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Analysis of behavioral variables across domains and strains in zebrafish: Role of brain monoamines

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Abstract

Important neurochemical variations between strains or linages which correlate with behavioral differences have been identified in different species. Here, we report neurochemical and behavioral differences in four common zebrafish wild-type strains (blue shortfin, longfin stripped, leopard and albino). *leo* zebrafish have been shown to display increased scototaxis in relation to the other strains, while both *nacre* and *leo* zebrafish show increased geotaxis. Moreover, *leo* displayed increased nocifensive behavior, while *nacre* zebrafish showed increased neophobia in the novel object task. *lof* zebrafish showed decreased turn frequency in both the novel tank and light/dark tests, and habituated faster in the novel tank, as well as displaying increased 5-HT levels. *leo* zebrafish showed decreased brain 5-HT levels and increased 5-HT turnover than other strains, and *nacre* had increased brain DA levels. Finally, specific behavioral endpoints co-varied in terms of the behavioral and neurochemical differences between strains, identifying cross-test domains which included response to novelty, exploration-avoidance, general arousal, and activity.

Keywords: Novelty; Exploration-Avoidance; Arousal; Activity; Anxiety; Monoamines

1. Introduction

Important genetic variations between strains or lineages which correlate with behavioral differences have been identified in different species. The degree with which these variations explain the behavioral differences is not fully understood, but the use of behaviorally distinct strains might represent an important model to understand human behavioral disorders [1–4]. In this direction, mouse and rat inbred strains have been shown to differ in anxiety-like behavior and impulsivity [5–9]. The use of genetically tractable organisms, including invertebrate models and non-mammalian vertebrates, could generate important information regarding the genetic architecture underlying these disorders [10].

In this sense, zebrafish (*Danio rerio* Hamilton 1822) represent important additions to this arsenal, presenting technical advancements such as optogenetics and transgenesis which facilitate the study of neural circuits [11,12], complex behavioral phenotypes [10], and the presence of outbred strains which show considerable genetic variability [13]. Zebrafish inbred

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strains have been shown to differ in many important traits which are relevant for their behavior, such as brain transcriptome [14] and neurochemistry [15]. Some of these strains have also been subjected to behavioral testing, such as the novel tank test, and shown to differ in terms of anxiety-like behavior [14,16–19] and associated functions, such as habituation to novelty [20,21] and boldness [22,23]. While these behavioral endpoints have been assessed mostly in animals from inbred and outbred strains such as AB, WIK, SH, Tü and TL (http://zfin.org), commonly found mutant phenotypes were also used, including skin mutant phenotypes such as leopard, albino and longfin [24]. For example, Egan et al. [24] demonstrated that, in relation to wild-type shortfin, albino and leopard zebrafish show increased bottom-dwelling; Kiesel et al. [16] demonstrated a similar profile for longfin mutants.

In the present work, we analyze the behavioral and neurochemical differences between the common WT phenotypes shortfin, longfin, leopard and albino.

2. Methods

2.1 Animals and housing

40 animals from the blue shortfin phenotype (*bsf*), 40 from the longfin stripped phenotype (*lof*), 40 from the albino phenotype (*nacre*) and 40 from the leopard phenotype (*leo*) were used in the present study. Animals were group-housed in mixed-phenotype 40 L tanks, with a maximum density of 25 fish per tank. Tanks were filled with deionized and reconstituted water at room temperature (28 °C) and a pH of 7.0-8.0. Lighting was provided by fluorescent lamps in a cycle of 14-10 hours (LD), according to the standard of care zebrafish [25]. All manipulations minimized their potential suffering, as per the recommendations of the Canadian Council on Animal Care [26]. All procedures complied with the Brazilian Society for Neuroscience and Behavior's (SBNeC) guidelines for the care and use of animals in research.

2.2. Novel tank test

The protocol for the novel tank diving test used was modified from Cachat et al. [27]. Briefly, animals were transferred to the test apparatus, which consisted of a 15 x 25 x 20 cm (width x length x height) tank lighted from above by two 25 W fluorescent lamps, producing an average of 120 lumens above the tank. As soon as the animals were transferred to the apparatus, a webcam was activated and behavioral recording begun. The webcam filmed the

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apparatus from the front, thus recording the animal's lateral and vertical distribution. Animals were allowed to freely explore the novel tank for 6 minutes, after which they were removed from it and exposed to the scototaxis tank. Video files were later analyzed by experimenters blind to the treatment using X-Plo-Rat 2005 (http://scotty.ffclrp.usp.br), and the images were divided in a 3 x 3 grid composed of 10 cm^2 squares. The following variables were recorded:

time on top: the time spent in the top third of the tank;

geotaxis habituation: calculated as single-minute habituation rates (SHR), defined as the modulus of the difference between the time on top in the sixth minute and in the first minute;

squares crossed: the number of 10 cm^2 squares crossed by the animal during the entire session;

erratic swimming: the number of "erratic swimming" events, defined as a zig-zag, fast, unpredictable course of swimming of short duration; and

freezing: the total duration of freezing events, defined as complete cessation of move-ments with the exception of eye and operculae movements.

homebase: For the establishment of homebases, the number of visits and time spent in each 10 cm 2 square were calculated and expressed as % of total; a zone qualified as a homebase based on the maximal percentages for individual
animals.

2.3 Light/dark test

Determination of scototaxis was carried as described elsewhere [28,29]. Briefly, animals were transferred to the central compartment of a black and white tank (15 cm X 10 cm X 45 cm h X d X l) for a 3-min. acclimation period, after which the doors which delimit this compartment were removed and the animal was allowed to freely explore the apparatus for 15 min. The following variables were recorded:

time on the white compartment: the time spent in the top third of the tank (percentage of the trial);

squares crossed: the number of 10 cm^2 squares crossed by the animal in the white compartment;

latency to white: the amount of time the animal spends in the black compartment

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before its first entry in the white compartment (s);

entries in white compartment: the number of entries the animal makes in the white compartment in the whole session;

erratic swimming: the number of "erratic swimming" events, defined as a zig-zag, fast, unpredictable course of swimming of short duration; and

freezing: the proportional duration of freezing events (in % of time in the white compartment), defined as complete cessation of movements with the exception of eye and operculae movements.

thigmotaxis: the proportional duration of thigmotaxis events (in % of time in the white compartment), defined as swimming in a distance of 2 cm or less from the white compartment's walls.

risk assessment: the number of "risk assessment" events, defined as a fast (<1 s) entry in the white compartment followed by re-entry in the black compartment, or as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline).

2.4 Novel object exploration test

The novel object task was adapted from Sneddon [30]. Animals were transferred to a 15 x 25 x 20 (width x length x height) tank and allowed to acclimate for 5 minutes. After that period, a novel object (made up of a combination of red, yellow, green, blue and black Lego® Duplo bricks such that the object was no longer than 9 cm in length and 6 cm in height) was slowly lowered into the tank (so as not to startle the fish) and placed at the center of a (previously defined) 10 cm diameter circle at the middle of the tank. A webcam filmed the apparatus from above, and the time spent within that circle and the number of squares crossed were recorded for 10 minutes.

2.5 Nocifensive behavior

To assess behavioral responses to a chemical, inescapable nociceptive stimulus, animals were acclimated to the test tanks (10 cm length X 10 cm width X 20 cm height Plexiglas tanks containing water from the home tank) for 30 min and then individual baseline (pre-treatment) locomotor responses (number of 3 x 3 squares crossed during the session) were monitored for 5 min. Each fish was then individually cold-anesthetized and injected in the anal fin with a

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1% solution of acetic acid. Afterwards, animals were returned to the original test tanks to recover from anesthesia, after which behavioral recording took place. The frequency of tail-beating events, in which the animal vigorously moves its tail but do not propel itself in the water [31], and the change in total locomotion in relation to the baseline [32], were recorded as variables pertaining nocifensive behavior.

2.6 HPLC analysis of monoamines

Serotonin, 5-HIAA, norepinephrine, dopamine, DOPAC, MHPG and 3,4-dihydroxybenzylamine (DHBA) (50 mg) were dissolved in 100 mL of eluting solution ($HClO_4$ 70% [0.2 N], 10 mg EDTA, 9.5 mg sodium metabissulfite) and frozen at -20 °C, to later be used as standards. The HPLC system consisted of a delivery pump (LC20-AT, Shimadzu), a 20 µL sample injector (Rheodyne), a degasser (DGA-20A5), and an analytical column (Shimadzu Shim-Pack VP-ODS, 250 x 4.6 mm internal diameter). The integrating recorder was a Shimadzu CBM-20A (Shimadzu, Kyoto, Japan). An electrochemical detector (Model L-ECD-6A) with glassy carbon was used at a voltage setting of +0.72 V, with a sensitivity set at 2 nA full deflection. The mobile phase consisted of a solution of 70 mM phosphate buffer (pH 2.9), 0.2 mM EDTA, 34.6765 mM SDS, 10% HPLC-grade methanol and 20% sodium metabissulfite as a conservative. The column temperature was set at 17 °C, and the isocratic flow rate was 1.6 ml/min. Brains were dissected on ice-cold (< 4 ºC) magnesium- and calcium-free phosphate-buffered saline (MCF) after sacrifice and homogenized in eluting solution, filtered through a 0.22 µm syringe filter, spiked with 0.22 µl of 2.27 mM DHBA (internal standard) and then injected into the HPLC system.

2.7 Cluster analysis

Raw data was first transformed into Maximum Predictive Values (MPV), following the approach of Linker et al. [33]. Briefly, taking the data from wild-type shortfin animals as reference, for each variable the MPV was calculated as the ratio of the mean difference between two groups and their pooled standard deviations as follows:

$$MPV = \frac{Mean_{Target\ strain} - Mean_{Shortfin}}{\frac{Pooled\ standard\ deviations}{\sqrt{2}}},$$

where

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$$Pooled\ standard\ deviations = \sqrt{(n_{shortfin} - 1 \times Variance_{shortfin}) + \frac{(n_{target\ strain} - 1 \times Variance_{target\ strain})}{n_{shortfin} + n_{target\ strain}}}.$$

Given the mathematical simplicity of these measures, MPV scores were automatically calculated by LibreOffice Calc 3.6.6.2.

These scores represent the intensity (positive or negative) that fish from a given strain displayed a given behavioral endpoint in relation to shortfin fish. Resulting scores were normalized by centering each endpoint around the mean. Hierarchical clustering was then performed across behavioral endpoints and strains ('arrays') with Cluster 3.0 (University of Tokyo, Japan) using uncorrected correlation as clustering method, and single linkage as similarity metric. Clustering results were visualized as a dendrogram and colored “array” in Java TreeView (University of Glasgow, UK).

3. Results

3.1 Novel tank test

nacre and *leo* fish spent less time in the top third of the novel tank than *bsf* zebrafish ($F_{[3, 39]} = 4.133$, $p = 0.0129$; Figure 1A). No differences were observed between *lof* zebrafish and other strains. *lof* zebrafish crossed more squares in the 6-min. session than *bsf* animals ($p < 0.05$), but no effects were observed between any other strain comparisons ($H_{[df = 4]} = 7.622$, $p = 0.05$; Figure 1B). In relation to *lof* zebrafish, but not in relation to other strains, *leo* displayed greater turn frequency ($H_{[df = 4]} = 17.45$, $p = 0.0006$; Figure 1C). *lof* and *nacre* froze more than *bsf*, and *leo* froze less than *lof* ($F_{[3, 39]} = 6.506$, $p = 0.0012$; Figure 1D). Homebase behavior did not differ across strains ($F_{[3, 39]} = 0.9261$, NS; Figure 1E). Habituation scores were higher in *lof* than *bsf*, smaller in *nacre* than *bsf*, and smaller in *nacre* and *leo* than in *lof* ($F_{[3, 39]} = 24.63$, $p < 0.0001$; Figure 1F). With the exception of *nacre*, all strains spent more time on the top in the last 3 min than in the first 3 min ($F_{[3, 72]} = 2.82$, $p = 0.0449$; Figure 1G).

3.2 Light-dark test

leo zebrafish spend less time in the white compartment than *bsf* and *lof* zebrafish; no other strain differences were observed ($F_{[3, 39]} = 12.66$, $p < 0.0001$; Figure 2A). The latency to enter the white compartment ($\chi^2 = 5.495$, NS) or the number of entires in the compartment ($H_{[df = 4]} = 1.414$, NS) did not differ between strains (Figures 2B and 2C). *nacre* and *leo* zebrafish

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showed increased risk assessment in relation to *bsf* and *lof* zebrafish ($H_{[df = 4]} = 16.92$, $p = 0.0007$; Figure 2D). No strain differences were observed in thigmotaxis in the white compartment ($F_{[3, 39]} = 1.116$, NS; Figure 2E). *leo* zebrafish displayed greater turn frequency in the white compartment than *lof* zebrafish ($H_{[df = 4]} = 9.561$, $p = 0.0227$; Figure 2F). Finally, *nacre* zebrafish froze more than *bsf*, *lof* and *leo* zebrafish ($F_{[3, 39]} = 16.20$, $p < 0.0001$; Figure 2G).

3.3 Novel object test

nacre zebrafish spent less time near the novel object than *bsf* ($F_{[3, 39]} = 3.918$, $p = 0.0161$; Figure 3A). Locomotion in the novel object test was not different between strains ($H = 6.406$, NS; Figure 3B).

3.4 Nocifensive behavior

After injection of acetic acid in the tail, *leo* zebrafish displayed more tail-beating than *bsf* and *nacre* animals ($H_{[df = 4]} = 16.30$, $p = 0.001$; Figure 4A). Likewise, *leo* decreased their activity to a greater extent than *bsf* zebrafish after this nociceptive manipulation ($F_{[3, 39]} = 3.193$, $p = 0.035$; Figure 4B).

3.5 Brain monoamines

nacre zebrafish showed higher brain dopamine levels in relation to *bsf* animals ($F_{[3, 11]} = 6.953$, $p = 0.0128$; Figure 5A). No differences were observed in DOPAC levels ($F_{[3, 11]} = 2.602$, NS) or dopamine turnover rates ($F_{[3, 11]} = 3.35$, NS; Figures 5D and 5G). *lof* zebrafish had higher 5-HT levels than other strains, while *leo* had lower levels than all strains ($F_{[3, 11]} = 21.94$, $p = 0.0003$; Figure 5B). *lof* and *leo* had lower 5-HIAA levels than other strains ($F_{[3, 11]} = 7.765$, $p = 0.0094$; Figure 5E), and *leo* had higher serotonin turnover than other strains (F[3, 11] = 12.16, $p = 0.0024$; Figure 5H). *bsf* animals had lower NE levels than *lof* and *leo* animals ($F_{[3, 11]} = 8.198$, $p = 0.008$; Figure 5C), while *leo* had higher MHPG levels than all other strains ($F_{[3, 11]} = 12.06$, $p = 0.0024$; Figure 5F). Finally, *bsf* zebrafish had higher NE turnover rates than *lof* animals ($F_{[3, 11]} = 4.251$, $p = 0.0451$).

3.6 Clustering

Cluster analysis using *lof*, *nacre* and *leo* zebrafish against a *bsf* “reference” produced four

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identifiable behavior clusters (Figure 6), the first including tail-beating, time on top, habituation score, time near novel object and NE levels ($r^2 = 0.579$); the second ("exploration-avoidance") including freezing in the light/dark test, time on white, change in activity after acid injection, number of entries on white and DA and 5-HT levels ($r^2 = 0.741$); the third ("general arousal") including turn frequency in both the novel tank test and the light/dark test, locomotion in the novel tank test, thigmotaxis, risk assessment, latency to white, DOPAC and MHPG levels, and the turnover of all monoamines ($r^2 = 0.767$); and the last ("activity") including time spent on the homebase, locomotion in the novel object test, freezing in the novel tank test, and 5-HIAA levels ($r^2 = 0.930$). *nacre* and *leo* clustered together ($r^2 = -0.243$), with *lof* as outgroup.

4. Discussion

In the present work, *leo* zebrafish have been shown to display increased scototaxis in relation to the other strains, while both *nacre* and *leo* zebrafish show increased geotaxis. Moreover, *leo* displayed increased nocifensive behavior, while *nacre* zebrafish showed increased neophobia in the novel object task. *lof* zebrafish showed decreased turn frequency in both the novel tank and light/dark tests, and habituated faster in the novel tank, as well as displaying increased 5-HT levels. *leo* zebrafish showed decreased brain 5-HT levels and increased 5-HT turnover than other strains, and *nacre* had increased brain DA levels. Finally, specific behavioral endpoints co-varied in terms of the behavioral and neurochemical differences between strains, identifying cross-test domains which included response to novelty, exploration-avoidance, general arousal, and activity.

In both the novel tank test and in the light/dark test, *nacre* zebrafish showed increased freezing. While this behavior is poorly understood in zebrafish, freezing behavior does seem to vary with genetic background. Cachat et al. (2011) observed a small difference between *bsf* and *leo* zebrafish in freezing in the novel tank test, while no differences between *lof* and *leo* zebrafish were observed both in the NTT and the light/dark test [34]. AB zebrafish selected for high freezing in the open field test show increased bottom-dwelling, increased alarm reaction, increased scototaxis and increased latency to feed in both disturbed and undisturbed conditions [19]. In another study, Blaser et al. (2010) demonstrated that zebrafish which consistently avoid the white compartment also freeze more after being confined to the white com-

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partment. Thus, freezing seems to reflect either a fear response or a response to stressful manipulations, and therefore strains which show prominent freezing in the novel tank and light/dark tests could represent 'reactive' strains. Interestingly, the response of adult zebrafish with a mutation in the glucocorticoid receptor (gr^{s357}) after transference to a novel environment is to freeze instead of explore, an effect which is reversed by acute diazepam or subchronic fluoxetine treatment [36]. Likewise, transient knockdown of *tyrosine hydroxylase 1* during development decreases freezing in adult zebrafish exposed to a novel tank [37], suggesting an important role for catecholamines in this response.

Lending credibility to such interpretations is the observation that *nacre* zebrafish also show decreased exploration of novel objects, as well as decreased habituation in the novel tank test and increased risk assessment in the light/dark test. In another study, differences between *nacre* zebrafish and *bsf* were observed in bottom-dwelling [24]. Thus, these animals show exaggerated stress responses to novelty, similarly to gr^{s357} mutants [36,38]. This response does not necessarily result from increased anxiety, as decreased 'novelty-seeking' could also be responsible for these results (Hughes, 1997; Hughes, 2007). A non-selective exaggerated responsiveness to stressors is discarded by the observation that *nacre* display normal nociceptive behavior after acetic acid injection. Thus, this common mutant may represent an important addition in behavioral genetics in the sense that it shows selective responsiveness to novelty, but not to nociceptive or simple anxiogenic stimuli.

In the literature, erratic or burst swimming has been defined as sharp changes in direction or velocity and repeated darting [39] which, in the novel tank test, are increased by 'anxiogenic' manipulations such as morphine withdrawal, alarm substance presentation and caffeine administration [18]; and decreased by acute fluoxetine and 5-HT_{1B} receptor antagonists [40]. These measures commonly, but not necessarily, include the fast turns quantified in the present article as 'turn frequency'. While not necessarily being equivalent to erratic swimming measures reported elsewhere in the literature, turn frequencies are of ecological relevance, because zebrafish can turn against the water current only until the current speed equals their routine maximum swimming speed [41,42]. In the present manuscript, turn frequency was higher in *leo* than in other zebrafish strains in both the novel tank and the light/dark test. Nonetheless, turn frequencies did not differ between *lof* and *bsf* zebrafish, which should be expected if this variable was controlled solely by metabolic and/or biomechanic constraints,

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as observed in routine swimming [41]. These results are also consistent with observations that erratic swimming does not differ between *bsf* and *lof* zebrafish [16], between *bsf* and *leo* [18] or between *lof* and *leo* [34].

From the neurochemical point of view, some observations call attention. First, all strains had higher norepinephrine levels than the reference *bsf*. In the multivariate analysis, NE levels grouped in the first cluster, which included tail-beating in the nocifensive behavior assay, time spent near the novel object, and time on top and habituation in the novel tank test. The third cluster extracted in our analysis shows behavioral endpoints more consistent with generalized arousal, such as turn frequency in both the novel tank test and the light/dark test, locomotion in the novel tank test, thigmotaxis, risk assessment and latency to white; moreover, the metabolites of dopamine and norepinephrine, DOPAC and MHPG, as well as the turnover rates of all neurotransmitters analyzed, clustered in this group. While NE has been proposed to mediate many different behaviors, in zebrafish noradrenergic drugs so far have been shown to modulate arousal [43]. Along with increased responsiveness to sensory stimuli and voluntary motor activity, increased arousal leads to increased emotional reactivity [44,45], and other neurotransmitter systems, including 5-HT [46], have been implicated in zebrafish arousal.

While NErgic neurotransmission was higher in *lof*, *nacre* and *leo*, 5-HT levels were lower in *leo*, which also show increased anxiety-like behavior in the light/dark test and in the novel tank test, as well as increased nocifensive behavior. *leo* also showed increased 5-HT turnover and increased MPHG levels, suggesting increased monoamine oxidase activity. Dopamine levels were altered only in *nacre*, reinforcing the hypothesis of elevated reactivity to novelty in these mutants. DA and 5-HT levels clustered together with change in activity in the nocifensive behavior assay, as well as freezing, time on white, and number of entries on white in the light/dark test. A role for serotonin in this assay has been proposed in zebrafish [34,40,47,48], but so far little is known about the role of this neurotransmitter in fish nociception, nor on the role of dopamine in scototaxis.

The heterogeneous nature of behavioral variation in this paper supports our anterior notion that behavioral tests of 'anxiety' in zebrafish do not necessarily measure the same dimensions [49]. The present results suggest that these tests fall under the aegis of 'domain interplay' [50], with different behavioral endpoints mapping to different behavioral domains. Using a similar approach to cluster analysis presented in this paper, Cachat et al. (2011) demonstrated

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the existence of two major clusters in the novel tank test, the first including (among others) latency to upper half of the tank, freezing and erratic swimming, and the second including time spent in the upper half, distance traveled and average velocity. Importantly, these clusters grouped in relation to the effects of 'anxiolytic' manipulations (which decrease behaviors from the first cluster and increase behavior from the second) and 'anxiogenic' manipulations (with the opposite effect); the latter include animals from the *leo* strain. Moreover, clustering based on habituation rates, instead of anxiety level, produces different results in the same assay [21], suggesting that anxiety and habituation are independent in the NTT. In this latter work, *leo* zebrafish were also shown to habituate freezing faster than *bsf*, but this effect was inversely affected by exposure to an alarm substance or to acute caffeine treatment, and freezing habituation was actually increased by anxiolytic treatments (chronic ethanol, chronic fluoxetine, acute nicotine, chronic morphine).

In conclusion, the present paper demonstrated that common wild-type zebrafish strains differ in their behavior in multiple behavioral assays, suggesting a genetic basis for conflict- and novelty-stress induced behavior, as well as in nocifensive behavior. Moreover, a monoaminergic substrate for these differences has also been described. In general, the identification of the genes and neural substrates underlying the behavioral variation of these common zebrafish mutants could represent important additions to the arsenal of tools to understand the neurogenetics of anxiety disorders.

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Figure 1 – Behavioral differences between zebrafish from the blue shortfin (bsf), longfin (lof), albino (nacre), and leopard (leo) phenotypes in the novel tank test (NTT). (A) Time spent on the top third of the tank in the whole 6-min session; (B) Total number of squares crossed in the 6-min session; (C) Number of turns per minute; (D) Total freezing duration; (E) Time spent in a "homebase"; (F) Single-minute habituation score; (G) Time spent in the top third of the tank in the first 3-min (gray bars) and last 3-min (white bars). Bars represent mean ± standard error. Boxplots represent median ± interquartile range, with Tukey whiskers. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

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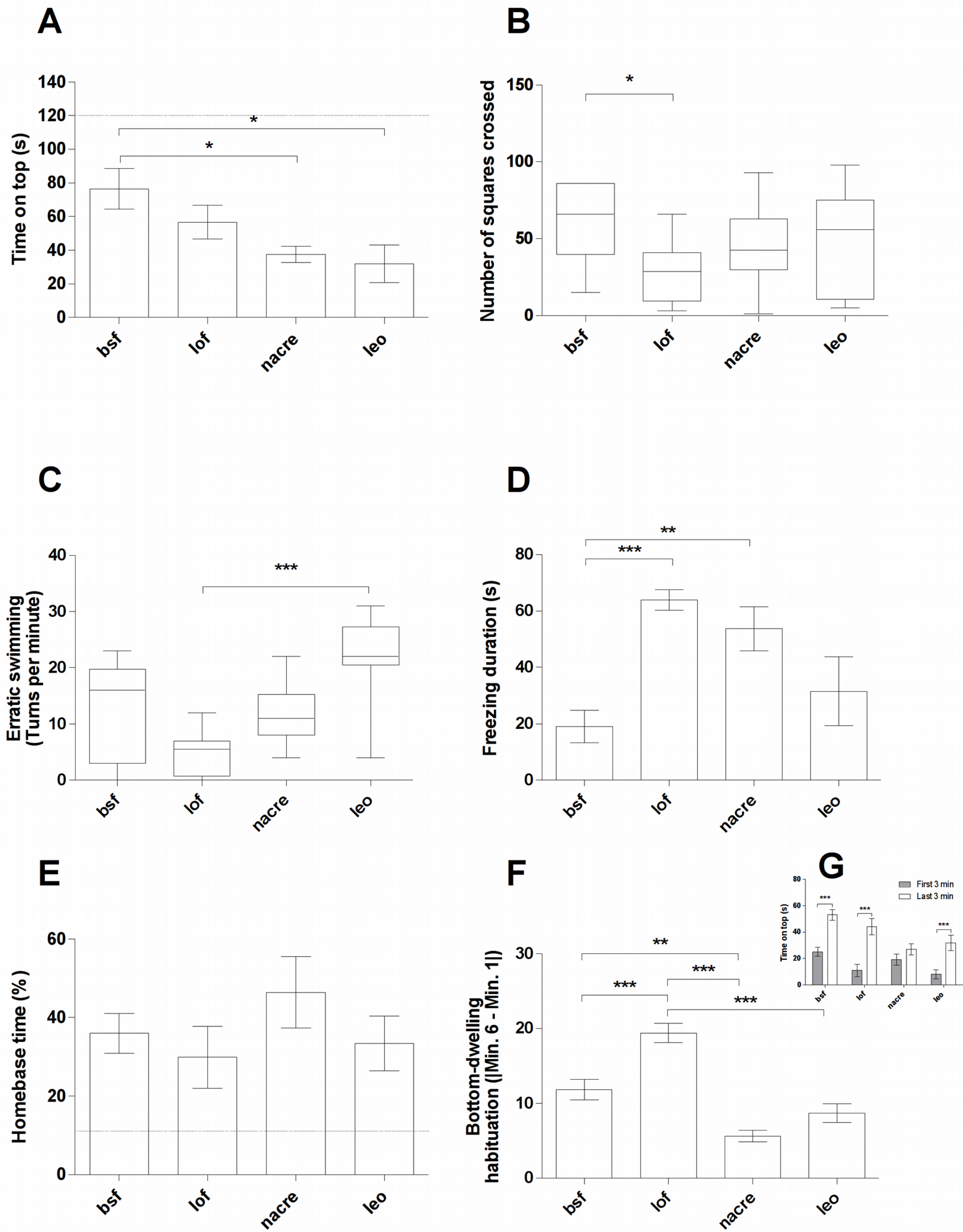

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Figure 2 - Behavioral differences between zebrafish from the blue shortfin (bsf), longfin (lof), albino (nacre), and leopard (leo) phenotypes in the light/dark test (LDT). (A) Time spent on the white compartment; (B) Latency to enter the white compartment; (C) Total number of entries in the white compartment; (D) Total number of risk assessment events; (E) Percent of the time on the white compartment spent in thigmotaxis; (F) Number of turns per minute on the white compartment; (G) Total duration of freezing in the white compartment. Bars represent mean ± standard error. Boxplots represent median ± interquartile range, with Tukey whiskers. Latencies are represented as Kaplan-Meier estimates of time until event. ***, $p < 0.001$; *, $p < 0.05$.

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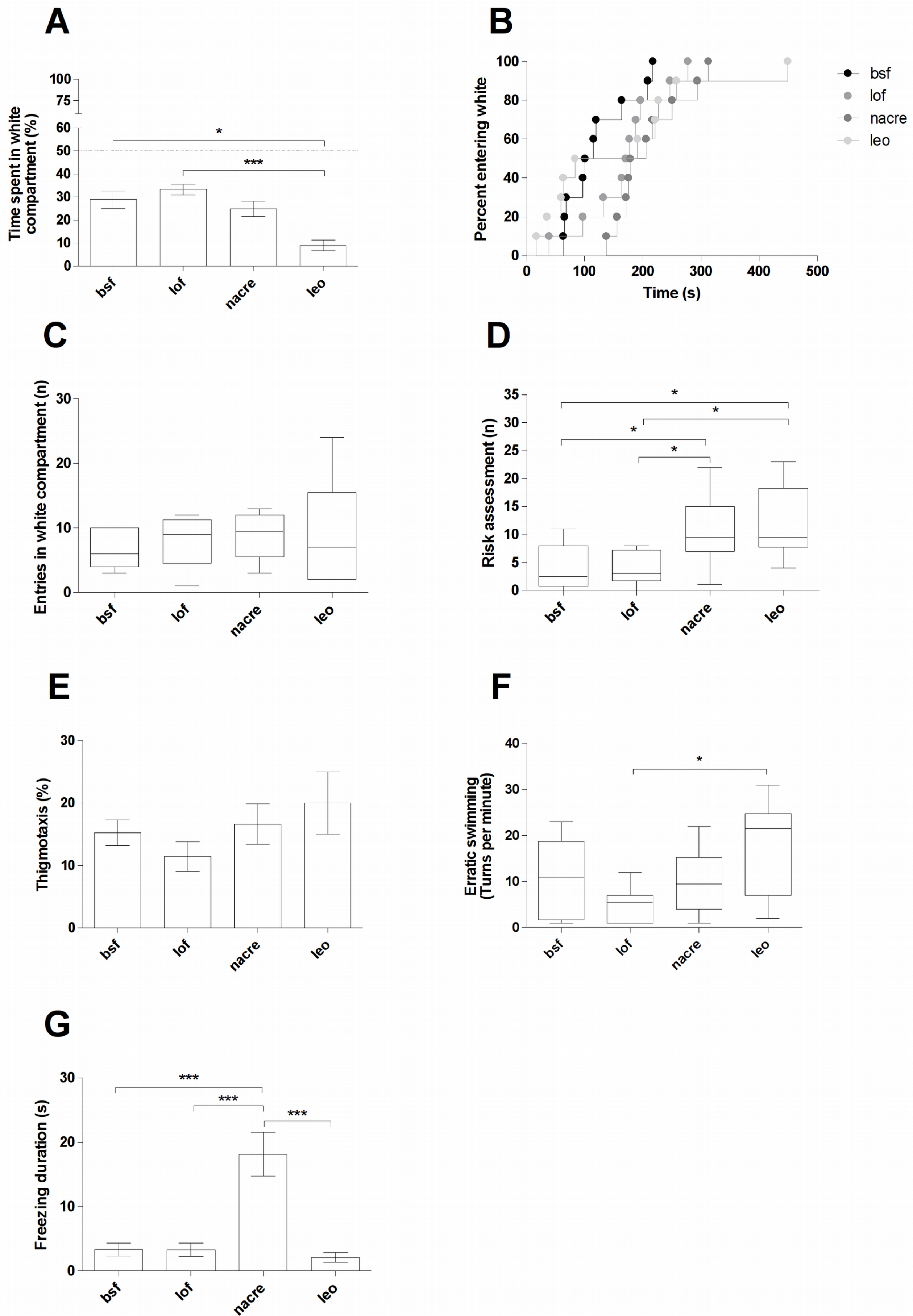

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Figure 3 - Behavioral differences between zebrafish from the blue shortfin (bsf), longfin (lof), albino (nacre), and leopard (leo) phenotypes in the novel object exploration test (NOET). (A) Time spent near the object in the whole 10-min session; (B) Total number of squares crossed in the 10-min session. Bars represent mean ± standard error. Boxplots represent median ± interquartile range, with Tukey whiskers. *, $p < 0.05$.

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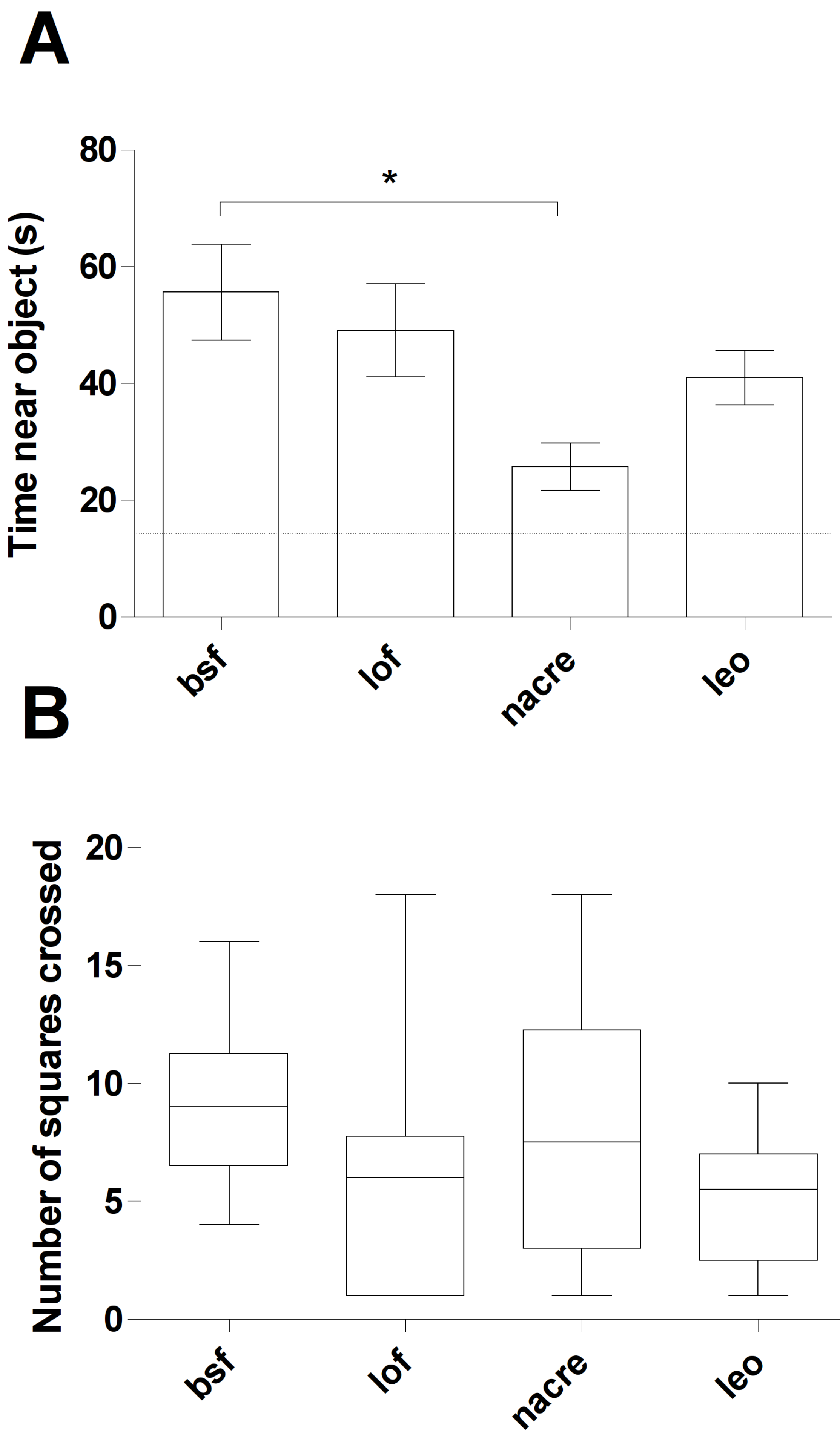

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Figure 4 - Behavioral differences between zebrafish from the blue shortfin (bsf), longfin (lof), albino (nacre), and leopard (leo) phenotypes in the nocifensive behavior assay. (A) Frequency of tail-beating events; (B) Change in baseline activity in relation to pre-injection levels. Bars represent mean ± standard error. Boxplots represent median ± interquartile range, with Tukey whiskers. **, $p < 0.01$; *, $p < 0.05$.

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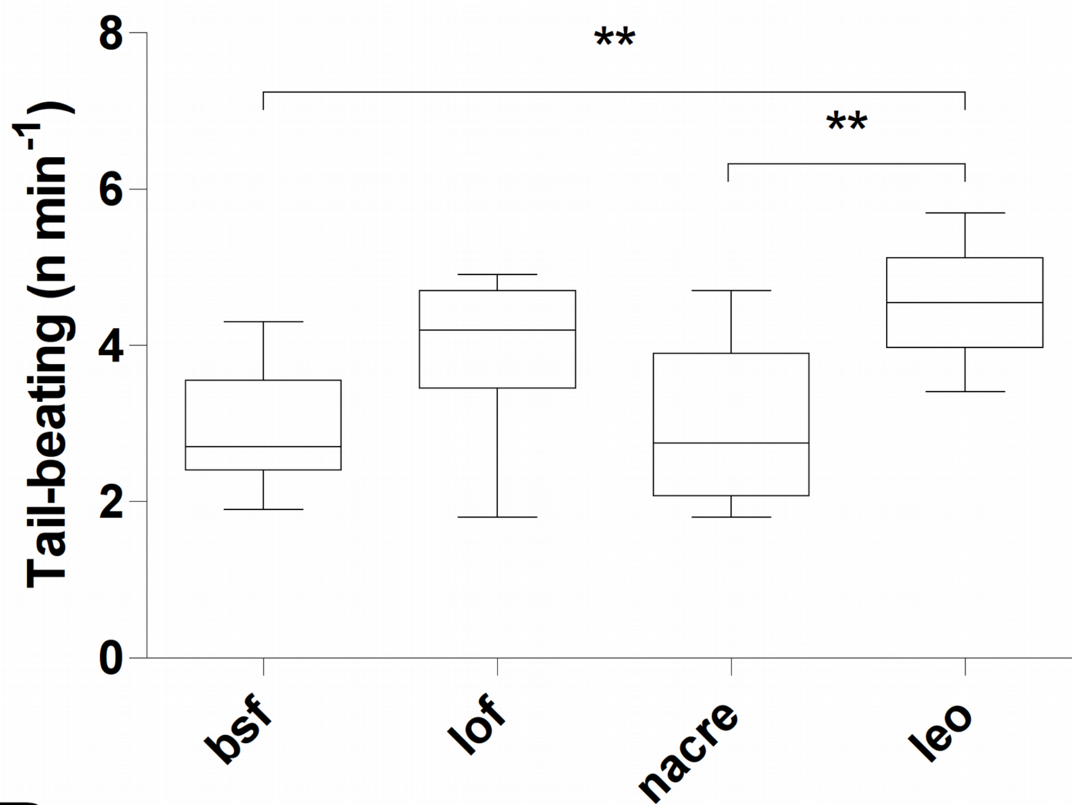

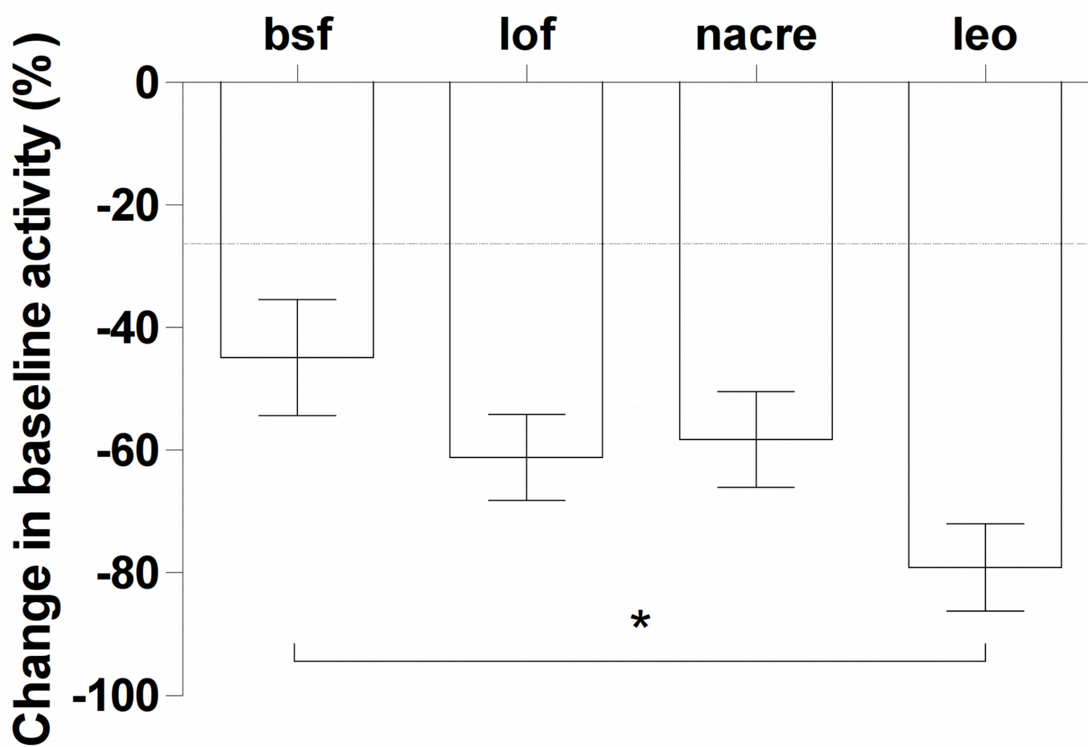

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Figure 5 – Monoamine levels in the brains of zebrafish from the blue shortfin (BSF), longfin (lof), albino (nacre), and leopard (leo) phenotypes. (A) Dopamine (DA) levels; (B) DOPAC levels; (C) Dopamine turnover (DOPAC:Dopamine ratios); (D) Serotonin (5-HT) levels; (E) 5-HIAA levels; (F) Serotonin turnover (5-HIAA:5-HT ratios); (G) Norepinephrine (NE) levels); (H) MHPG levels; (I) Norepinephrine turnover (MHPG:NE ratios). Bars represent mean ± standard error. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

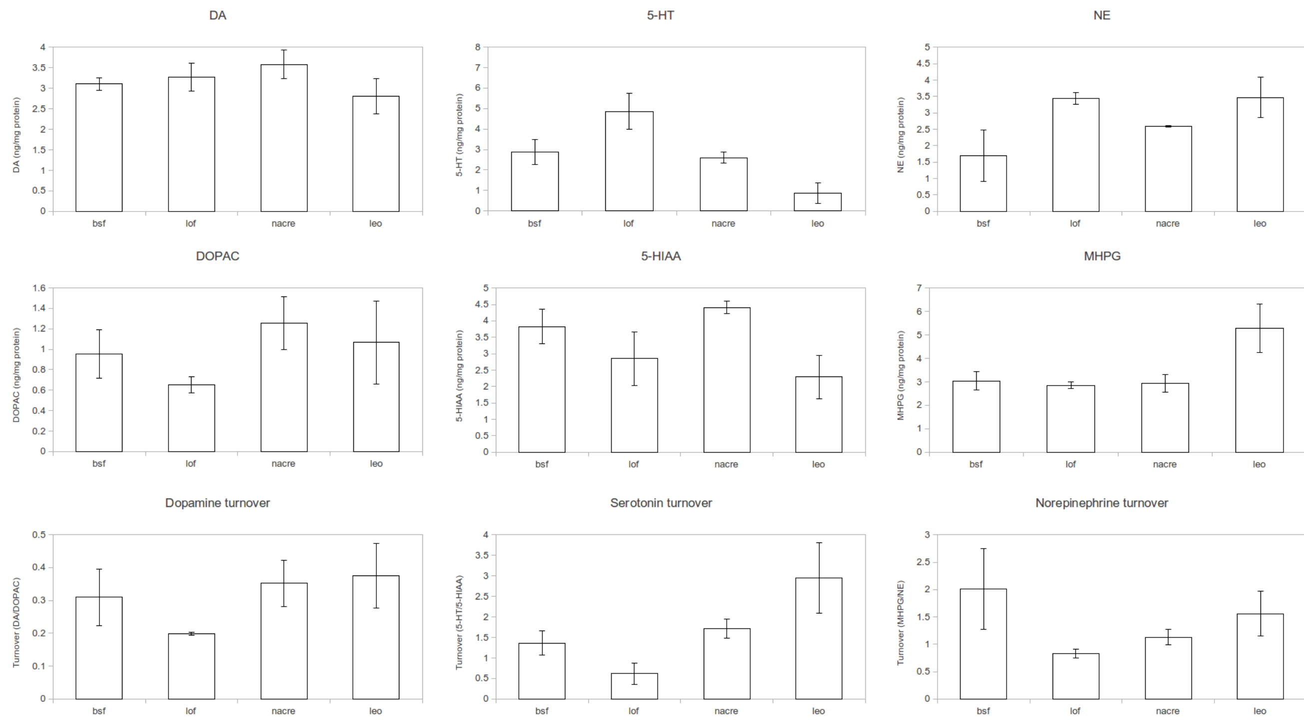

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Figure 6 – Hierarchical clustering of behavioral and neurochemical variables (rows) vs. phenotypes (columns). Clustering was made by calculating Maximum Predictive Values in relation to a reference phenotype (blue shortfin).