Supplementary information for the article:

**Brain dysfunction during warming is linked to oxygen limitation in larval zebrafish**

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**Script for statistical analyses, figures, and the corresponding data:**
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This supplementary file includes

- Supplementary figures (Figure S1-S4)
- Supplementary movies (Movie S1-S3)
- Supplementary statistical analyses
- Supplementary tables (Table S1-S10)
Supplementary figures (Figure S1-S4)

Supplementary Figure S1. Behavioural responses to heat ramping.

A. Average swimming speed of five-day-old control (n = 14, empty circles) and heat ramp fish (n = 12, black circles) as a function of temperature. The swimming speed decreased from 2.9 ± 0.4 to 1.7 ± 0.4 mm/s during the assay. There was no difference in the change in swimming speed between the control and the heat ramp treatment (linear mixed-effects model, time (end): $\beta \pm$ S.E = -1.2 ± 0.4 mm/s, t(100) = -2.4, p = 0.015; $R^2$ = 0.1, see Table S1 for full model output and model including the effect of treatment).

B. Proportion of spiral swimming events (black), and loss of equilibrium (grey) in control (dashed) and heat ramp fish (solid).

C. Number of spiral swimming events (see Methods and Movie S1) recorded during the 12 minutes preceding CT max for five-day-old heat ramp fish (black circles, n = 14), or during the corresponding period in the control fish (empty circles, n = 12; Chi-squared test: $X^2_{(df=1;N=26)} = 15.6$, p $<$ 0.001, Table S2).

D. Loss of equilibrium (% time, see Methods and Movie S2) in heat ramp and control fish during the same period as in C (Wilcoxon Rank Sum test: $r = 0.58$, p = 0.004, Table S2). The bars and error bars indicate the group mean and S.E. in A. and C, and median and inter quartile range in D. E. Frequency of individuals that reached the loss of response criterion showing that none of the control fish (n = 14, white bar) and all the heat ramp fish (n = 12, black bar) lost escape response.
Supplementary Figure S2. Frequency of calcium peaks during heat ramping over temperature

A. Frequency of medulla calcium peaks in heat ramp fish (n = 11, same data as displayed in Figure 2F) aligned to temperature during the heat ramping. The data is displayed as average (black line) and S.E. (shaded area). B. Frequency of medulla calcium peaks in laterally mounted heat ramp fish (n = 9, corresponding to Figure 4). The higher medullar activity compared to A can be attributed to startle due to the blue excitation light being turned on at the beginning of each short recordings in B, whereas the light was continuously on during A.

Supplementary Figure S3. Effect of oxygen level on the temporal dynamics of the brain-wide depolarization

Effects of oxygen availability on medulla activity and the temporal dynamics of the brain wide depolarization. A. Change in brain fluorescence aligned to the peak depolarization in hypoxia (n = 14), normoxia (n = 12) and hyperoxia (n = 14). B. Amplitude of the brain-wide depolarization in hypoxia (n = 14), normoxia (n = 12) and hyperoxia (n = 14; Table S8). C. Recovery time between peak depolarization and the return to baseline fluorescence in hypoxia (n = 14), normoxia (n = 12) and hyperoxia (n = 13; Table S8). D. Frequency of brainstem calcium events recorded during the 30 minutes preceding the brain-wide depolarization in hypoxia (n = 14), normoxia (n = 12) and hyperoxia (n = 14). A-D. Results for hypoxia (60 %, magenta), normoxia (100 %, black) and hyperoxia (150 %, cyan) are presented with mean and S.E. (A, D: solid lines and shaded area; B, C: bars and error bars;).
Supplementary Figure S4. Sensory response in the optic tectum during warming at different oxygen levels.

In A, the amplitude of the optic tectum response (ΔF/F₀) is aligned on temperature during the heat ramping, and in B, it is aligned time before depolarization. C. Proportion of responses to light flashes in the optic tectum during heat ramping aligned on time at depolarization (same data as in Figure 6C). A-C. Results for hypoxia (60 %, magenta), normoxia (100 %, black) and hyperoxia (150 %, cyan) are presented with mean (solid lines) and S.E. (shaded area).

Supplementary movies (Movie S1-S3)

Supplementary Movie S1. Spiral swimming in a five-day-old zebrafish heat ramp larva. Speed 1X.

Supplementary Movie S2. Loss of equilibrium in the same heat ramp larva as in Supplementary Movie 1. Speed 1X.

Supplementary Movie S3. Representative illustration of the depolarization spreading in the brain of a five-day-old Tg(elavl3:GCaMP6s) larva exposed to a heat ramp (same fish as in Figure 2E). Speed 1X.

Supplementary statistical analyses

CTₘₐₓ and behavioural response to heat ramping

To analyse the swimming velocity during heat ramping and in the control group a linear mixed-effects model was performed with treatment and time step as predictor variables and the fish identity as random factor (Table S1). The proportion of spiral swimming events was analysed using a Chi-square test to handle the lack of variance in the control group (Table S2). A Wilcoxon rank sum test was used to test the treatment effect on loss of equilibrium (Table S2). To test the difference in CTₘₐₓ throughout development, a linear regression model was used with CTₘₐₓ as a function of the age. The CTₘₐₓ value...
of one adult fish was considered an extreme outlier and results from models with and without this fish are included in the supplements (Table S3).

Change in frequency of calcium peaks with temperature
The effect of heat ramping on the frequency of calcium peaks averaged from the medullae in both brain halves was analysed with a linear mixed-effects model with fish identity as a random factor. Temperature was standardized so that the start of the trial was in the intercept of the model (42 minutes before depolarization) (Table S4).

Temperature of CTmax and depolarization
The temperature at which depolarization and CTmax occurred in freely swimming fish was compared with a mixed-effects model with temperature as response variable, a categorical fixed effect of response type (CTmax or depolarization) and fish identity as a random factor (Table S5).

Change in cardiac function during heat ramping
Effect of temperature on heart rate was tested with a linear mixed-effects model where fish identity was defined as a random factor. The model was releveled to check for differences between the different temperature steps and the p-value significance criterion was accordingly reduced to p<0.001 for this analysis (Table S6).

Effect of oxygen level on CTmax and the brain-wide depolarization
The effect of oxygen level on CTmax was analysed with an ANOVA testing the effect of the treatments normoxia, hypoxia and hyperoxia on CTmax. Two outliers were removed from the hyperoxia group and one from the hypoxia (< Q1-3 x IQR). The model results are presented with and without the outliers (Table S7). Experiment was included as a factor in initial models to test if there was an effect of the two data collection periods. The effect of oxygen on depolarization onset, amplitude and recovery time was assessed using the same approach (Table S8).

Effect of oxygen level on neural activity
The frequency of calcium peaks during heat ramping averaged across the two medullae was analysed with linear mixed-effects model with an interaction between temperature and treatment and fish identity as a random factor (Table S9). Temperature was included with quadratic and cubic effects to model the different treatment groups response to temperature, and temperature was standardized so that the starting temperature (28 °C) was in the intercept. The neural response to light stimuli in the optic tecti were tested using a mixed-effects model with binomial data (response, no response) and the logit link function (Table S10). During initial observations of the data, complex relationships between the effects of temperature, oxygen and their interaction were detected. Temperature was therefore centered around the mean temperature to model these effects. Fish identity was included as a random factor.
Supplementary tables (Table S1-S10)

Supplementary Table S1. Linear mixed-effects model on swim speed
Results of mixed-effects model for swim speed in larval zebrafish (Figure S1A) during heat ramping (0.3°C/min) and over an equally long period for control fish (without heat ramping), including fish identity as a random factor. The models include the effect of five time-intervals (activity measured for approximately 3 minutes at five intervals during the trial) and the second model includes the effect of treatment (control or heat ramp). The last measurement for each fish (Time 5) corresponds to the one-degree elevation preceding CT_{max} for the heat ramp fish. The first measurement (Time 1) is in the intercept (and control treatment for the second model) and the units of the estimates are in mm/s.

| Parameter         | Swim speed model | Model including treatment |
|-------------------|------------------|---------------------------|
|                   | Estimate (β)     | S.E. | t-value | p-value | Estimate (β) | S.E. | t-value | p-value |
| Intercept (Time 1)| 2.90             | 0.44 | 6.62    | <0.001  | 2.55         | 0.52 | 4.88    | <0.001  |
| Time 2            | -0.59            | 0.48 | -1.23   | 0.222   | -0.59        | 0.48 | -1.23   | 0.222   |
| Time 3            | 0.08             | 0.48 | 0.18    | 0.861   | 0.08         | 0.48 | 0.18    | 0.861   |
| Time 4            | -0.99            | 0.48 | -2.05   | 0.043   | -0.99        | 0.48 | -2.05   | 0.043   |
| Time 5            | -1.18            | 0.48 | -2.43   | 0.017   | -1.18        | 0.48 | -2.43   | 0.017   |
| Treatment         |                  |      |         |         | 0.76         | 0.62 | 1.22    | 0.233   |

Random Effects

| Parameter | Estimate | S.E. |
|-----------|----------|------|
| σ²        | 3.04     |      |
| τ₀₀       | 1.94 FishID | 1.89 FishID |
| N         | 26 FishID | 26 FishID |
| Observations | 130       | 130   |
| Marginal R² / Conditional R² | 0.050 / 0.420 | 0.076 / 0.430 |

Supplementary Table S2. Model results for spiral swimming events and loss of equilibrium.
Comparisons between the two treatments (heat ramp and control) was done using a Chi-squared test on the proportion of individuals in each treatment that experienced spiral swimming events during the last 12 minutes preceding CT_{max} (0 out of 14 control fish, and 10 out of 12 heat ramp fish). The percentage of time during the same period that the individuals experienced loss of equilibrium was compared with a Wilcoxon rank sum test.

| Parameter | Spiral swimming | Loss of equilibrium |
|-----------|-----------------|---------------------|
|           | X²   | df | p-value | W | Estimate (r) | p-value |
| Treatment | 15.6 | 1  | <0.001  | 27 | 0.58         | 0.004  |

N = 26: n_{control} = 14, n_{heat ramp} = 12
Supplementary Table S3. ANOVA testing the effect of age on CT<sub>max</sub>.
Results for the statistical test for CT<sub>max</sub> in five-day-old, nine-day-old, and adult zebrafish (Figure 1C) testing the effect of life-stage on the upper thermal limit. Model 1 (<i>F</i>(2,37) = 2.73, <i>p</i> = 0.08) is including all fish whereas in Model 2 (<i>F</i>(2,36) = 1.89, <i>p</i> = 0.17) one extreme outlier (<i>Q1-3 x IQR</i>) was removed from the adult group. The five-day-old group is in the intercept and units are in °C.

| Parameter | Model 1 | Model 2 |
|-----------|---------|---------|
|           | Estimate (β) | S.E. | t-value | p-value | Estimate (β) | S.E. | t-value | p-value |
| Intercept (5dpf) | 41.36 | 0.19 | 219.54 | <0.001 | 41.36 | 0.17 | 250.19 | <0.001 |
| Age (9dpf) | -0.10 | 0.27 | -0.39 | 0.698 | -0.10 | 0.23 | -0.45 | 0.659 |
| Age (Adult) | -0.54 | 0.25 | -2.16 | 0.038 | -0.41 | 0.22 | -1.84 | 0.073 |

Observations 40 39
R<sup>2</sup> / R<sup>2</sup> adjusted 0.129 / 0.082 0.095 / 0.045

Supplementary Table S4. Linear mixed-effects model on the frequency of calcium events during warming.
Results on the effect of heat ramping on the average frequency of calcium peaks from the two medullae of larvae zebrafish. The time is standardized so that start of the measuring period is in the intercept. The Time effect is given per minute approaching the depolarization (Figure 2F) and includes a quadratic effect to model the thermal effects on the frequency of calcium peaks.

| Parameter | Frequency of calcium peaks (min) |
|-----------|----------------------------------|
|           | Estimate (β) | S.E. | t-value | p-value |
| Intercept (Control) | 1.52 | 0.28 | 5.52 | <0.001 |
| Treatment (Heat ramp) | -0.40 | 0.36 | -1.09 | 0.281 |
| Time | 0.02 | 0.02 | 0.86 | 0.392 |
| Time<sup>2</sup> | -0.00 | 0.00 | -1.10 | 0.274 |
| Time * Heatramp | 0.23 | 0.03 | 8.00 | <0.001 |
| Time<sup>2</sup> * Heatramp | -0.01 | 0.00 | -8.81 | <0.001 |

Random Effects
| Parameter | Estimate |
|-----------|----------|
| σ<sup>2</sup> | 0.53 |
| τ<sub>00 FishID</sub> | 0.35 |
| N<sub>FishID</sub> | 19 |

Observations 266
Marginal R<sup>2</sup> / Conditional R<sup>2</sup> 0.413 / 0.646
Supplementary Table S5. Mixed-effects model comparing CT$_{\text{max}}$ and depolarization temperature. 
Results of the statistical test comparing the CT$_{\text{max}}$ and brain-wide depolarization temperatures (Figure 3D). The mixed-effects model includes fish identity as a random factor. CT$_{\text{max}}$ is in the intercept and units are in °C.

| Parameter | Estimate (β) | S.E. | t-value | p-value |
|-----------|--------------|------|---------|---------|
| Intercept (CT$_{\text{max}}$) | 40.86 | 0.17 | 240.01 | <0.001 |
| Brain-wide depolarization | 0.50 | 0.11 | 4.49 | 0.004 |

Random Effects

|  |  
|---|---|
| $\sigma^2$ | 0.04 |
| $\tau_{\text{0 FishID}}$ | 0.16 |
| $N_{\text{FishID}}$ | 7 |

Observations: 14
Marginal R$^2$ / Conditional R$^2$: 0.249 / 0.840

Supplementary Table S6. Mixed-effects model on the effect of warming on heart rate. 
Results of the statistical test for the effect of increasing temperature on heart rate in larval zebrafish (Figure 4C) averaged over 1 min intervals during heat ramping. The mixed-effects model includes fish identity as a random factor. The first temperature interval is in the intercept and the units are in Hz.

| Parameter | Estimate (β) | S.E. | t-value | p-value |
|-----------|--------------|------|---------|---------|
| Intercept (28°C) | 4.39 | 0.16 | 27.00 | <0.001 |
| Temperature (31°C) | 0.91 | 0.19 | 4.91 | <0.001 |
| Temperature (34°C) | 1.27 | 0.19 | 6.88 | <0.001 |
| Temperature (37°C) | 0.36 | 0.19 | 1.90 | 0.065 |
| Temperature (Depolarization) | 0.26 | 0.19 | 1.40 | 0.170 |
| Temperature (After) | -1.38 | 0.20 | -6.98 | <0.001 |

Random Effects

|  |  
|---|---|
| $\sigma^2$ | 0.14 |
| $\tau_{\text{0 FishID}}$ | 0.07 |
| $N_{\text{FishID}}$ | 9 |

Observations: 50
Marginal R$^2$ / Conditional R$^2$: 0.753 / 0.836
Supplementary Table S7. ANOVA testing the effect of oxygen availability on CT_{max}.
Results of the statistical test for CT_{max} in hypoxia, normoxia and hyperoxia in larval zebrafish testing the effect of oxygen level on the upper thermal limit and the effect of experimental period (Figure 5A). Model 1 (F_{(3,72)} = 1.68, p=0.18) is including all fish, whereas Model 2 (F_{(3,69)} = 7.99, p<0.001) is excluding three extreme outliers (< Q1-3 x IQR), two from the hyperoxia group and one from the hypoxia group. The final model, Model 2.2 (F_{(2,78)} = 11.67, p<0.001), is without Experiment as a fixed factor. The normoxia treatment is in the intercept and units for the estimates are in °C.

| Parameter          | Model 1     | Model 2.1    | Model 2.2   |
|--------------------|-------------|--------------|-------------|
|                    | Estimate (β) | S.E.         | t-value     | p-value | Estimate (β) | S.E.         | t-value     | p-value | Estimate (β) | S.E.         | t-value     | p-value |
| Intercept (Control)| 39.59       | 0.39         | 101.85      | <0.001   | 39.81       | 0.24         | 164.70      | <0.001   | 39.65       | 0.14         | 282.63      | <0.001   |
| Hypoxia            | -0.64       | 0.39         | -1.66       | 0.102    | -0.68       | 0.24         | -2.82       | 0.006    | -0.58       | 0.21         | -2.79       | 0.007    |
| Hyperoxia          | 0.02        | 0.39         | 0.04        | 0.968    | 0.40        | 0.24         | 1.68        | 0.098    | 0.49        | 0.21         | 2.29        | 0.025    |
| Experiment         | 0.06        | 0.34         | 0.17        | 0.862    | -0.18       | 0.21         | -0.85       | 0.399    |             |             |             |          |
| Observations       | 76          | 73           | 73          |          |             |             |             |          |
| R² / R² adjusted   | 0.066 / 0.027| 0.258 / 0.226| 0.250 / 0.229|          |             |             |             |          |

Supplementary Table S8. Effect of oxygen availability on depolarization onset, amplitude, and recovery.
Results of the ANOVAs on 1) brain-wide depolarization onset (F_{(3,36)} = 45.5, p<0.001), 2) amplitude of depolarization (F_{(3,36)} = 7.45, p<0.001), and 3) recovery time after depolarization(F_{(3,35)} = 22.83, p<0.001), in normoxia, hypoxia and hyperoxia in larval zebrafish (Figure 5B, Figure S3B, C) testing the effect of oxygen level on attributes of the brain-wide depolarization. This experiment was conducted in two replicates (different time of year and experimenter and Experiment is included as a fixed effect. Importantly, the treatment effect on depolarization temperature remained strong across the experiments. The normoxic treatment (control) is in the intercept and units are in °C, % ΔF/ΔF₀, and minutes, respectively.

| Parameter          | Depolarization onset | Amplitude of depolarization | Recovery time |
|--------------------|-----------------------|-----------------------------|---------------|
|                    | Estimate (β)         | S.E. | t-value | p-value | Estimate (β) | S.E. | t-value | p-value | Estimate (β) | S.E. | t-value | p-value |
| Intercept (Control)| 41.03                 | 0.33 | 125.85 | <0.001 | 79.48        | 12.35 | 6.43    | <0.001 | 2.54         | 0.37 | 6.95    | <0.001 |
| Hypoxia            | -2.69                 | 0.39 | -6.90  | <0.001 | 14.88        | 14.80 | 1.00    | 0.322  | 2.57         | 0.44 | 5.86    | <0.001 |
| Hyperoxia          | 1.22                  | 0.39 | 3.13   | 0.003  | -10.60       | 14.73 | -0.72   | 0.476  | -0.71        | 0.44 | -1.60   | 0.119  |
| Experiment(2)      | -1.76                 | 0.32 | -5.55  | <0.001 | 53.71        | 12.04 | 4.46    | <0.001 | -0.35        | 0.36 | -0.97   | 0.341  |
| Observations       | 40                    | 40   | 39     |        |             |       |         |        |             |       |         |        |
| R² / R² adjusted   | 0.791 / 0.774         | 0.383 / 0.332               | 0.662 / 0.633 |
Supplementary Table S9. Mixed model testing the effect of oxygen level on neural activity during warming. Results of the statistical test for frequency of medulla calcium peaks during heat ramping in larval zebrafish (Figure 5D) testing the effect of oxygen level (normoxia, hypoxia and hyperoxia) on neural activity. Fish identity is included as a random factor. The model includes temperature with quadratic and cubic effects to model the thermal effects, and an interaction between treatment and temperature. The temperature is standardized so that 28 °C (starting temperature) is in the intercept of the model. The estimates are the average frequency of peaks between the two medullae.

| Parameter                                      | Estimate (β) | S.E. | t-value | p-value |
|------------------------------------------------|--------------|------|---------|---------|
| Intercept (Control)                            | 1.67         | 0.21 | 8.02    | <0.001  |
| Treatment (Low)                                | 0.46         | 0.26 | 1.75    | 0.085   |
| Treatment (High)                               | 0.07         | 0.26 | 0.28    | 0.782   |
| Temperature (standardized)                     | -0.19        | 0.08 | -2.50   | 0.013   |
| Temperature (standardized)^2                   | 0.08         | 0.01 | 5.64    | <0.001  |
| Temperature (standardized)^3                   | -0.01        | 0.00 | -7.87   | <0.001  |
| Treatment (Low)^*Temperature (standardized)    | -0.16        | 0.03 | -5.66   | <0.001  |
| Treatment (High)^*Temperature (standardized)   | 0.09         | 0.02 | 3.75    | <0.001  |

Random Effects

|                |              |
|----------------|--------------|
| \( \sigma^2 \) | 0.49         |
| \( \sigma_{\text{FishID}} \) | 0.23 |
| \( N_{\text{FishID}} \)      | 33           |

Observations 391

Marginal \( R^2 \) / Conditional \( R^2 \) 0.318 / 0.533
Supplementary Table S10. Binomial mixed model on the neural responses to visual stimuli during warming.

Effect of oxygen availability on optic tectum response to light stimuli during heat ramping with water oxygen level (normoxia, hypoxia and hyperoxia; Figure 6C). Binomial data (response, no response) was tested in a mixed model and the results are on logit scale. The results can be interpreted in terms of probability of responding to light by using the inverse of the logit function ($\text{logit}^{-1}=\frac{e^x}{1+e^x}$). At mean temperature control group had 26 % probability of responding, high had 37 % and low had half of control (13%) (calculated using the inverse logit function).

| Parameter                                      | Estimate (β) | S.E. | t-value | p-value |
|-----------------------------------------------|--------------|------|---------|---------|
| Intercept (Control)                           | -1.04        | 0.13 | -7.68   | <0.001  |
| Treatment (Low)                               | -0.85        | 0.22 | -3.92   | <0.001  |
| Treatment (High)                              | 0.49         | 0.18 | 2.67    | 0.008   |
| Temperature (centered)                        | -0.13        | 0.02 | -5.28   | <0.001  |
| Temperature (centered)$^2$                    | -0.02        | 0.01 | -2.35   | 0.019   |
| Treatment (Low)$^*$Temperature (centered)     | 0.06         | 0.05 | 1.20    | 0.230   |
| Treatment (High)$^*$Temperature (centered)    | 0.07         | 0.03 | 2.17    | 0.030   |
| Treatment (Low)$^*$Temperature (centered)$^2$ | 0.04         | 0.01 | 3.01    | 0.003   |
| Treatment (High)$^*$Temperature (centered)$^2$| 0.00         | 0.01 | 0.53    | 0.598   |

Random Effects

| $\sigma^2$ | 3.29 |
| $r_{00}$ FishID | 0.06 |
| N FishID | 38 |

Observations 2763

Marginal R$^2$ / Conditional R$^2$ 0.080 / 0.083