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# Isothermal calorimetry reveals that successful regeneration after a spinal cord injury in larval zebrafish is associated with an increase in energy expenditure

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Unlike mammals, larval zebrafish recover very rapidly from a complete spinal cord injury (SCI). Zebrafish larvae recover normal appearing swimming behavior 2 days after a complete SCI at 3 days-postfertilization (dpf) [1]. At the cellular level, this fast and successful recovery process involves the generation of new spinal cord neurons [1,2] and axon regeneration through the site of injury [3]. In contrast, neurons in the mammalian central nervous system (CNS) are incapable of regenerating after a SCI (see [4]).

Cell regeneration (involving cell proliferation and differentiation) and axon regeneration are cellular processes with high energetic costs requiring an efficient production of ATP [5–8]. Problems with mitochondrial dynamics and energy deficits limit regeneration in the CNS [4,8]. For example, ARMCX1, a protein that mobilizes mitochondria, promotes axon regeneration in retinal ganglion cells after an optic nerve injury [9]. Deleting syntaphilin, a mitochondria-anchoring protein [10], promotes axon growth and functional improvements after SCI [6,10]. In *C. elegans*, single-neuron analyses revealed that axons in which mitochondria do not increase fail to regenerate [5]. In *Drosophila*, co-activation of PI3K and EGFR in glia increases aerobic glycolysis and promotes axon regeneration through glia-derived metabolites like L-lactate [11]. Moreover, local L-lactate administration promotes the regeneration of corticospinal axons after SCI [11]. In the zebrafish CNS, cAMP enhances mitochondrial trafficking and axon regrowth [3]. These studies are just a few examples from invertebrate and vertebrate species revealing the importance of energy metabolism for neuronal regeneration. Thus, it is of great interest to develop new methods to study energy demands and expenditure after CNS injuries and during regeneration.

Measuring the power required to regenerate the spinal cord after a complete SCI could be reported as the rate of free energy dissipation with an enthalpic contribution (which is dissipated as heat) [12]. Interestingly, recent studies have applied the use of calorimeters to measure heat dissipation in early developing zebrafish embryos [13,14]. Implementation of this method to the larval zebrafish model of SCI could offer a convenient vertebrate system to study the energetic costs of successful spinal cord regeneration in whole animals. Here, we used an isothermal calorimeter to measure thermal dissipation in zebrafish larvae after a complete SCI. Our data show that the successful regenerating process activated in larval zebrafish after a complete SCI increases thermal dissipation (i.e., energy expenditure; see below).

In this study, wild-type adult zebrafish were kept and raised under standard conditions in the fish facilities of the area of Genetics of the University of Santiago de Compostela (facility code: AE-LU-003,

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ES270280346401). All experiments were approved by the Bioethics committee of the University of Santiago de Compostela and the Xunta de Galicia (project license no.: 01/20/LU-003) and were carried out in accordance with the European Union and Spanish guidelines for animal experiments. Embryos were collected from breeding tanks and were divided into Petri dishes at a density of 80 to 100 embryos per dish in E3 medium (for a 60× solution in dd$H_2O$: NaCl 0.3 M, KCl 10.2 mM, $CaCl_2 \cdot 2\ H_2O$ 19.8 mM, $MgSO_4 \cdot 7\ H_2O$ 19.8 mM) until 3 dpf. For this study, a total of 340 zebrafish larvae were used.

For the SCI surgery, 3 dpf zebrafish larvae were anaesthetized in E3 medium containing 0.02 % aminobenzoic-acid-ethyl methyl-ester (MS-222, Sigma). Larvae were transferred to a Petri dish with a roughened surface as described. Following removal of excess water, the larvae were placed in a lateral position, and the tip of a sharp 30 G × 1/2″ hypodermic needle was used to transect the dorsal part of the trunk at the level of the urogenital pore. Care was taken to avoid injuring the notochord. After the transection SCI, larvae were returned to E3 medium. Injured animals were checked 1 h after surgery to confirm that they were correctly injured (i.e. they had a full transection SCI but did not have bulging tissue coming from an injured notochord).

Control non-injured 3 dpf animals or injured 3 dpf animals were transferred to plastic vials in groups of 5 or 10 larvae per vial with 3 ml of E3 medium (Suppl. Fig. 1A, B). The vials were placed in calorimeter cells of the I-Cal Flex isothermal calorimeter (Calmetrix, Needham, MA, USA) (Suppl. Fig. 1C, D). Values of heat dissipation (power in mW) from each vial in each cell were recorded during 42 h after 5 h of acclimatation/stabilization in the calorimeter.

Heat dissipation was measured every minute during those 42 h. A Two-Way ANOVA was used to compare heat dissipation between groups of 5 or 10 non-injured animals or between groups of 10 control non-

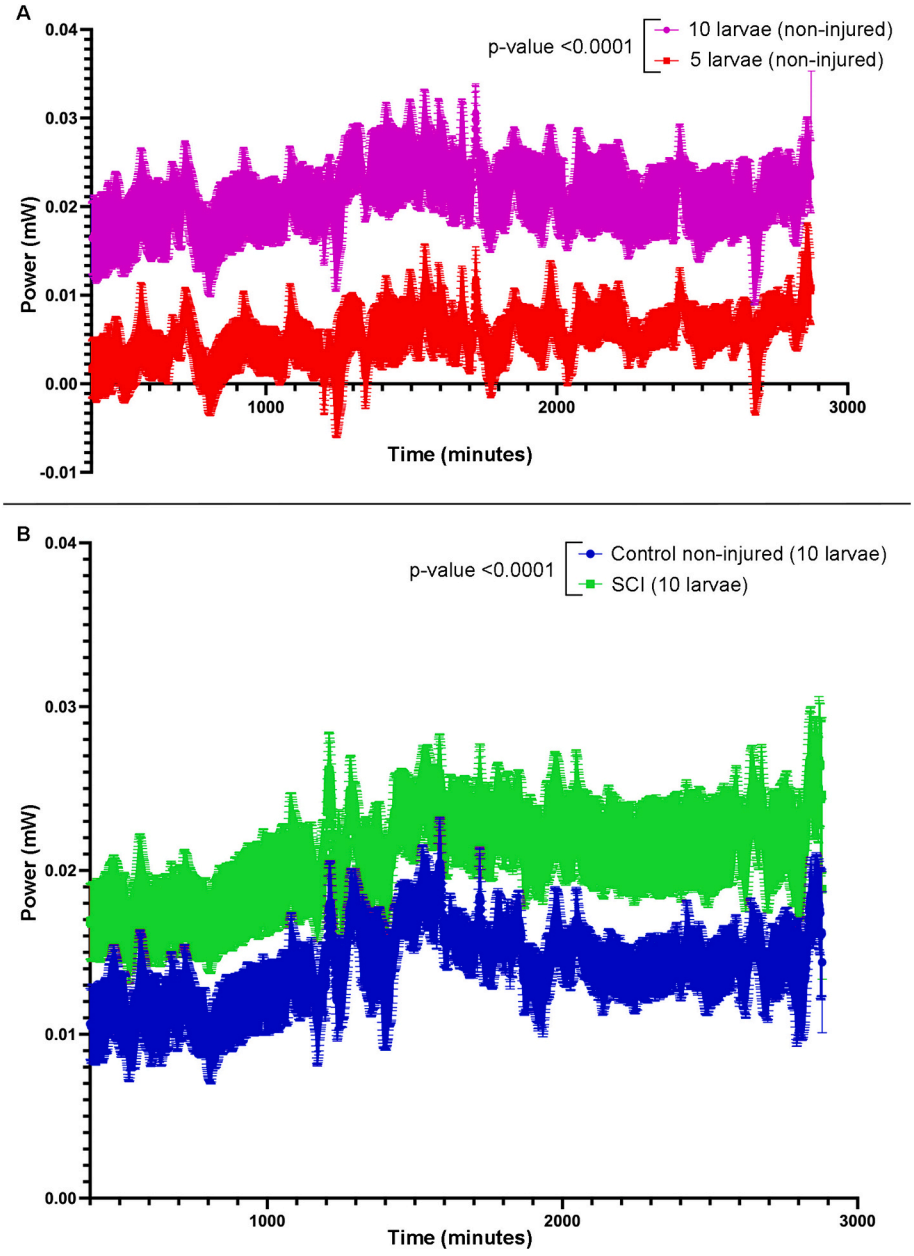


**Fig. 1.** Heat dissipation from non-injured controls and from zebrafish larvae with a complete SCI. A. Comparison of heat dissipation from groups of 5 (6 groups) or 10 (7 groups) zebrafish larvae (3 to 5 dpf). A Two-Way ANOVA analysis revealed a significantly higher heat dissipation in tubes containing 10 zebrafish larvae ($p < 0.0001$). B. Comparison of heat dissipation from groups of 10 control non-injured (13 groups) or 10 injured (14 groups) zebrafish larvae (3 to 5 dpf). A Two-Way ANOVA analysis revealed a significant increase in heat dissipation during recovery after a complete SCI ($p < 0.0001$).

injured animals and 10 injured animals. Significance level was set at $p < 0.05$.

First, as a control of the method to determine heat dissipation from zebrafish larvae using an isothermal calorimeter, we compared groups of 5 (6 groups) and 10 (7 groups) non-injured larvae from 3 to 5 dpf. As expected, we observed higher heat dissipation ($p < 0.0001$) in tubes containing 10 larvae (Fig. 1A). Thus, the isothermal calorimeter can detect differences in heat dissipation from zebrafish larvae (3 to 5 dpf). Since groups of 5 larvae are close to the detection limit of the calorimeter (Fig. 1A), we decided to use groups of 10 larvae for the control vs SCI comparison. So, next, we compared heat dissipation between groups of 10 control non-injured (13 groups) and 10 injured (14 groups) larvae from 3 to 5 dpf. Our results revealed a significant increase in heat dissipation (i.e., energy expenditure) during successful regeneration after a complete SCI in zebrafish larvae ($p < 0.0001$; Fig. 1B).

The heat dissipated by whole developing vertebrate embryos has been measured in frogs and fish [13–15]. These studies showed that heat dissipation rate increases rapidly during embryonic development until or soon after gastrulation, and that this increase coincides with the period of successive cell divisions [13]. Indeed, pharmacological treatments blocking cell proliferation in developing zebrafish abolish the increase in heat dissipation rate [14]. Rodenfels et al. [14] used a mathematical model to propose that increasing costs of maintaining or building plasma membrane during cell division underlie the increasing heat dissipation rate in developing embryos. Thus, the increased heat dissipation that we detected after SCI in larval zebrafish could also be related to processes of cell proliferation, neuronal regeneration, and axonal growth, which are cellular processes that require building new plasma membranes. It should be considered that a limitation of our heat dissipation measurements is, as previously indicated for developing embryos [12], that we are obtaining a global measurement with contributions from many cellular processes occurring at the same time at different locations within the regenerating larvae. Another limitation is that we are measuring heat dissipation from 10 larvae at the same time, although this limitation can be overcome in the future by using isothermal calorimeters with enhanced sensitivity. In any case, our results indicate that the enhancement of cellular energetics supports successful regeneration after a complete SCI in zebrafish. Our study provides a new method to study energy expenditure during regenerative processes in whole animals with the advantages of zebrafish larvae transparency, rapid regeneration, and amenability for transgenesis, mutagenesis or in vivo drug testing.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2024.149498.

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## CRediT authorship contribution statement

**Laura González-Llera:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Álvaro J. Arana:** Investigation. **Laura Sánchez:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Carmen Alvarez-Lorenzo:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Antón Barreiro-Iglesias:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## References

[1] J. Ohnmacht, Y. Yang, G.W. Maurer, A. Barreiro-Iglesias, T.M. Tsarouchas, D. Wehner, D. Sieger, C.G. Becker, T. Becker, Spinal motor neurons are regenerated after mechanical lesion and genetic ablation in larval zebrafish, Development 143 (9) (2016 May 1) 1464–1474, https://doi.org/10.1242/dev.129155.

[2] L. Cavone, T. McCann, L.K. Drake, E.A. Aguzzi, A.M. Oprişoreanu, E. Pedersen, S. Sandi, J. Selvarajah, T.M. Tsarouchas, D. Wehner, M. Keatinge, K.S. Mysiak, B.E. P. Henderson, R. Dobie, N.C. Henderson, T. Becker, C.G. Becker, A unique macrophage subpopulation signals directly to progenitor cells to promote regenerative neurogenesis in the zebrafish spinal cord, Dev. Cell 56 (11) (2021 Jun 7) 1617–1630.e6, https://doi.org/10.1016/j.devcel.2021.04.031.

[3] Y. Xu, M. Chen, B. Hu, R. Huang, B. Hu, *In vivo* imaging of mitochondrial transport in single-axon regeneration of zebrafish Mauthner cells, Front. Cell. Neurosci. 24 (11) (2017 Jan) 4, https://doi.org/10.3389/fncel.2017.00004.

[4] B. Zheng, M.H. Tuszynski, Regulation of axonal regeneration after mammalian spinal cord injury, Nat. Rev. Mol. Cell Biol. 24 (6) (2023 Jun) 396–413, https://doi.org/10.1038/s41580-022-00562-y.

[5] S.M. Han, H.S. Baig, M. Hammarlund, Mitochondria localize to injured axons to support regeneration, Neuron 92 (6) (2016 Dec 21) 1308–1323, https://doi.org/10.1016/j.neuron.2016.11.025.

[6] Q. Han, Y. Xie, J.D. Ordaz, A.J. Huh, N. Huang, W. Wu, N. Liu, K.A. Chamberlain, Z.H. Sheng, X.M. Xu, Restoring cellular energetics promotes axonal regeneration and functional recovery after spinal cord injury, Cell Metab. 31 (3) (2020 Mar 3) 623–641.e8, https://doi.org/10.1016/j.cmet.2020.02.002.

[7] X.T. Cheng, N. Huang, Z.H. Sheng, Programming axonal mitochondrial maintenance and bioenergetics in neurodegeneration and regeneration, Neuron 110 (12) (2022 Jun 15) 1899–1923, https://doi.org/10.1016/j.neuron.2022.03.015.

[8] L. Cheng, B. Cai, D. Lu, H. Zeng, The role of mitochondrial energy metabolism in neuroprotection and axonal regeneration after spinal cord injury, Mitochondrion 69 (2023 Mar) 57–63, https://doi.org/10.1016/j.mito.2023.01.009.

[9] R. Cartoni, M.W. Norsworthy, F. Bei, C. Wang, S. Li, Y. Zhang, C.V. Gabel, T. L. Schwarz, Z. He, The mammalian-specific protein Armcx1 regulates mitochondrial transport during axon regeneration, Neuron 92 (6) (2016 Dec 21) 1294–1307, https://doi.org/10.1016/j.neuron.2016.10.060.

[10] Q.Y. Wu, H.L. Liu, H.Y. Wang, K.B. Hu, P. Liao, S. Li, Z.Y. Long, X.M. Lu, Y. T. Wang, Syntaphilin mediates axonal growth and synaptic changes through regulation of mitochondrial transport: a potential pharmacological target for neurodegenerative diseases, J. Drug Target. 31 (7) (2023 Aug) 685–692, https://doi.org/10.1080/1061186X.2023.2230522.

[11] F. Li, A. Sami, H.N. Noristani, K. Slattery, J. Qiu, T. Groves, S. Wang, K. Veerasammy, Y.X. Chen, J. Morales, P. Haynes, A. Sehgal, Y. He, S. Li, Y. Song, Glial metabolic rewiring promotes axon regeneration and functional recovery in the central nervous system, Cell Metab. 32 (5) (2020 Nov 3) 767–785.e7, https://doi.org/10.1016/j.cmet.2020.08.015.

[12] S. Ghosh, A. Körte, G. Serafini, V. Yadav, J. Rodenfels, Developmental energetics: energy expenditure, budgets and metabolism during animal embryogenesis, Semin. Cell Dev. Biol. 30 (138) (2023 Mar) 83–93, https://doi.org/10.1016/j.semcdb.2022.03.009.

[13] J. Rodenfels, K.M. Neugebauer, J. Howard, Heat oscillations driven by the embryonic cell cycle reveal the energetic costs of signaling, Dev. Cell 48 (5) (2019 Mar 11) 646–658.e6, https://doi.org/10.1016/j.devcel.2018.12.024.

[14] J. Rodenfels, P. Sartori, S. Golfier, K. Nagendra, K.M. Neugebauer, J. Howard, Contribution of increasing plasma membrane to the energetic cost of early zebrafish embryogenesis, Mol. Biol. Cell 31 (7) (2020 Mar 19) 520–526, https://doi.org/10.1091/mbc.E19-09-0529.

[15] Y. Nagano, K.L. Ode, Temperature-independent energy expenditure in early development of the African clawed frog *Xenopus laevis*, Phys. Biol. 11 (4) (2014 Aug) 046008, https://doi.org/10.1088/1478-3975/11/4/046008.