travelled **Neurochemical outcomes** ↑ *cox-2* expression = *tnf-α* expression ↑ *IL-6* expression = *IL-10* expression

Zimmermann *et al.*, 2016	7	10	1	Adult	M	**Neurochemical outcomes** ↓ Membrane-bound Adenosine Deaminase = Cytosolic Adenosine Deaminase = *ada1* expression = *ada2.1* expression = *ada2.2* expression = *adal* expression = *adaasi* expression = ATP hydrolysis = ADP hydrolysis = AMP hydrolysis
Benneh *et al.*, 2017	14	8	1, 3	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time in the upper zone = Entries in the upper zone ↑ Latency to upper zone (3 days post UCS) ↓ Time spent in light region (1 day post UCS) ↓ Entries in the light region (1 day post UCS) **Social behaviour** ↓ Shoal average area (3 days post UCS)
Fulcher *et al.*, 2017	15	6	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Distance to bottom (1-3 minutes of test)

						↓ Freezing duration (1-3 minutes of test) **Locomotor function** ↑ Distance travelled (1-3, 6-10 minutes of test) ↑ Absolute turn angle (1-3, 11-15 minutes of test) **Morphometric measurements** ↑ Bodyweight **Neurochemical outcomes** = Dopamine levels = DOPAC levels = Serotonin levels = 5-HIAA levels **Social behaviour** = Distance to stimulus ↑ Variance of distance to stimulus (1-3 minutes of test)
Grzelak *et al.*, 2017	10	5	Unclear	Adult	Unclear	**Cortisol** ↑ Serum cortisol **Leukogram** ↓ Lymphocytes differential count ↑ Monocytes differential count

						= Neutrophils differential count = Eosinophils differential count
Jayamurali & Govindarajulu, 2017	15	7	1	Adult	M:F	**Neurochemical outcomes** ↑ *crf* expression ↓ *gr* expression ↑ *p53* expression ↑ *NOXA* expression ↓ *bcl2* expression ↑ *casp3* expression
Rambo *et al.*, 2017	7	7	1	Adult	M+F	**Aggression** ↑ Relative time spent close to the mirror (male) **Cortisol** ↑ Whole-body cortisol (male) **Locomotor function** = Total distance travelled = Mean speed = Crossings
dos Santos Sampaio *et al.*, 2018	15	6	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↑ Latency to upper zone ↑ Freezing duration **Locomotor function** ↓ Total distance travelled

						↓ Quadrants crossed ↑ Erratic swimming
Marcon *et al.*, 2018a	7	7	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↓ Entries in the upper zone ↑ Time in the bottom **Cortisol** ↑ Trunk cortisol **Locomotor function** = Total distance travelled **Neurochemical outcomes** ↑ Reactive oxygen species (ROS) levels - DCF fluorescence
Marcon *et al.*, 2018b	7	6	1	Adult	M:F	**Neurochemical outcomes** ↑ TBARS levels ↑ Reactive oxygen species (ROS) levels - DCF fluorescence ↓ NPSH levels = SH total levels ↓ SOD activity = CAT activity
Reddy *et al.*, 2018	7	10	1	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↑ Latency to upper zone

						↑ Freezing duration **Locomotor function** ↓ Crossings **Social behaviour** ↓ Interaction time
Song *et al.*, 2018	35	>10	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↓ Entries in the upper zone = Freezing bouts **Cortisol** ↑ Whole-body cortisol **Dendritic spines** ↑ Average number of spines **Locomotor function** = Total distance travelled ↓ Mean meander moved = Low mobility duration = Low mobility frequency = Regular mobility duration = Regular mobility frequency = Highly mobility duration = Highly mobility frequency ↓ Mean velocity

						= Mean maximal velocity **Neurochemical outcomes** = *bdnf* expression = *p75* expression = *trkB* expression = *gfap* expression **Peripheral outcomes** ↑ Whole-body *IL-1β* ↑ Whole-body *IL-6* ↑ Whole-body *IL-10* ↑ Whole-body *bdnf*
Costa de Melo *et al.*, 2019	15	6	1	Adult	F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↑ Latency to upper zone ↑ Freezing duration **Locomotor function** ↓ Total distance travelled ↓ Quadrants crossed ↑ Erratic swimming
Huang, Butler & Lubin, 2019	14	6	1	Adult	M+F	**Anxiety/fear-related behaviour** = Percent at bottom **Cortisol** ↑ Trunk cortisol (15 min after the last stressor)

						Locomotor function = Total distance travelled **Neurochemical outcomes** ↑ *ache* expression (female) ↑ *nr3c1* expression ↑ *hsd11b2* expression = *npy* expression
Marcon *et al.*, 2019	14	6	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone = Time in the middle zone ↑ Time in the bottom ↓ Entries in the upper zone **Locomotor function** = Total distance travelled = Crossings **Neurochemical outcomes** ↑ TBARS levels ↓ NPSH levels = SH total levels ↓ SOD activity = CAT activity
Mocelin *et al.*, 2019	14	6	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time in the upper zone ↓ Entries in the upper zone

						↑ Time in the bottom = Entries in the bottom **Locomotor function** = Total distance travelled = Crossings **Neurochemical outcomes** ↑ TBARS levels ↑ Reactive oxygen species (ROS) levels - DCF fluorescence ↓ NPSH levels ↓ SOD activity = CAT activity
Reddy *et al.*, 2019	7	10	1,4	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↑ Latency to upper zone ↑ Freezing duration (social behaviour test, before drug treatment) **Locomotor function** ↓ Crossings **Social behaviour** ↓ Interaction time ↑ Latency to interaction

Demin *et al.*, 2020	34	>10	7, 14, 21, 28, 35	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↓ Time spent in the light zone (1, 2, 3 weeks of UCS) ↓ Distance to the surface (1 week of UCS) ↑ Distance to the surface (2 weeks of UCS) ↓ Time spent active (1 week of UCS) ↑ Time spent active (3 weeks of UCS) **Locomotor function** ↑ Distance travelled (5 weeks of UCS) **Neurochemical outcomes** = Whole-brain serotonin ↑ 5-HIAA levels (2 weeks of UCS) ↓ 5-HIAA levels (4 weeks of UCS) ↑ 5-HIAA/5HT ratio (2 weeks of UCS) ↓ 5-HIAA/5HT ratio (3,4 weeks of UCS) = Norepinephrine = *saga* expression ↓ *isg15* expression

						↓ *otx5* expression ↑ *tpm4b* expression **Social behaviour** ↓ Interfish distance (5 weeks of UCS)
Golla *et al.*, 2020	8	5	1, 2, 3, 8	Larval	Unclear	**Anxiety/fear-related behaviour** = Thigmotaxis index = Scototaxis index ↓ Vertical position (1-3 days post UCS) ↑ Ratio of fish in bottom third (1-3 days post UCS) **Locomotor function** ↑ Total distance travelled (Light-dark test; 2 days post UCS) ↑ Mean velocity (Light-dark test; 2 days post UCS) **Morphometric measurements** ↓ Size **Social behaviour** = Nearest neighbour distance = Interfish distance

O'Daniel & Petrunich-Rutherford, 2020	7	7	1, 8	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone (1 day post UCS) ↑ Entries in the upper zone (7 days post UCS) ↑ Distance travelled in the upper zone (7 days post UCS) = Freezing duration **Cortisol** ↓ Trunk cortisol (1 day post UCS) **Locomotor function** = Total distance travelled = Mean ambulatory velocity **Morphometric measurements** = Trunk weight
Thomson *et al.*, 2020	7	3	0	Adult	F	**Anxiety/fear-related behaviour** ↓ Time spent in the bottom **Locomotor function** ↓ Velocity = Fractal dimension
Bertelli *et al.*, 2021	14	6	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone = Entries in the upper zone = Time in the centre zone

						↑ Freezing duration **Locomotor function** ↓ Total distance travelled (open tank test) = Absolute turn angule = Crossings **Morphometric measurements** ↓ Weight **Neurochemical outcomes** ↑ TBARS levels ↓ NPSH levels **Peripheral outcomes** ↑ Blood glucose **Social behaviour** = Time in the interaction zone = Interaction time = Number of interactions
Biney *et al.*, 2021	14	8	4	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↓ Entries in the upper zone ↓ Time spent in the light zone = Entries in the light zone

						Social behaviour = Shoal cohesion
Chen *et al.*, 2021	35	Unclear	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↓ Time spent in the light zone ↑ Latency to the dark zone **Cortisol** ↑ Peripheral cortisol **Neurochemical outcomes** ↑ *bdnf* expression ↑ *tnf-α* expression ↑ *IL-1β* expression ↑ *IL-10* expression **Morphometric measurements** ↓ Body mass index
Demin *et al.*, 2021	77	>10	1	Adult	M:F	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone **Learning and memory** ↓ Time spent in the light zone **Locomotor function** = Mean velocity **Neurochemical outcomes**

						↑ Norepinephrine levels = Dopamine levels = Serotonin levels = 5HIAA to 5HT ratio **Social behaviour** ↓ Interfish distance
Fontana *et al.*, 2021a	7, 14	8	~ 180	Larval / Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the bottom (7 days of UCS protocol) **Cortisol** = Whole-body cortisol **Learning and memory** = Time spent close to the object = Entries to the object zone **Locomotor function** = Total distance travelled
Fontana *et al.*, 2021b	3, 7, 14	8	1, 120	Larval / Juvenile, Adult	M:F	**Anxiety/fear-related behaviour** ↑ Time spent in the upper zone (7 days of UCS protocol/ Adult) ↓ Time spent in the dark zone (7 days of UCS protocol/ Adult) ↑ Thigmotaxis (7 days of UCS protocol/ Juvenile) = Preference index

						Cortisol = Whole-body cortisol **Learning and memory** = Total turns = Alternations = Repetitions **Locomotor function** = Total distance travelled **Social behaviour** = Interfish distance = Shoal average area
Fontana *et al.*, 2021c	3	3	> 75	Juvenile / Adult	Unclear	**Anxiety/fear-related behaviour** = Time spent in the bottom **Learning and memory** ↑ Average of turns ↑ Relative alterations ↓ Relative repetitions = Relative right turns = Relative left turns **Locomotor function** = Total distance travelled

						Social behaviour = Shoal cohesion
Kirsten *et al.*, 2021	14	9	0.5	Adult	M:F	**Neurochemical outcomes** = *bdnf* expression ↑ *tnf-α* expression ↑ *IL-1β* expression = *IL-4* expression = *IL-6* expression ↑ *IL-10* expression ↓ *c-FOS* expression = *INF-γ* expression
Reddy *et al.*, 2021	10	10	1, 2	Adult	M:F	**Anxiety/fear-related behaviour** ↑ Time spent in the bottom ↓ Transitions to upper zone ↑ No movement duration ↑ Latency to feed ↓ Feeding frequency ↓ Latency to freeze ↑ Freezing bouts ↑ Freezing duration ↓ Time spent in the pheromone zone **Locomotor function** ↓ Total distance travelled ↓ Mean velocity ↓ Movement duration ↓ Highly mobile duration

						↓ Duration of erratic movements **Neurochemical outcomes** ↓ *bdnf* expression ↑ *crf* expression ↑ *calcineurin* expression ↓ *B-III tubulin* expression = *blbp* expression ↓ *pmTOR/mTOR* ratio ↓ *sox2* expression ↓ *sox2* positive cells **Proliferative index** ↑ Proliferative index telencephalon (Dm) ↓ Proliferative index telencephalon (Dld + Dlv) **Social behaviour** ↓ Duration of interaction (with target fish in the interaction zone) ↓ Interaction frequency (with target fish in the interaction zone)
Rosdy *et al.*, 2021	14	10	Unclear	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↓ Time spent in the light zone
Shams, Khan & Gerlai, 2021	15	6	1	Adult	M:F	**Cortisol** ↑ Whole-body cortisol

Zhang *et al.*, 2021	28	8	1	Adult	Unclear	**Anxiety/fear-related behaviour** ↓ Time spent in the upper zone ↓ Latency to the upper zone ↓ Freezing bouts ↓ Freezing duration ↑ Immobility time **Locomotor function** ↓ Total distance travelled ↓ Mean velocity ↑ Meandering ↑ Absolut turn angle ↑ Angular velocity

IV. DISCUSSION

Ten years after the publication of the first study of UCS conducted using zebrafish as the model animal (Piato *et al.*, 2011), we performed a systematic review and meta-analysis of the literature to evaluate and synthetize the behavioural and neurochemical effects of this protocol. Despite the relatively low number of studies carried out with far fewer animals than the rodent literature, the main findings of our study show that UCS increases anxiety-like behaviour and cortisol levels while decreasing locomotor activity in zebrafish. On the other hand, no effects on social behaviour and other biomarkers (*bdnf* and *crf*) were observed in this species.

Such results somewhat correlate with the findings gathered from experiments conducted with rodents. As mentioned before, although the stress regimen is shown to consistently induce anhedonic behaviour in rodents, several variables intrinsic to the organisms such as species, sex, age, and resilience or the protocol itself have a great impact on the outcomes measured, leading to the heterogeneity seen in the literature (Antoniuk *et al.*, 2019). Results for anxiety-like behaviour (Kompagne *et al.*, 2008; Cox *et al.*, 2011; Zhu *et al.*, 2014), locomotor function (Kumar, Kuhad & Chopra, 2011; Sequeira-Cordero *et al.*, 2019), and social behaviour (Boxelaere *et al.*, 2017) vary considerably depending on the conditions applied in the experiments and are still in need of a thorough systematic review to determine effect direction. The same can be said for the hormonal regulation of the stress response and related neurochemical outcomes. It is also expected to observe an increase in corticosterone and an imbalance of neurochemical markers driven by the UCS in rodents, but many reports reveal behavioural alterations in the absence of detectable modifications in these other parameters as reviewed elsewhere (Willner, 2017a; Lages *et al.*, 2021).

Many factors might explain the high heterogeneity revealed between included studies and the behavioural response of fish. The number and classes of stressors used to generate stress differ substantially between studies. This information is crucial since different stressors have been shown to trigger different patterns of behavioural and biochemical responses in rodents (Antoniuk *et al.*, 2019). The majority of experiments have been conducted using mixed samples of both male and female zebrafish without reporting individualised effects of UCS by sex. Unfortunately, it is still difficult to evaluate these differential impacts since more studies are required to conduct analyses grouped by sex; however, a few experiments have already shown that stress can elicit different responses in male and female zebrafish (Rambo *et al.*, 2017; Huang, Butler & Lubin, 2019).

Subgroup analyses indicate that the duration of the stress protocol might also influence the outcome of the UCS protocol, corroborating what was shown within previous works (Piato *et al.*, 2011; Palucha-Poniewiera *et al.*, 2020; Fontana *et al.*, 2021a). When grouping experiments by this variable, no significant effects of the stress are observed in anxiety/fear-related behaviour, locomotor function, and cortisol levels for stress regimens of up to 7 days despite the overall effects of UCS for these domains. Protocols with more than 7 days, on the other hand, show a significant effect of UCS for the same variables, indicating that regimens of more than a week of stress are necessary to reveal the deleterious consequences of stress in zebrafish. It is important to note that most experiments designed to evaluate the long-lasting effects of UCS in zebrafish were included in the group with shorter stress times. In these cases, stress sessions occur in early developmental stages and tests usually take place later in the animal life. This allows for a long washout period between the stress and outcome assessment that might explain the lack of effects of stress when such

designs are used. Capturing UCS effects heavily depends on assessment timing (Willner, 2017a; Bosch *et al.*, 2022), and tests should be scheduled to avoid observing acute effects of a single stressor as well as losing the effects of the intervention as a whole since animals are likely to eventually recover, unless the stressors coincide with a window of developmental vulnerability (Jankord *et al.*, 2011).

The results of this review should be interpreted with caution considering that the main effects of the analyses were influenced by studies with a high risk of bias. Although many efforts have been made to improve the reporting quality of pre-clinical research (Sert *et al.*, 2020), the publication of studies adhering to measures designed to mitigate the risk of bias associated with methodological conduct is still low (Baker *et al.*, 2014; Macleod *et al.*, 2015). These problems hamper the correct analysis of results and contribute to the reproducibility crisis in the biomedical field (Samsa & Samsa, 2019; Gerlai, 2019), encouraging researchers to question the validity of animal models (Worp *et al.*, 2010). By excluding studies with a high risk of bias in the sensitivity analysis it was possible to visualise the direct impacts of these on distorting the main effects found in the meta-analyses for anxiety/fear-related behaviour, locomotor activity, and especially for cortisol, for which effect direction was inverted in sensitivity analysis. Conclusions should also be conservative for *bdnf* and *crf* since, as mentioned before, all of the studies included presented a high risk of bias, revealing the alarming need to improve internal validity and reporting quality.

In the same way, publication bias plays a part in generating misleading assumptions even in meta-analyses based on broad and rigorous systematic reviews (Worp *et al.*, 2010). There is evidence of selective publishing of studies for the domains tested based on funnel plot inspection and Egger's test evaluation, pointing to the need

to conduct well-delineated experiments using this model, as these results denote a possible overestimation of the effects of chronic stress in zebrafish.

V. CONCLUSIONS

(1) The overall results of our meta-analysis reveal the effects of UCS in increasing anxiety/fear-related behaviour and cortisol levels in stressed animals while decreasing locomotor function.

(2) No effects of stress were found on social behaviour and the expression of *bdnf* and *crf*, but the literature reporting these outcomes is limited and with evidence of bias.

(3) The risk of bias was considered generally high for the studies included in this review, indicating poor methodological and reporting quality of studies conducted using zebrafish.

(4) We found moderate to high heterogeneity in the data, suggesting that several variables could influence the results obtained. Given the small number of studies included, it is difficult to point out the sources of variation other than the duration of the stress protocol.

(5) Protocols of more than a week of stress seem to be better suited to induce behavioural and biochemical alterations that are expected to occur with UCS.

(6) The analyses conducted stress the need to conduct well-designed experiments using the UCS model to assess its effects on zebrafish behaviour and neurochemical parameters, further exploring the sources of variation that might influence the results, such as the nature of stressors and sex.

(7) Overall, this review corroborates the need for improvement in methodological and reporting conduct across preclinical research.

VI. AUTHOR CONTRIBUTIONS

Matheus Gallas-Lopes: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualisation, and writing - original draft; **Leonardo M. Bastos:** conceptualization, investigation, methodology, and writing – review & editing; **Radharani Benvenutti:** conceptualization, investigation, methodology, and writing – review & editing; **Alana C. Panzenhagen:** conceptualization, formal analysis, methodology, visualisation, and writing - review & editing; **Angelo Piato:** conceptualization, investigation, methodology, and writing – review & editing; **Ana P. Herrmann:** conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, visualisation, and writing – review & editing;

VII. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

VIII. DATA AVAILABILITY

All data is available in Open Science Framework (https://osf.io/j2zva/).

IX. ACKNOWLEDGEMENTS

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