

Rearing temperatures have lasting effects on the somatic development and swimming kinematics of larval zebrafish

By

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Abstract:

In ectothermic species such as the zebrafish (*Danio rerio*), rearing temperature is known to affect a host of physiological and behavioral processes. In the present study, we set out to disentangle the effects of somatic size, rearing temperature, and nervous system development on wild-type zebrafish. We first demonstrated that somatic sizes of zebrafish reared at 25°C, 28°C, and 32°C converge at 6-7 days post-fertilization (dpf). Observed differences in swimming at this age are therefore more likely to be attributed to lasting differences in the nervous system – which generates signals responsible for swimming – as opposed to differences in somatic size. Subsequent analyses of fish at these ages revealed lasting impacts of rearing temperature on several kinematic parameters including bout displacement (mm), bout duration (s), and swim rate (Hz). In particular, relatively higher swimming rates and higher bout displacements at night were observed in 32C fish when compared to lower temperature counterparts. Therefore, our results suggest an isolatable circadian swimming deficit in larvae reared at high temperatures.

Introduction:

Homeostasis, broadly defined as the maintenance of a stable internal environment, is a keystone concept in the physiology, behavior, and development of living things. Of the abiotic factors implicated in homeostatic processes, temperature is especially relevant to the Zebrafish (*Danio rerio*), a model organism used to investigate questions of physiology, behavior, and development owing to their small size, low cost, and transparency in early developmental stages (1). In particular, Zebrafish are ectotherms in that the regulation of their internal body temperature depends largely on the surrounding aquatic environment (2). A plethora of previous studies have demonstrated that rearing temperatures have broad effects that span somatic, behavioral, and nervous system development. Their findings will be briefly discussed below.

Somatic Size:

With regard to the effect of chronic rearing temperature on somatic size, Schmidt and Starck found significant differences in the length of zebrafish embryos raised at 24°C, 27°C, and 30°C between 10-26 hours-post fertilization (hpf). These size measurements were obtained in intact zebrafish embryos prior to hatching (Fig 1) (3). The precise temperature values selected for cool, control, and warm experimental conditions were motivated by the observation in a seminal paper by Kimmel et. al 1995 that found sharp increases in mortality occurring for temperatures outside of 25-33°C (5). Their results suggest temperature has a broad effect on the rate of somatic development for zebrafish in the embryonic stage.

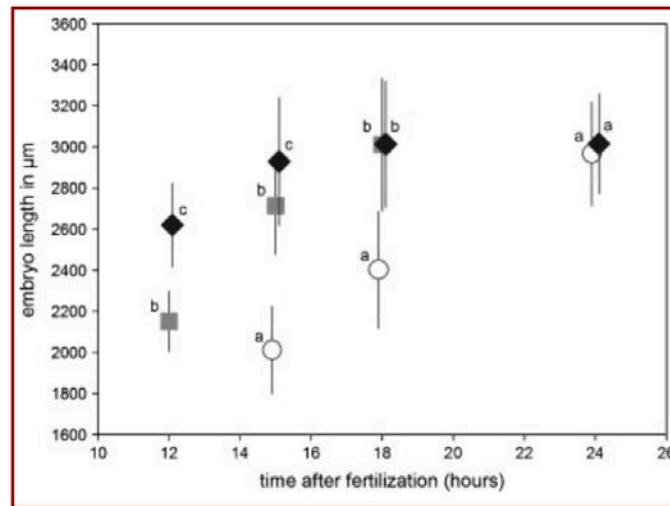


Figure 1: Temperature dependence of somatic size in embryonic (10-26 hpf) zebrafish.

White circles correspond to embryos raised at 24°C, grey squares correspond to embryos raised at 27°C, and black diamonds correspond to embryos raised at 32°C. Significant differences in sizes are indicated through the use of different letters. Excerpted from Schmidt, K. and Starck, J.M. (2010), Developmental plasticity, modularity, and heterochrony during the phylotypic stage of the zebra fish, *Danio rerio*. *J. Exp. Zool.*, 314B: 166-178.

In a separate study, Schnurr and Scott sought to quantify the effect of early temperature perturbation in the embryonic phase on the resulting somatic phenotype in zebrafish. They initially raised zebrafish embryos at 22°C, 27°C, and 32°C respectively until the embryos hatched. At that point, larvae were transferred to a controlled 27°C environment for the remainder of their lifespan. The results of this study are summarized in Fig. 2. Significant differences were observed between fish raised at these temperature groups at 56, 84, and 140 days post-fertilization (dpf) (4). These data, in tandem with the results of several enzymatic assays, suggest that the effects of acute, early temperature perturbation (i.e. in the embryonic stage) have consequences on somatic and metabolic development at time points far beyond the conclusion of the temperature perturbation.

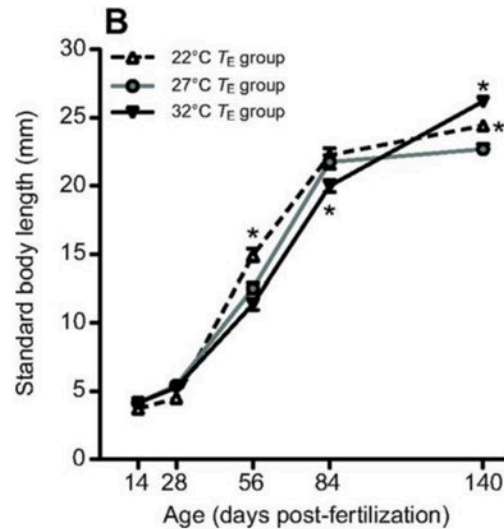


Figure 2: Effect of early temperature perturbation on somatic size in larval, juvenile, and adult zebrafish. Excerpted from Schnurr, M. E., Yin, Y., & Scott, G. R. (2014). Temperature during embryonic development has persistent effects on metabolic enzymes in the muscle of zebrafish. *The Journal of Experimental Biology*, 217(Pt 8), 1370–1380. <https://doi.org/10.1242/jeb.094037>

The effect of *chronic* temperature perturbation on the somatic development of the zebrafish, however, is relatively unknown and has not been adequately investigated in the literature. Moreover, to the best of our knowledge, there has not been literature that has surveyed the temperature dependence of somatic size between 2-14 dpf. To address these gaps in the literature, we elected to quantify and track the somatic development of fish reared at 25°C, 28°C, and 32°C under a *chronic* rearing condition. We hypothesized that fish reared at higher temperatures would have a generally higher rate of somatic growth, with fish raised at higher temperatures measuring longer than their lower temperature counterparts over the course of this developmental timeframe.

Swimming Kinematics:

In the larval stage, zebrafish swim in discrete bouts interspersed with near-pauses. In particular, larval zebrafish are front-heavy and adjust swimming kinematics as a corrective measure in response to postural instability. This constitutes a key developmental aspect of the zebrafish's ability to balance (6). A schematic of a typical swimming pattern is provided in Fig. 3, where bouts were defined as any activity that exceeded a *5 mm/sec threshold* (dotted green line), and pauses defined as periods between bouts. Quantitative measures of swimming behavior include bout displacement (mm), bout duration (s), and swim rate, where swim rate (Hz) measures the frequency of bouts per second.

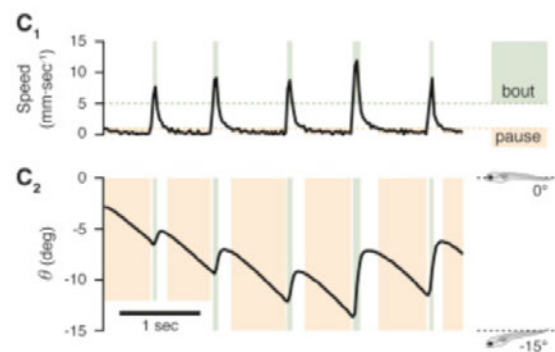


Figure 3: Zebrafish swim in discrete bouts interspersed with near pauses: Excerpted from Ehrlich, D. E., & Schoppik, D. (2017). Control of Movement Initiation Underlies the Development of Balance. *Current biology : CB*, 27(3), 334–344. <https://doi.org/10.1016/j.cub.2016.12.003>

Swimming behavior in the zebrafish is influenced by a plethora of other relevant factors, however, including age, morphology, and neurological development. In the present study, somatic length in mm was used as a broad proxy for morphology owing to its relative ease and quickness of acquisition. ‘Neurological development’, then, broadly encompasses any cause of zebrafish swimming behavior that is *not* dependent on morphology and which can be isolated to

the nervous system, which generates signals responsible for swimming. In the present study, we sought to disentangle the effects of somatic size, neurological development, age, and rearing temperature on the swimming behavior of zebrafish.

Methods

Animals -

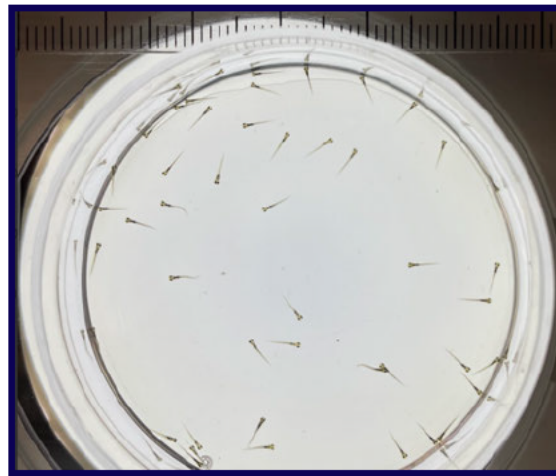
Naturally spawned eggs were initially collected, cleaned, and maintained in E3 solution (0.30 M NaCl, 0.01 M KCl, 0.03 M CaCl₂, 0.02 M MgSO₄, pH 7.1) on a 14/10 hour light/dark cycle at a maximum density of 30 per 100-mm diameter Petri dish. Overnight crosses were such that egg spawning and fertilization were synchronized upon lights on; as such, embryogenesis corresponded to Zeitgeber time (ZT) = 0. Larvae were generated from an in-cross of the *AB* wild-type line and eggs within each clutch were randomly divided into three treatments corresponding to incubators set at different rearing temperatures: 25°C, 28°C, and 32°C. Larvae were housed under static conditions with daily water changes until 5 days post-fertilization (dpf), at which point they were transferred to temperature-controlled tanks containing aquarium system water maintained at 700-750µS conductivity. Upon transfer, larvae were fed twice daily with a single serving of *Otohime A* Marine Fish larval and weaning feed. Animal handling and experimental protocols were approved by the University of Wisconsin-Madison College of Letters and Sciences Institutional Animal Care and Use Committee (IACUC)

Imaging and Somatic Size measurements:

Images of larvae were captured with an iPhone 12 camera set at 1x magnification positioned at a height of 7.5 cm above the level of the Petri dish base. Images were captured for

all dishes that had hatched eggs, even if not all larvae in a given dish had hatched. There were no measurements for 2 dpf larvae at 25°C as no larvae raised at this temperature hatched at that age. Effort was made to capture clear, well-contrasted images of fish against a clear background (Fig. 4a) with a thin ruler positioned underneath the Petri dish.

Processed images were imported into ImageJ and the freehand line tool was used to measure, for each image, a pixel/mm conversion factor (Fig. 4b). Length measurements (in pixels) were taken of each individual larvae (Fig. 4c). Larvae that were actively swimming, bent, and out-of-focus were excluded from measurements. The pixel/mm conversion factor was then used, via an Excel function, to convert length measurements of larvae from pixels to mm. For each dish, anywhere between 15-40 larvae would be imaged depending on the number of larvae remaining in a single clutch at a given age.



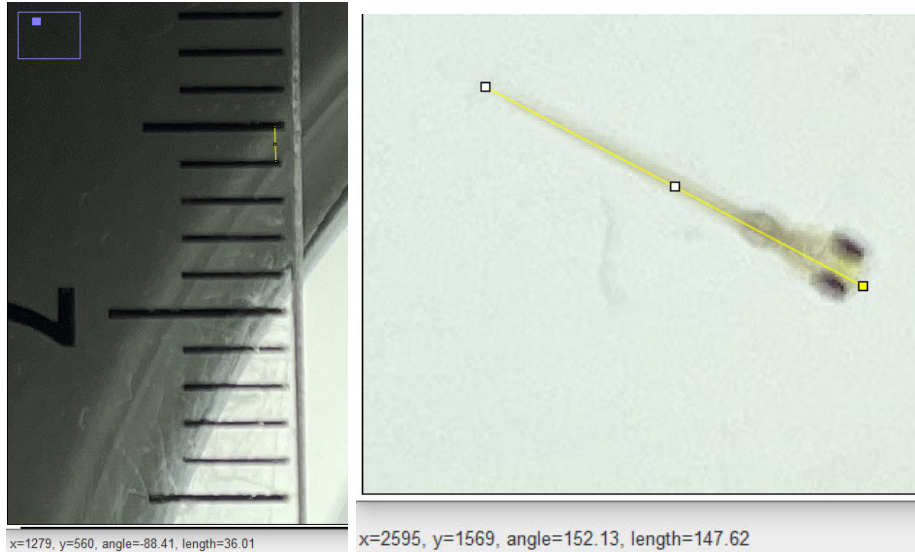


Figure 4: Acquisition of images and quantification of larval somatic size. *Top (3a):* representative image used to obtain measurements of somatic size. *Bottom left (3b):* sample measurement of pixel/mm conversion factor. *Bottom right (3c):* sample measurement of somatic size for one larvae, obtained from head to tail.

Free swimming apparatus

Swimming kinematics were measured within a water column and performed as reported previously (6). Six larvae from single clutches corresponding to specific temperature groups were filmed in glass tanks (52x45x12 mm, G208,m Azzota Corp.) using a digital camera (GS3-U3-23S6M-C, FLIR) with a close-focusing, manual zoom lens (18-108 mm Macro Zoom 7000 Lens, Navitar). Four filming setups were mounted in parallel and enclosed within a light-tight box with an exhaust fan to cool to 28°C. Video was acquired at 40 Hz and the framewise position and orientation of larvae in the field-of-view was computed in real-time using the NI-IMAQ vision acquisition environment of Lab-VIEW (National Instruments). Swim bouts were detected with a 5 mm/sec speed threshold, bout duration was measured as the number of frames exceeding this threshold, and bout displacement (mm) was calculated as the Euclidean distance between positions at the start and end of this window using custom code

in Matlab (Mathworks). Larvae were left in the filming setup for 48 hours and recordings were initiated at the same time across all clutches.

Results

Experiment 1 - Somatic Size

Somatic size measurements were obtained for $n = 50$ -100 larvae at ages 2-14 dpf for fish reared at 25°C, 28°C, and 32°C. The results are plotted in Fig. 5. In particular, a one-way ANOVA revealed a significant effect of temperature on length [$F(3,32)=181.2$, $p=6.34E-34$] at 3 dpf and no significant difference between temperature groups for 6 dpf.

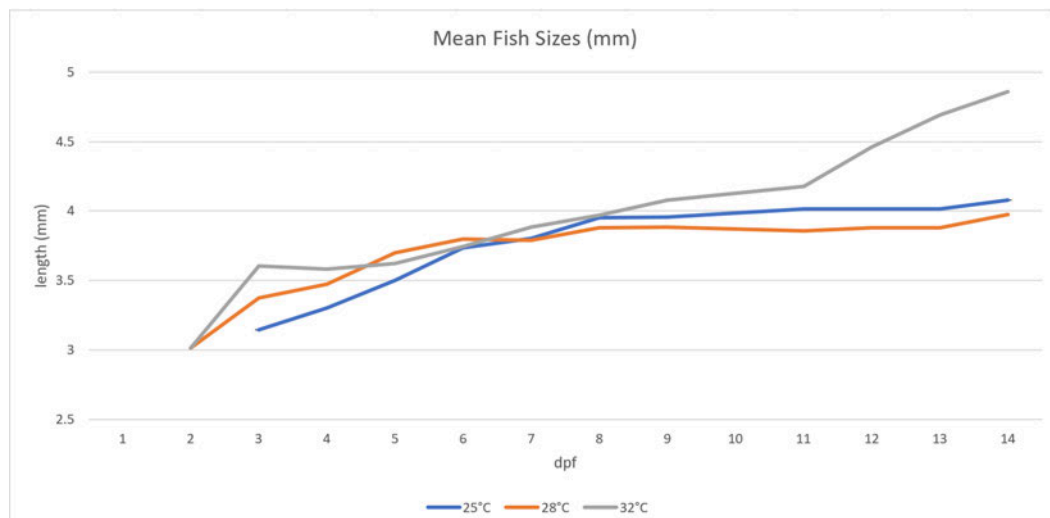
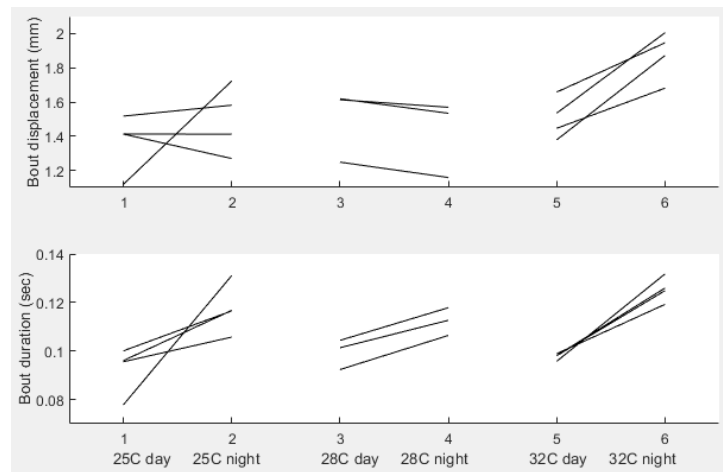


Figure 5. Somatic size of zebrafish larvae in chronic temperature condition between 2-14 dpf. Graph of somatic size across age for fish reared at 25°C, 28°C, and 32°C, respectively. There was a significant effect of temperature on length for the 3 dpf group [$F(3,32)=181.2$, $p=6.34E-34$] and no significant difference between temperature groups at 6 dpf.

The rates of growth from 3 to 7 dpf for larvae in the three different temperature groups were estimated by the slope of the best-fit line for length vs age (dpf) data with 95% confidence intervals. The results are as follows: 175 ± 10 $\mu\text{m/day}$ for the 25°C group; 116 ± 10 $\mu\text{m/day}$ for the 28°C group; 67 ± 13 $\mu\text{m/day}$ for the 32°C group. Non-overlapping 95% confidence intervals indicated significant differences at $\alpha = 0.05$.

Experiment 2: Swimming Kinematics

After observing this convergence of somatic size at 6 dpf, we sought to quantify various kinematic parameters of fish swimming at this age; namely, we quantified bout duration (s), bout displacement (mm), and swim rate (Hz). In particular, we observed circadian-dependent changes in swimming kinematic parameters as depicted in Fig. 6. Each line in the figure below depicts kinematic parameters corrected for night and day for a single clutch (i.e. $n = 4$ clutches at 25°C, $n = 3$ clutches at 28°C and $n = 4$ clutches at 32°C). Across all temperature groups, bout duration increased during the night relative to the day (Fig. 6b). Larvae reared at 32°C, however, tended to displace further at night when compared to their lower temperature counterparts. Larvae reared at 25°C and 28°C decreased their swim rate at night relative to day (Fig 6c); peculiarly, larvae raised at 32°C did *not* decrease their swim rates at night to the same extent as 28°C or 25°C larvae. 32°C fish also had significantly higher bout displacements at night when compared to their lower temperature counterparts. Though statistical analyses have not been performed on these parameters yet, the general trends are salient and consistent with qualitative observation.



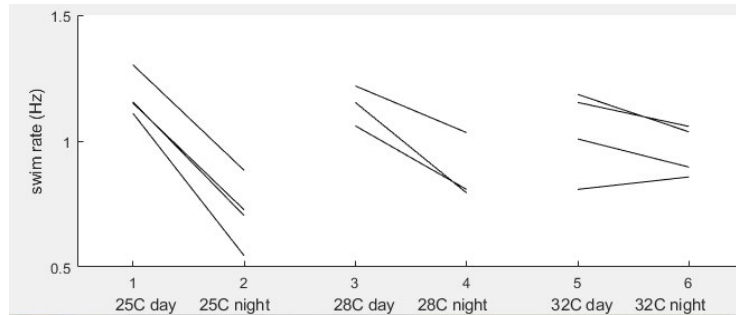


Figure. 6: Swimming Kinematics: plots of **6a.) top:** bout displacement (mm), **6b.) middle:** bout duration (sec), and **6c.) bottom:** swim rate (Hz) as a function of chronic rearing temperature and circadian time (14h light, 10h dark). Graphs courtesy of David Ehrlich.

Discussion

Experiment 1

The somatic sizes (mm) of zebrafish larvae reared at different temperatures, though significantly different between temperature groups at 3 dpf, converges at 6-7 dpf. This result, though initially inconsistent with my initial hypothesis where fish raised at 32°C were projected to be consistently larger than lower temperature counterparts throughout the entire 2-14 dpf time frame, proved indispensable to the conclusion that somatic size is *less likely* contributing to observed differences in swimming behavior at 6-7 dpf. Instead, these observed differences in swimming are more likely attributed to lasting changes in the nervous system.

The observed convergence is also consistent with statistically significant differences in rates of somatic growth between 25°C, 28°C, and 32°C larvae. In particular, the rate of growth between 3-7 dpf tracked inversely with rearing temperature: i.e. the 25°C group had the fastest growth, the 28°C group had slower growth, and the 32°C group had the slowest growth. A plausible explanation for this phenomenon relates to the relative rates of yolk metabolism between fish raised at different temperature groups. A previous mathematical model revealed that metabolism of yolk area over time can be modeled using a decreasing sigmoidal function.

The model reveals a nonconstant rate of yolk metabolism where the rate of utilisation increases to a maximum (i.e. the inflection point), but then proceeds to decrease and asymptote towards yolk area = 0 (Fig. 7). The same authors modeled fish length as an increasing logistic function (Figure 8) and found a strong association between yolk usage and length change between 4 and 5 dpf ($P=0.017$) (7). It is plausible, then, that fish reared at higher temperatures expedite both yolk metabolism and corresponding rates of somatic growth – graphically, both graphs are ‘shifted to the left’ – such that reductions in growth rates and the corresponding asymptotes in fish length are observed *sooner*. This is consistent with our observation that 32°C fish have the slowest rates of growth when compared to their lower temperature groups.

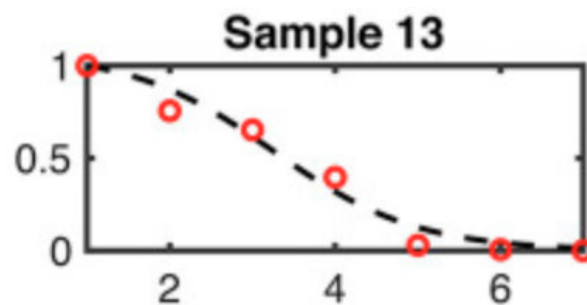


Figure 7: Yolk metabolism can be modeled using a decreasing sigmoidal curve. The x-axis represents age (dpf) whereas the y-axis represents Normalized Yolk Area. The rate of yolk metabolism increases to a maximum (in this graph, somewhere between 3 and 4 dpf) and then declines as yolk area asymptotes towards area = 0. Excerpted from Schwartz, A. V., Sant, K. E., Navarrete, J., & George, U. Z. (2021). Mathematical modeling of the interaction between yolk utilization and fish growth in zebrafish, *Danio rerio*. *Development* (Cambridge, England), 148(9), dev193508. <https://doi.org/10.1242/dev.193508>

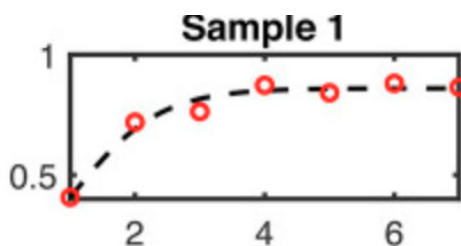


Figure 8: Somatic growth in larval fish can be modeled as an increasing logistic function: the x-axis represents age (dpf) whereas the y-axis represents Normalized Length. The rate of somatic growth reaches an asymptote around 6-7 dpf. Excerpted from Schwartz, A. V., Sant, K. E., Navarrete, J., & George, U. Z. (2021). Mathematical modeling of the interaction between yolk utilization and fish growth in zebrafish, *Danio rerio*. *Development* (Cambridge, England), 148(9), dev193508. <https://doi.org/10.1242/dev.193508>

Limitations of this particular study included variable quality of photos. Some of the imaged larvae were pixelated and it was occasionally difficult to delineate the exact point at which the tail of a fish ended due to inadequate contrast. Future work will involve obtaining length measurements, as well as tracing yolk area, using light microscopy where more detailed and focused images will be possible.

Experiment 2

We observed isolatable, circadian-related deficits for fish reared at 32°C. In particular, these fish reduce their swimming rate to a lesser extent during the night when compared to the lower temperature (25°C and 28°C) counterparts, with one group actually *increasing* swim rate during the night relative to day. In addition, 32°C fish displaced substantially further at night compared to corresponding bout displacements for 25°C and 28°C fish. Since displacement is a function of both velocity and time, and bout duration (time) increases during the nighttime for all *three* temperature groups, it can be concluded that 32°C fish swim markedly faster at night compared to the day.

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