PI: **Rohner, Nicolas**	Title: Starvation resistance and resilience of metabolic dysfunction in cavefish	
Received: 08/20/2019	FOA: RM19-006 Clinical Trial:Optional	Council: 05/2020
Competition ID: FORMS-E	FOA Title: NIH Directors New Innovator Award Program (DP2 Clinical Trial Optional)	
1 DP2 AG071466-01	Dual: OD,RM	Accession Number: 4339954
IPF: 4323301	Organization: STOWERS INSTITUTE FOR MEDICAL RESEARCH	
Former Number: 1DP2OD028806-01	Department:	
IRG/SRG: ZRG1 MOSS-R (70)R	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 1,500,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Y Early Stage Investigator: Y
Senior/Key Personnel:	*Organization:*	*Role Category:*
Nicolas Rohner	STOWERS INSTITUTE FOR MEDICAL RESEARCH	PD/PI

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION*

○ Pre-application ● Application ○ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

2. DATE SUBMITTED | **Application Identifier**

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION

Legal Name*: STOWERS INSTITUTE FOR MEDICAL RESEARCH
Department:
Division:
Street1*: [redacted]
Street2:
City*:
County:
State*:
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*:

Person to be contacted on matters involving this application
Prefix: Dr. First Name*: Michelle Middle Name: Last Name*: Lewallen Suffix:
Position/Title: Grants Information Manager
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email:

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)**

7. TYPE OF APPLICANT*

M: Nonprofit with 501C3 IRS Status (Other than Institution of Higher Education)

Other (Specify):
Small Business Organization Type ○ Women Owned ○ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ○ Resubmission
○ Renewal ○ Continuation ○ Revision

If Revision, mark appropriate box(es).
○ A. Increase Award ○ B. Decrease Award ○ C. Increase Duration
○ D. Decrease Duration ○ E. Other *(specify)* :

Is this application being submitted to other agencies?* ○Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Starvation resistance and resilience of metabolic dysfunction in cavefish

12. PROPOSED PROJECT
Start Date* 08/15/2020
Ending Date* 06/30/2025

13. CONGRESSIONAL DISTRICTS OF APPLICANT
MO-005

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Nicolas Middle Name: Last Name*: Rohner Suffix:
Position/Title: Assistant Investigator
Organization Name*: STOWERS INSTITUTE FOR MEDICAL RESEARCH
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*:
Phone Number*: [redacted] Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$1,500,000.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$1,500,000.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Dr. First Name*: David Middle Name: M Last Name*: Chao Suffix:
Position/Title*: President & CEO
Organization Name*: Stowers Institute for Medical Research
Department: Executive
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*:
Phone Number*: [redacted] Fax Number: [redacted] Email*: [redacted]

Signature of Authorized Representative*
Michelle Lewallen

Date Signed*
08/20/2019

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:Cover_Letter_with_signature.pdf

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: STOWERS INSTITUTE FOR MEDICAL RESEARCH

Duns Number: [redacted]

Street1*: [redacted]

Street2: [redacted]

City*: [redacted]

County:

State*: [redacted]

Province:

Country*: [redacted]

Zip / Postal Code*: [redacted]

Project/Performance Site Congressional District*: [redacted]

Additional Location(s) File Name:

Funding Opportunity Number: RFA-RM-19-006. Received Date: 2019-08-20T13:13:52.000-04:00

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ● No

IACUC Approval Date: 11-01-2018

Animal Welfare Assurance Number [redacted]

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	Project_summary.pdf
8. Project Narrative*	Project_Narrative.pdf
9. Bibliography & References Cited	
10.Facilities & Other Resources	Facilities_and_Other_Resources.pdf
11.Equipment	

Funding Opportunity Number: RFA-RM-19-006. Received Date: 2019-08-20T13:13:52.000-04:00

Project Summary

The overarching goal is to understand the complex genetic network regulating energy metabolism. This is a critically important problem from the standpoint of human health, and a great deal has already been learned through previous physiological and genetic studies of normal and disease conditions, primarily in humans and rodents. Many of these studies have taken advantage of patients or laboratory animals with extremely impaired metabolic responses. In these cases, errors in metabolic pathways result in severe deleterious consequences, leading to morbidity and mortality. In nature, however, there is a second kind of extreme variation: there are many cases where animals have adapted to extreme environments or extreme lifestyles through changes in metabolic regulation. In these instances, the metabolic changes are adaptive, not pathological, meaning they occur in such a way that the effects are not deleterious and/or other physiological changes occur to compensate for any detrimental consequences. In principle, this provides an opportunity for identifying resilience mechanisms that may have escaped notice through study of the circuits disrupted in disease. However, to fully harness this natural variation for gene discovery, one wants to be able to compare metabolic regulation in such animals with related organisms that have not undergone such extreme adaptation. Yet, in many cases there is no closely related animal with a more “normal” metabolism for comparison. An important exception is the cave-dwelling Mexican cave tetra, *Astyanax mexicanus*, which thrives under essentially starvation conditions, and its river cousins who are of the same species. Together they offer a unique and exciting opportunity to examine the metabolic adaption to an extreme living condition in an organism ripe for genetic and genomic analysis. This proposal builds on published data showing that cavefish have evolved a dramatically altered metabolism, including accumulation of high body fat levels and fatty livers, insulin resistance and unstable blood glucose levels, without apparent adverse effects on their health. Use of state of the art genomic, genetic and gene editing technologies now offers the possibility of identifying signaling pathways that underlie starvation resistance and resilience mechanisms under extreme nutritional situations. The proposal leverages the practical advantages of the model system to be amenable to comparative approaches such as RNA-Seq, chromatin architecture analysis, mapping strategies such as QTL-analysis, gene modification techniques such as CRISPR/Cas9, and novel transgenic lines, all combined with its unique ecological adaptation scenario. This research proposal, therefore, aims to take advantage of this resource to understand more fully the metabolic changes that allow the cavefish to survive in their bleak environment and to start to dissect the regulatory changes that underlie the resilience mechanisms allowing these fish to tolerate what in most animals, including us humans, would be considered a disease.

Project Narrative:

This work will identify the genetic pathways underlying the changes in metabolic regulation in cavefish adapted to living in what would otherwise be starvation conditions and the resilience mechanisms that accompany these. This represents a unique opportunity to uncover unexpected mechanisms and novel pathways underlying fat storage metabolism that may have been at play in human evolution and/or infer therapeutic targets for developing strategies for treating obesity and resultant diseases.

FACILITIES & RESOURCES

Lab Resources: Dr. Rohner's laboratory has 1,220 sq. ft. of space, including personal bench and desk space for 8-10 researchers. The laboratory has two Leica stereoscopes (M205C), one Zeiss Axioplan 2 imaging microscope, 4 Eppendorf PCR machines (vapo-protect) and one Biorad CFX96 real-time PCR system. Additionally, common equipment and lab space, tissue culture rooms, and a cold room are available as a shared space near the laboratory.

Stowers Institute Resources: The Stowers Institute is a technologically advanced biomedical research facility that houses approximately 210,000 sq. ft. of finished laboratory space including a large share of advanced technology facilities that support scientific research. Each facility is headed by an expert in the field who works with research teams across the institute to offer access to the latest technology and techniques, enabling Stowers scientists to accomplish in days and weeks what would take months and years elsewhere. Particularly relevant to this grant are the following facilities:

Reptile and Aquatics Facility: The Reptile & Aquatics Facility provides the institute's research staff with the highest quality laboratory animal care and support services for non-mammalian species. A knowledgeable, specially trained staff of dedicated technicians caters to the needs of the researchers. The facility is fully AAALAC-accredited. All housing, husbandry practices, and veterinary care for the animals are in compliance with the Guide for the Care and Use of Laboratory Animals and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, as well as all other institutional, state, and local regulations. Staff offer a variety of technical services based on researcher's needs, including breeding, embryo harvest, tissue sampling, genotyping, histology preparation, cryopreservation, and identification services. The state-of-the-art cavefish facility, which was custom-built for the Rohner Lab, is located next to the lab space for convenient access by the researchers. It consists of 6 Pentair G-Hab racks (G36X-STOW), 4 Pentair Z-Hab racks (S56C-STOW) and one "dark rack", which provides the ability to raise and manipulate animals in complete darkness. The room has a total capacity of approximately 10,000 adult individuals. For micromanipulation, the room is equipped with two Leica stereoscopes (LED 2500) that are attached to high-throughput micro-injectors and compressors.

Molecular Biology: The Molecular Biology Facility supports researchers by providing high-quality services, collaborative project potential and access to state-of-the-art technology. Services include DNA sequencing, single-cell RNA-Seq, ChIP-Seq, real-time quantitative PCR, droplet digital PCR, CRISPR-Cas9 genome engineering, custom automation, and plasmid preparation. Genome sequencing experts consult with researchers to design the best approach for their projects. They have experience using the CRISPR-Cas9 system to generate mutations by non-homologous end joining (NHEJ) or homology-directed repair (HDR) using donor constructs. The group designs guide RNAs, synthesizes donor constructs, works closely with animal core facilities and provides a screening service to detect mutations. The facility utilizes the latest next-generation sequencing technology.

Computational Biology: The Computational Biology group at the Stowers Institute assists investigators with the analysis of biological data. The group offers in-pipeline development, secondary and tertiary analysis, data interpretation and training. The bioinformatics experts primarily work with next-generation high-throughput sequence data. Supported NGS data types include RNA-seq (including single-cell RNA-Seq), ChIP-Seq, DNA-Seq, and uncommon data types such as ATAC-Seq, HiC-Seq, Methyl-Seq, etc. The Bioinformatics Center operates several computational servers, Linux clusters and myriad programs, packages, and programming environments for analysis of biological data.

Intellectual Environment: The Stowers Institute provides an enriching and collaborative environment, including but not limited to Robb Krumlauf, Alejandro Sánchez-Alvarado, Matt Gibson, Paul Trainor, and Sarah Zanders who are working on the interface of development, evolution and genetics. Additionally, the principal investigator of this grant has an assistant professor position at Kansas University Medical Center with the Department of Molecular & Integrative Physiology, providing expertise in a wide range of physiological model systems and approaches.

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Nicolas Middle Name Last Name*: Rohner Suffix:

Position/Title*: Assistant Investigator
Organization Name*: STOWERS INSTITUTE FOR MEDICAL RESEARCH
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*:
Zip / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number:

E-Mail*: nro@stowers.org

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: PHD Degree Year: 2010

Attach Biographical Sketch*: File Name: Biosketch.pdf

Attach Current & Pending Support: File Name: Current_and_Pending_Support.pdf

Tracking Number: GRANT12922831 Funding Opportunity Number: RFA-RM-19-006 . Received Date: 2019-08-20T13:13:52.000-04:00

BIOGRAPHICAL SKETCH

Prov de the fo ow ng nformat on for the Sen or/key personne and other s gn f cant contr butors.
Fo ow th s format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Nicolas Rohner

POSITION TITLE: Assistant Investigator / Assistant Professor

eRA COMMONS USER NAME (credent a , e.g., agency og n): [redacted]

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education such as nursing include postdoctoral training and residency training if applicable Add/delete rows as necessary)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Comp et on Date MM/YYYY	FIELD OF STUDY
Friedrich-Alexander-University, Erlangen	B.Sc.	05/2002	Biology
Friedrich-Alexander-University, Erlangen	M.Sc.	04/2005	Genetics
Max-Planck-Institute for Developmental Biology, Tübingen	Ph.D.	05/2010	Genetics, Developmental Biology
Harvard Medical School, Boston	Postdoctoral	08/2015	Genetics, Genomics, Evolutionary Genetics, Developmental Biology

A. Personal Statement

I have the expertise, training and motivation necessary to successfully carry out the proposed research project. I was educated as a general biologist (B.Sc.) and a geneticist (M.Sc.) in Germany. For my Ph.D. and during my postdoc, I was trained as a developmental biologist and geneticist. My research interest focuses on the interface of developmental biology, genetics and evolution with a long-term interest in understanding the genetic basis and molecular mechanisms of adaptation. For the last ten years, I have been working with the system that will be used for this grant, the cavefish *Astyanax mexicanus*. I have a strong network of collaborations with most other scientists using this emerging model system. Together, we have established the cavefish as a strong genetic model system, addressing the question of how these fish have adapted to their extreme habitat. In the last five years, I have channeled my expertise on one particularly striking aspect of cave habitats – the nutrient scarcity – and the extreme metabolic adaptations and resilience mechanisms that have resulted due to this environmental factor. This endeavor has laid the groundwork for this grant application. In 2015, I started as a new faculty at the Stowers Institute for Medical Research, where I have established collaborations with core research teams who have expertise in bioinformatics, cytometry, proteomics and robotics. Through my affiliation with [redacted] I have established collaborations with leading experts in the field of Diabetes and Obesity, providing me with the necessary expertise to conduct the metabolic studies in the established model systems.

B. Positions and Honors:

PROFESSIONAL EXPERIENCE:

2005-2010 Graduate Student [redacted] Max-Planck-Institute
2010-2015 Postdoctoral Fellow [redacted], Harvard Medical School
2015-date Assistant Investigator, Stowers Institute for Medical Research
2015-date Assistant Professor, Department of Physiology KU Medical Center

AWARDS AND HONORS:
2006 Best Poster Award at the 1st European Meeting of Evolution and Development, Prague
2011 Stipend from the GSO (German Scholars Organization)
2011 Two-year Postdoctoral Fellowship from the German Science Foundation (DFG)
2012 Selected for the AAAS Program for Excellence in Science
2012 Postdoc Research Day Best Poster Award at Harvard Medical School
2014 Speaker Prize at the Harvard Medical School Department of Genetics Retreat
2014 Young Investigator Travel Award for the SMBE Meeting, Puerto Rico
2014 EMBO Travel bursary to attend the FEBS EMBO 2014 Meeting, Paris
2018 Edward Mallinckrodt Foundation Grantee

C. Contribution to Science

1. Using fish as a model for molecular genetics and adult form
During my Ph.D. with [redacted] at the Max-Planck-Institute and in a recently completed follow-up study in collaboration with the Harris Lab at Harvard Medical School, I have used the zebrafish as a forward genetic model to study the molecular and genetic basis of adult-specific structures, such as fins, skull and scales. This work was part of the first large-scale forward genetic screen on adult form conducted in vertebrates, contrasting the majority of previous work focusing on early developmental phenotypes. Given that adult form is under strong selection in nature, we were able to use this knowledge to uncover genetic mechanisms underlying morphological adaptations in other fish species, both in domestication and in natural species.

1. Harris MP, **Rohner N**, Schwarz H, Perathoner S, Konstantinidis P, and Nüsslein-Volhard C (2008) *Zebrafish eda and edar mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. PLoS Genetics* 4(10):e1000206.
2. **Rohner N**, Bercseny M, Orban L, Kolanczyk ME, Linke D, Brand M, Nüsslein-Volhard C, and Harris MP (2009) *Duplication of fgfr1 Permits Fgf Signaling to Serve as a Target for Selection during Domestication. Current Biology* 19(19):1642-7.
    - Recommended by Faculty1000, Editors choice Science, Editors choice Nature, Highlighted by Maderspacher F. Curr Biol. 2009 Oct 13;19(19):R902-4.
3. Daane JM, **Rohner N**, Konstantinidis P, Djuranovic S, Harris MP (2016) *Phylogenomic evidence for epistasis between fgfr1 and fgf20 in skeletal evolution. Mol Biol Evol* 33(1):162-73.

2. Substantiated *Astyanax mexicanus* as a tractable genetic model for adaptation and evolution
In recent years, I have substantially contributed to further establishing the cavefish system *A. mexicanus* as an important evolutionary model with tractable genetic, genomic, and functional tools. In two genetic mapping studies, we have characterized and mapped two important behavioral traits in different cavefish populations, representing the first detailed QTL study on behavioral traits in this system. In a study published in *Science*, I worked on the role of canalization through HSP90 and the impact of standing genetic variation on evolution. Furthermore, I was integral in the first cavefish genome paper, establishing *A. mexicanus* as a genomic model system. I have since established the use of CRISPR/Cas9 in *A.* and developed new transgenic lines.

1. Kowalko JE, **Rohner N**, Rompani SB, Peterson BK, Linden TA, Yoshizawa M, Kay EH, Weber J, Hoekstra HE, Jeffery WR, Borowsky R, Tabin CJ (2013) *Loss of Schooling Behavior in Cavefish through Sight-Dependent and Sight-Independent Mechanisms. Current Biology* 23(19): 1874-83.
2. Kowalko JE, **Rohner N**, Linden TA, Rompani SB, Warren WC, Borowsky R, Tabin CJ, Jeffery WR, Yoshizawa M (2013) *Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of Astyanax mexicanus. Proc Natl Acad Sci USA* 110(42): 16933-8.
3. **Rohner N**, Jarosz DF, Kowalko JE, Yoshizawa M, Jeffery WR, Borowsky RL, Lindquist S, Tabin CJ (2013) *Cryptic Variation in Morphological Evolution: HSP90 as a Capacitor for the Adaptive Loss of Eyes in Cavefish Science* 342(6164):1372-5.
    - Highlighted by Pennisi E. Science. 2013 Dec 13;342(6164):1304 and by Burgess DJ. Nat Rev Genet. 2014 Feb;15(2):64. Featured in National Geographic and Scientific American.
4. McGaugh SE, Gross JB, Aken B, Blin M, Borowsky RL, Chalopin D, Hinaux H, Jeffery WR, Keene AC, Ma L, Minx P, Murphy D, O'Quin KE, Rétaux S, **Rohner N,** Searle SMJ, Stahl B, Tabin C, Volff JN, Yoshizawa M, Warren WC (2014) *The cavefish genome reveals candidate genes for eye loss. Nature Communications* 5:5307.
5. Klaassen H, Wang Y, Adamski K, **Rohner N**, Kowalko JE (2018) *CRISPR mutagenesis confirms the role of oca2 in melanin pigmentation in Astyanax mexicanus. Dev Biol.*
6. Stahl BA, Peuß R, McDole B, Kenzior A, Jaggard JB, Gaudenz K, Krishnan J, McGaugh SE, Duboue ER, Keene AC, Rohner N (2019) *Stable transgenesis in Astyanax mexicanus using the Tol2 transposase system. Dev.Dyn.* (Epub ahead of print).

3. Establishing *A. mexicanus* as a functional model for the study of starvation resistance and resilience against metabolic syndrome-like phenotypes

In the last five years, I have established the cavefish system as a model for metabolic evolution. I have found that as a response to the nutrient-poor environment in the cave, cavefish have acquired hyperphagia, higher fat metabolism, and better starvation resistance, in part due to a shift in fat development and mutations in MC4R. Additionally, I found that cavefish carry a structural mutation in the insulin receptor that leads to decreased binding efficiency of insulin. Interestingly, both identified mutations result in similar phenotypes (similar to that observed in obesity and diabetic patients) but without obvious pathological consequences (e.g. advanced glycation end-products) or altered longevity (cavefish live for more than 15 years). These findings raise the question of whether these resilience mechanisms are part of the physiological adaptations in cavefish that result in resistance to extreme starvation. To functionally validate the identified mutations, I have established *in vitro* and *in vivo* functional testing approaches in cell culture, zebrafish, and cavefish.

1. Aspiras A***, Rohner N***, Martineau B, Borowsky R, Tabin CJ (2015) *Loss of function mutations in MC4R drive adaptation of Astyanax mexicanus through hyperphagia*. *PNAS* 112(31):9668-73 * contr buted equa y
    - Featured by National Geographic, BBC Radio and the New York Times.
2. Riddle MR, Aspiras AC, Gaudenz K, Peuß R, Sung JY, Martineau B, Peavey M, Box AC, Tabin JA, McGaugh S, Borowsky R, Tabin CJ, **Rohner N** (2018) *Insulin resistance in cavefish as an adaptation to a nutrient-limited environment. Nature* 555(7698):647-651.
    - From the Cover. Recommended Faculty1000. Highlighted by Rétaux S. Nature. 2018 Mar 29;555(7698):595-597 and Kling J.Lab Anim (NY). 2018 Jun;47(6):152. Featured in Nature Podcast, the National Geographic and the New York Times.

3. Xiong S, Krishnan J, Peuß R, **Rohner N** (2018) *Early adipogenesis contributes to excess fat accumulation in cave populations of Astyanax mexicanus. Dev Biol*. 441(2):297-304.

Complete List of Published Work in my Bibliography:

http://www.ncbi.nlm.nih.gov/sites/myncbi/1hu2_a5G98cko/bibliography/49332521/public/?sort=date&direction=ascending

http://www.ncbi.nlm.nih.gov/pubmed/?term=nicolas+rohner

D. Research Support
Ongoing Research Support

Stowers Institute for Medical Research — Rohner (PI) — 09/01/2015-08/31/2021
Role: PI

[redacted] — McGaugh (PI) — 05/15/2018-04/30/2022
NIH
Genetic architecture underlying natural variation in sleep loss and obesity
Role: Subcontractor

Edward Mallinckrot Foundation — Rohner (PI) — 08/01/2018-07/31/2021
High Blood Sugar Homeostasis as a Non-Pathological Adaption in Cavefish
Role: PI

Juvenile Diabetes Research Foundation — Rohner (PI) — 05/01/2019-04/31/2021
Molecular basis of resistance to hyperglycemia in the natural model, *Astyanax mexicanus* (cavefish)
Role: PI

Enabling Discovery through GEnomic Tools (EDGE) [redacted] — Duboué (PI) — 09/01/2019-08/31/2022
NSF-BSF: EDGE CT: Functional Genotype-Phenotype Mapping in the Mexican Blind Cavefish, *Astyanax mexicanus*
Role: Co-PI

Completed Research Support
None

[redacted]

[redacted]

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 03/31/2020

1. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ○ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ○ Yes ● No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes ○ No

5. Change of Investigator/Change of Institution Section

❑ Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

Tracking Number: GRANT12922831 Funding Opportunity Number: RFA-RM-19-006 . Received Date: 2019-08-20T13:13:52.000-04:00

OMB Number: 0925-0001
Expiration Date: 03/31/2020

Introduction	
1. Introduction to Application (for Resubmission and Revision applications)	
Research Plan Section	
2. Specific Aims	
3. Research Strategy*	Research_Strategy_Essay.pdf
4. Progress Report Publication List	
Other Research Plan Section	
5. Vertebrate Animals	Vertebrate_Animals_Statement.pdf
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support	
10. Resource Sharing Plan(s)	
11. Authentication of Key Biological and/or Chemical Resources	Authentication_of_Key_Resources.pdf
Appendix	
12. Appendix	

RESEARCH STRATEGY/ESSAY

Project science areas:

8 HIB; 6 MCB

Project description:

A detailed experimental plan and extensive preliminary data are not provided in order to comply with the FOA.

Scientific opportunity. Adaptation to new environments has shaped evolution over the entire history of life. Understanding the genetic basis of adaptation has broad applications not only for a basic understanding of evolution but also for human pathologies given that many modern human diseases (e.g. diabetes, allergies, many types of cancer) are a secondary consequence of present humans not being adapted to modern lifestyles and western diets. Relative to this issue, some animals have evolved physiological phenotypes that resemble human diseases (e.g. fatty liver or insulin resistance) but intriguingly they do not exhibit the same underlying pathophysiological consequences. The phenotypes in these animals are part of the natural physiological response required for them to survive under certain nutritional and metabolic states. For example, migrating birds can store large amounts of fat in the liver in order to fuel their long journeys without the pathological consequences associated with liver fat in humans [1]. A deeper understanding of evolution and adaptation may yield a more complete understanding of such phenotypes, their links to pathology and the mechanisms underlying resilience (e.g. why does a fatty liver in birds not impair metabolism or cause inflammation?). **My long-term goal is to elucidate the genetic basis and molecular mechanisms underlying adaptive changes in animal physiology and use this knowledge to better understand what distinguishes health and disease.** This work will address the following questions:

1. How do animals adapt to periods of nutrient abundance followed by long starvation periods?
2. What are the mechanisms of extreme fat accumulation and starvation resistance?
3. What are the resilience mechanisms that prevent pathologies accompanying such extreme metabolic states?

I have developed Mexican cavefish as a powerful genetic model to investigate resilience to metabolic dysfunction. This system has important and unique advantages that make it ideal to address the questions of this application (see details below), and I am uniquely positioned to fully exploit this research organism. This work will lead to a greater understanding of how naturally occurring genetic variation results in metabolic adaptation to varying nutritional requirements. Studying the extremes will lead to a better understanding of the regulation of complex physiological interactions between different tissues involved in fat storage, molecular signaling pathways, and systemic metabolic homeostasis.

Biomedical importance of understanding fat storage and resilience to metabolic dysfunction. Most modern societies suffer from a variety of obesity-related diseases, including heart disease, non-alcoholic fatty liver disease, and diabetes mellitus. It is estimated that over 100 million people in the United States have some form of fatty liver disease; while worldwide more than 400 million people are estimated to suffer from diabetes [2]. Despite the application of high-throughput human genetics to identify genotypes associated with disease, there is still remarkable variability in how disease express themselves. While the majority of individuals with obesity develop insulin resistance and are of increased risk for type 2 diabetes, hypertension and cardiovascular disease, approximately 10-25% of obese individuals are metabolically healthy [3]. Defining this genetic variation between individuals may provide fundamental insights into the underlying basis of pathology and offer new avenues for treatment and personalized medicine approaches.

Similar observations have been made in animals. While pets and animals in captivity can suffer from excessive weight gain and associated diseases [4 5], there are animal species that display extreme fat accumulation or high blood sugar as part of their natural physiology. For example, arctic ground squirrels increase their fat mass 7-8-fold before entering hibernation [6], and hooded seals have blood sugar levels up to 430 mg/dL (4-6 times the amount of healty humans) [7], all without negative effects on metabolic health. These species must have evolved compensatory mechanisms to allow them to tolerate such metabolic conditions. A better understanding of these adaptative processes could in principle provide additional clues into resilience

mechanisms in humans and animal species. There are important differences between pathological processes and normal physiological adaptations. While humans are evolutionary adapted to different environments, our current sedentary lifestyles combined with an over abundance of high caloric foods is a rather new situation to which human evolution has not had time to respond appropriately. Hence, valuable insights maybe obtained by studying situations in nature where animals have evolved resilience mechanisms over a much longer evolutionary time period. This is particularly relevant for metabolic processes as there is an increasing recognition that responses to nutritional stress are regulated by highly conserved signaling pathways, such as insulin signaling and MTOR signaling in diverse organisms from *Drosophila* to mammals [8]. This means that insights from studying adaptations in the cavefish system are likely to apply much more generally to other systems. In this respect, it is important to note that fish metabolism is similar to the physiology of other vertebrates. Fish share a considerable homology with humans at anatomical, molecular, and pharmacological levels. Key organs (adipose, liver, muscle) required for metabolic control in humans are similar between mammals and fish [9]. Furthermore, mechanisms controlling glucose homeostasis are comparable in mice and fish; and the same tissues, primarily the liver, skeletal muscle, and adipose, are sensitive to insulin [10]. Moreover, like mammals, fish store excess nutrients in the form of lipid droplets in white adipocytes and liver tissues [11]. There are also important examples where research on emerging model systems in fish has proven to be a valuable tool for understanding human pathological processes e.g. [12].

Major challenges in the field of extreme metabolic adaptation. There is a large body of experimental work and field studies on starvation resistance in *Drosophila* species [8]. The underlying signaling mechanisms are conserved between invertebrates and vertebrates, but the tissues for fat storage are fundamentally different. For example, liver and white adipose tissues are vertebrate inventions and will require a vertebrate model. There have been comparatively few examples where natural metabolic adaptations to extreme nutritional conditions have been studied. In those cases where molecular analyses have been carried out, such as the small number of innovative studies of hibernation (e.g. [13]), research has been hampered by the lack of a closely related species that does not display the extreme metabolic phenotype. To find novel pathways or unanticipated cross-talk involved in regulating metabolic adaptations, one needs an unbiased genetic approach (e.g. forward genetic mapping). Genetic studies requiring hybrid crosses are not feasible when all members of a given species share the relevant phenotypes, such as migration or hibernation. Moreover, most mammals and birds are relatively large, have long generation times and few offspring per generation, making genetic screens difficult, if not impossible. In this regard, fish have many advantages over other vertebrate systems due to their large number of clutch sizes and the relative ease of housing and breeding many individuals.

The cavefish system (*Astyanax mexicanus*) provides a unique vertebrate system to study this in both an experimentally and genetically tractable manner. There are distinct, yet still inter-fertile populations of this species living in both river and cave environments. While the river ("surface") fish live in a rich ecological environment and have a relatively typical fish physiology, the cave populations have adapted to survive in conditions of extreme starvation through much of the year, punctuated by brief floods that bring excessive nutrients. They have evolved a dramatically altered metabolism, including massive capacity for fat storage (e.g. fatty livers) and glucose regulation (e.g. insulin resistance) (described in detail below). As discussed below, the genetic and genomic tools are now in place to exploit these fish, in a manner similar to those that have allowed the zebrafish to serve as a powerful system for scientific discovery. My track record and contributions to developing tools in this system and the state of the art facilities at the Stowers Institute make me uniquely poised to exploit this system to study metabolic adaptations. Moreover, my active collaboration with [redacted] (translational expertise in glucose/lipid metabolism and fatty liver) provide additional resources that will lead to discoveries relatable to human health.

The cavefish model system. *A. mexicanus* is a small river-dwelling species that invaded the limestone cave system of the Sierra del Abra Mountains in Mexico at least four independent times around 150,000 years ago [14]. Under similar ecological pressures, the fish have evolved very similar traits in each of the caves they colonized (Fig. 1). This allows us to study repeated evolution and determine whether the same genes and genetic pathways versus different pathways are utilized to achieve the desired phenotypes. Moreover, the ecological conditions of the caves have been well studied. These include a total absence of light, a lack of predators, and – most relevant for the current study – no internal food sources (due to a lack of light/energy entering the system). Almost all the available food originates in seasonal floods that occur during the wet summer season. During the rest of the year, the fish are under starvation or near-starvation conditions, setting

the stage for physiological adaptations that allow them to nonetheless thrive in the cave environment. Importantly, the fish of the different caves and the surface fish that live nearby are of the same or very closely related species. This is critical for two main reasons: 1) The populations remain interfertile, allowing genetic analysis of the inheritance of the cave-specific and surface-specific traits, thus enabling study of genetic complementation. 2) Comparative transcriptomic or metabolomic studies can be more reliably interpreted (RNA-seq comparisons between different species are often problematic due to species-specific effects).

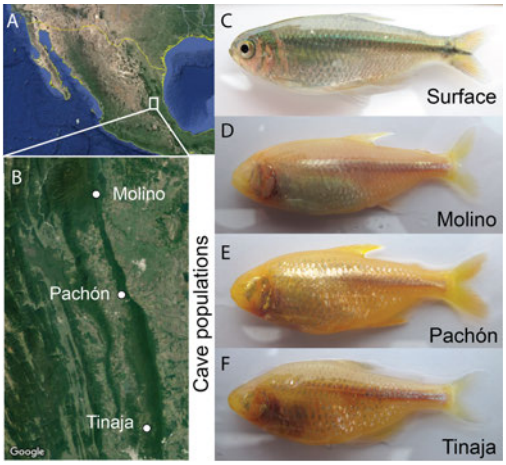

Fig. 1. The research organism. (A,B) Location of *A. mexicanus* in Mexico (C) Surface form (present throughout the entire region). (D-F) Three independently derived cave populations.

Development of tools for functional analysis: The cavefish system is perfectly suited to take advantage of large clutch sizes, external fertilization and the ability to use existing zebrafish Tol2 constructs. Several labs, including my own, have devoted considerable effort to building the tools needed to exploit this unique system. There are now high-resolution genetic maps [15], transcriptome databases [16], standardized breeding and in-vitro fertilization protocols [17 18] transgenic lines [18 19], methods for altering gene expression [20], and, recently, gene editing [21-23]. The genome of *A. mexicanus* has also recently been assembled [24], further developing it as a tractable model system.

Cavefish are a model for studying resilience to metabolic dysregulation. It has long been appreciated, that there are robust metabolic differences in cavefish, but until recently almost nothing has been known about the molecular nature of these differences. As might be expected for organisms adapted for living in an environment where food is scarce, we found that the cavefish are extremely starvation resistant [25]. Conversely, when food supplies are available, some of the cavefish populations display increased appetites (hyperphagia) and consequently achieve much higher body fat levels than do the surface fish [25]. We found that at least part of the genetic cause of hyperphagia in the cavefish is the presence of coding mutations in the melanocortin 4 receptor (MC4R) gene. MC4R loss-of-function mutations are known to cause hyperphagia in mice, and the amino acid mutated in the cave populations correlates with the location of causal mutations in cases of congenitally obese patients [26]. Moreover, we directly demonstrated that the cavefish MC4R allele is less sensitive to its ligand, MSH, than is the allele found in the surface fish and genetically mapped the starvation resistance in cavefish to this genomic locus. These observations not only establish the feasibility of our approach, but they also support our hypotheses that fundamental metabolic regulatory mechanisms are conserved between fish and humans. These results are in line with zebrafish studies showing that physiological functions such as energy balance and appetite control by the hypothalamus are conserved in vertebrates [27].

In a second study [28], we found that the cavefish populations have elevated blood sugar levels compared to the surface forms when fed a regular diet in the laboratory. We have studied this phenotype in detail and found that cavefish are glucose intolerant and have insulin-resistant skeletal muscles due to a mutation in the insulin receptor. The same mutation is known to cause insulin resistance in humans. We have functionally validated that the mutation causes a reduction in binding of insulin to the receptor and is sufficient to cause insulin resistance when introduced into zebrafish. In contrast to human patients, however, the mutation causes the fish to grow faster under a food-limited diet, giving them a potential adaptive advantage in the nutrient-limited cave environment. Interestingly, some of these cavefish populations do not develop elevated advanced glycation end products (AGEs), a common complication in patients with elevated blood sugar. Furthermore, cavefish live long and healthy lives (>15 years), similar to surface fish that do not carry the mutation that causes insulin resistance. We hypothesized that cavefish have evolved resilience mechanisms that allow them to tolerate the negative effects of high blood sugar and take advantage of the growth phenotypes associated with the insulin receptor mutation. The mechanism underlying both the growth advantage and the protective phenotypes are not understood. Both the similarities and differences between the fish physiology and human physiology will be the subject of further interrogation in this grant.

Physiology of starvation resistance. One can postulate three types of strategies to increase starvation resistance: 1) increasing the ability to sequester higher energy reserves, 2) lowering the use of these energy reserves, and 3) lowering the threshold of required energy for maintaining homeostasis. We have found evidence for all three of these in the cavefish populations of *A. mexicanus*. The aim of this proposal is to provide detailed insight into the genetic and molecular mechanisms underlying these strategies. We have compelling published and unpublished data suggesting that cavefish have distinct physiological responses to the availability of food, which include a dramatically altered glucose metabolism and a predisposition to store large amounts of lipid in white adipose tissues and in the liver. At the same time, they appear to be protected against fat-induced inflammation (bioRxiv 647255 https://doi.org/10.1101/647255) and the consequences of elevated blood sugar levels [28]. Despite the identification of some genetic (coding) changes that serve as a proof of principle that the genetic basis can be studied, it is clear that many more genomic loci (including regulatory changes) are involved in the drastic changes and more comprehensive methods are needed to untangle the molecular and genetic networks in use. This will not only be important for characterizing the physiology of these fish but also will pave the way for subsequent gene discovery to get at the underlying regulatory mechanisms at play in their altered response to glucose and fat acquisition.

This proposal has two primary goals:

(1) We will leverage state-of-the-art genomic, genetic, and functional tools to determine the molecular mechanisms of how cavefish are able to withstand such long starvation periods.

(2) We will identify the genetic pathways and strategies associated with resilience to metabolic dysfunction.

Drawing on our lab's extensive genetic experience, I propose to use a variety of integrative approaches and strategies to leverage the full innovative potential of the *A. mexicanus* model system: (1) Transcriptome and chromatin occupancy analysis (i.e. RNA-seq and ATAC-seq) combined with lipidomics and metabolomics analysis, (2) genetic mapping and complementation approaches (e.g. QTL), (3) functional testing (e.g. CRISPR/Cas9) and (4) mechanistic studies using novel transgenic lines.

Analysis of regulatory networks and transcriptional and metabolic states. In preliminary experiments, we have used RNA-seq to interrogate liver samples from both surface and cavefish, examining both fish fed *ad libitum* and those under a 3-month starvation regime. We found the greatest expression differences between transcripts in livers of fed and starved surface fish and the smallest differences were observed between the livers of starved and fed cavefish (Fig. 2). This data underscores our previous observation that cavefish are more starvation-resistant than surface fish. While our preliminary work has mostly focused on known candidate genes, this dataset provides an unbiased approach to discover novel genes and pathways involved both in starvation resistance and in the resilience phenotypes of cavefish.

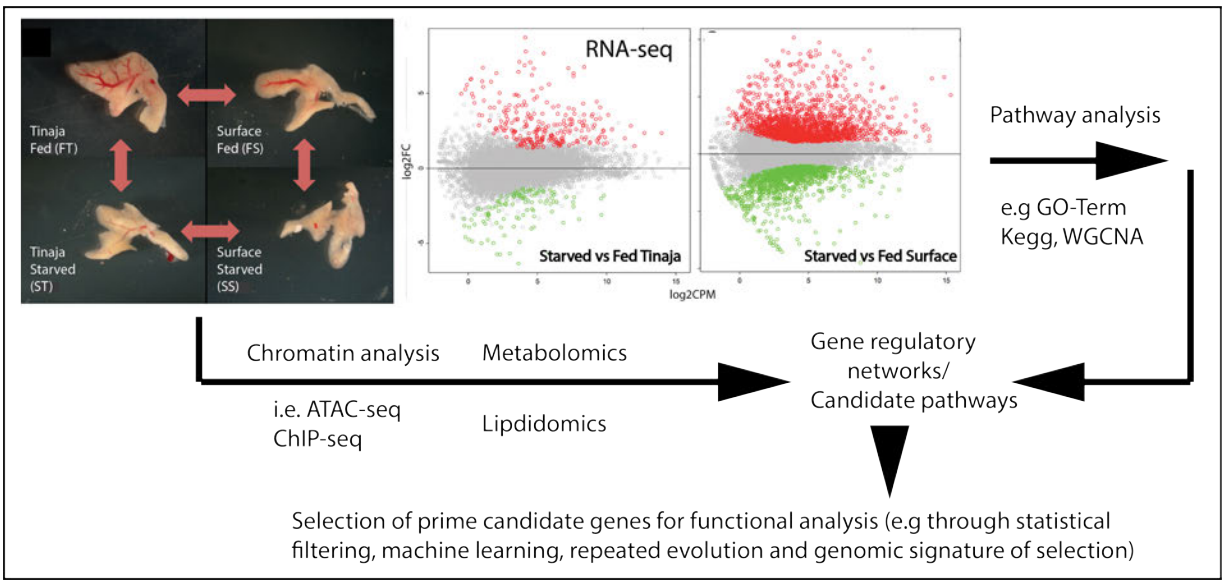

Fig. 2. Transcriptomics, chromatin analysis, & metabolomics.
Workflow of RNA-seq, ChIP-seq and lipidomics of cavefish and surface fish tissues (e.g. the liver) will provide insight into the regulatory networks of cavefish metabolic adaptation.

GO-term analysis revealed important candidate pathways that are involved in lipogenesis, cholesterol synthesis, and autophagy that can be systematically tested and functionally validated *in vivo* (see below). In this respect, it is worth mentioning that 82% of human disease genes in OMIM (Online Mendelian Inheritance in Man) have a fish orthologue [29]. To get at the regulatory networks and to narrow down to key pathways, I propose to further analyze the dataset bioinformatically using Kegg pathway, SNP, and weighted correlation network analysis (WGCNA). In addition I want to integrate it with data on chromatin states. As regulatory elements are often associated with characteristic epigenetic marks like histone modifications or binding of certain transcription factors, I propose to create genome-wide maps for the epigenetic states and binding properties of putative regulatory factors. We will use ATAC-seq (**A**ssaying **T**ransposon **A**ccessible **C**hromatin) to map open chromatin. As ATAC-seq can give thousands of open chromatin regions – many of which may not be functional enhancers or promoters – we will supplement the data with low-cell ChIP-seq against histone modifications of H3K4me3 and H3K27ac and enhancer-binding protein p300, which will enhance identification of active promoters and enhancers. By combining the data from these experiments, we will be able to generate genome-wide maps comparing gene expression profiles and putative regulatory regions that are poised or active in a tissue under starved versus fed experimental conditions. This work will be performed in adult liver tissue, the most metabolically active organ in fish. In parallel, we will supplement the genomic analyses with lipidomics and metabolomics of liver and fat tissues. Cavefish provide a perfect application for metabolics approaches given that the two very closely related surface and cavefish populations display substantial differences in their metabolism and fat deposition. We will analyze liver, fat, and muscle of three independently derived *A. mexicanus* cavefish populations (Pachón, Tinaja, Molino) and surface fish using the core services of the West Coast Metabolomics Center. In discussions with them we have determined the numbers of animals and tissues necessary to obtain statistically valid and interpretable results. We will use the following assays: analysis of primary metabolism by GC-TOF MS (carbohydrates, sugar phosphates, amino acids, hydroxyl acids, free fatty acids, purines, pyrimidines, aromatics, and exposome-derived chemicals) and complex lipids by CSH-QTOF MS/MS (ceramides; sphingomyelins; cholesteryl esters; oxysterols; lyso- and phospholipids; mono-, di- and tri-acylglycerols; and galactosyl- and glucuronyl-lipids).

Epigenetic and metabolomic approaches are susceptible to environmental variations. To minimize environmental noise, all experiments will be performed in laboratory-raised fish under the same water conditions and feeding regimes. We will also use biological replicates (in the case of the metabolomic studies, we will use up to ten age-, size- and sex-matched individuals). However, it is still likely that such a large-scale genome-wide analysis will provide us with an overly long list from which to narrow down individual candidate pathways to functionally test will be challenging. One strategy we will use to minimize false positives is to focus on cases where there is a correlation between changes in the chromatin states with differential gene expression in the liver-specific RNA-seq data, which would represent putative examples of cis-changes in expression. We will also take advantage of the different signatures observed in the three cavefish populations in our analyses. As the different cavefish populations have undergone parallel and convergent evolution, such a comparison can identify genes that show similar patterns in all the cave populations. The ability to have independent evolutionary experiments available is a unique and strong advantage of the system. These measures should increase false negatives and reduce false positives. For this approach to be effective, we will be using a variety of statistical approaches (e.g. Gene set enrichment analysis using correlation-weighted Kolmogorov–Smirnov statistics) and we will use machine learning to integrate the large datasets with other available datasets including large scale human GWAS studies on population differences in diet preferences (e.g. Greenlandic inuit [30]). I hypothesize that these integrated datasets will lead to the identification of a small number of key pathways and networks of target genes that are involved in the metabolic adaptation of cavefish to the unique cave environment. If all these measures still result in a large list of candidate genes we will prioritize analysis of genes by chosing genes with clear human orthologs and we will focus on genes that show signatures of selection in the cavefish genome from population sequences that we have access to [14].

Mapping the physiological phenotypes of cavefish to the genome, including the ability to withstand the formation of advanced glycation end products in cavefish populations. While “omics” approaches are important in providing a comprehensive overview of differentially regulated genes and gene products, ultimately, identifying the genetic changes responsible for the phenotypic differences between surface and cavefish requires a genetic approach. Quantitative trait locus (QTL) analysis is a useful method for model systems in which fertile hybrids can be generated, and it permits identification of genetic regions linked to the

phenotypes being studied. Not only is it possible to identify these genetic intervals, it is also possible to dissect the influence of different genetic factors (multiple genes, epistasis). It also provides information about genetic convergence when used in different populations. *Astyanax* has been a prime system for QTL analysis over the last decade, and I have extensive experience in using this technique [31 32]. I propose to map a variety of different metabolic traits. In particular, we will map and quantify: liver size and weight, liver lipid, visceral fat, insulin and glucagon, postprandial blood glucose, fasting blood glucose, glucose tolerance, body weight, appetite, and size – both under fast and fed conditions. These are traits that can be obtained semi-automatically from a single fish in consecutive measurements. One additional trait that I am particularly excited about is the resistance of cavefish to developing advanced glycation end-products (AGEs). While the other traits will be mapped using surface/cave hybrids, this particular trait will take advantage of the ability to generate fertile hybrids between different cave populations that show different levels of AGEs under high sugar levels (Fig. 3). The Tinaja and Pachón populations do not develop elevated AGEs under high blood sugar levels while another cavefish population (Molino) does [28]. The ability to generate fertile hybrids between these three populations in principle allows mapping of the genomic loci involved. I have already generated F1 Tinaja/Molino and Pachón/Molino hybrids, and I am currently generating 2000 F2 adult fish in the cavefish facility at the Stowers Institute. This proposed sample size should allow detection of loci that explain ≥3% of the variance for the trait (at a LOD significance threshold of 4) within a narrow genomic range (< 4cM for 95% confidence intervals). The F2 will be exposed to a high sugar diet for 3 months, genotyped, and the levels of AGEs measured. While QTL mapping alone may not yield the identity of specific genes, combined with the above-mentioned RNA-seq and ChIP-seq data, it will allow me to unravel the genetic networks of these traits.

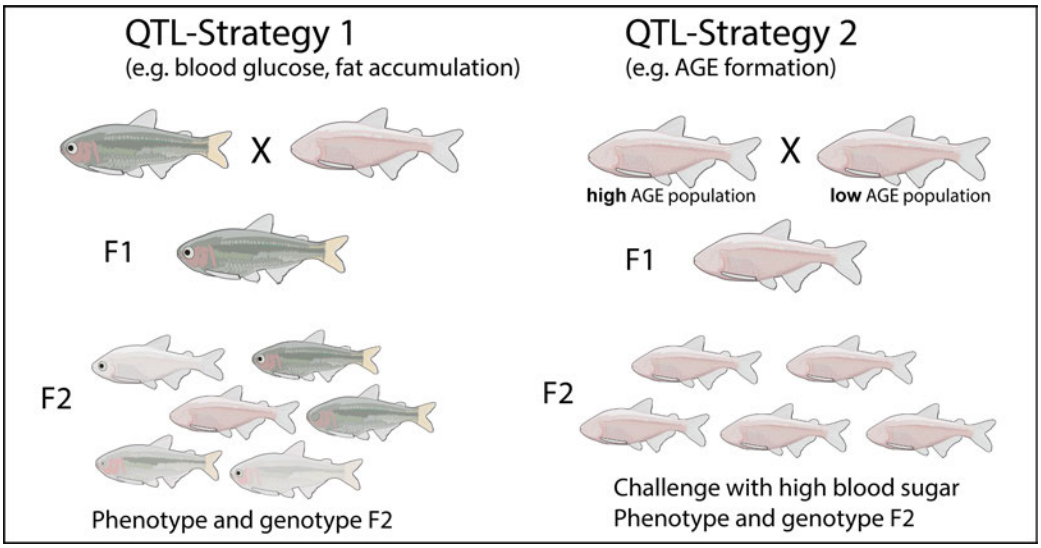

Fig. 3. Quantitative trait loci analysis (QTL). QTL analysis of metabolic traits that differ between surface fish and cavefish (left) or between different cavefish populations (right) will allow for the identification of genomic regions that are genetically linked to the metabolic adaptations of cavefish. Gene editing will allow the linking of genotype to phenotype using functional validation.

Since we previously were able to detect QTLs (even of rather variable traits such as behavioral adaptations) with a sample size of less than 300, the ability to precisely measure metabolic traits combined with the large brood sizes of cavefish, will allow us not only to uncover a smaller effect size QTL but also reveal a reasonably small interval size of major QTL. However, we know from our own experience as well as work in other labs that not all traits will prove mappable. For example, the genetic architecture of some traits involves a large number of genes, each of small effect, and others may be extremely difficult to take to the level of specific genes. Nonetheless, the ability to precisely quantify the traits of interests combined with the well-established linkage maps makes a mapping approach practical and worthwhile. If we are not able to obtain QTLs or the interval sizes are too large to pinpoint individual genes, we are prepared to increase the number of F2s and use backcrosses to increase the frequency of recombination events. The chances of success are enhanced because we are mapping the trait in three independently derived populations (Tinaja, Pachón and Molino). In addition we can integrate the data with population data we have access to [14] in order to perform genome-wide scans for selection to detect loci under natural selection, using differences in allele frequencies between populations.

Functional analysis using gene editing approaches. The ultimate goal in comparative strategies is to functionally test the identified genes and pathways *in vivo* and to integrate the results with other animal

systems. There are undoubtedly many changes in the cavefish genomes that contribute to these adaptations. Determining the phenotypic effect of each of these genetic changes requires placing them into the background of the surface fish genome in the absence of any other cave-specific mutations. In my lab at the Stowers Institute, we are set up to conduct such functional studies. The method of choice is currently the CRISPR/Cas9 system. We have established CRISPR mediated knockouts in cavefish [23], and we currently expanded the repertoire of CRISPR in cavefish by establishing homologous recombination. This will allow us to introduce specific cavefish alleles into the endogenous loci of the surface fish. These studies are important in laying the foundation for studying whether the observed mutations are sufficient or necessary for the respective phenotypes. These studies will be performed with the help of the advanced fish and molecular biology facilities at the Stowers Institute. In initial collaborations, we have successfully established protocols to introduce single nucleotide changes into the zebrafish genome using homologous recombination via CRISPR/Cas9 [28], and we will continue to collaborate with the facilities in order to establish the latest advances in the field of gene editing in the cavefish system. We will use the same technique we used before for swapping zebrafish alleles. In addition, we will test engineered and novel nucleases to expand the targeting coverage (e.g. Cas12a) and will explore a variety of new base editors (e.g. adenine base editors) [33]. If there are technical challenges that delay the feasibility of the technique, we are prepared to do preliminary characterizations in zebrafish. It is possible to introduce an entire surface fish and cavefish gene into the zebrafish genome and compare gene function outside of its species-specific context. To extend the relevance of our findings between fish and mammals, we can also test the candidate genes in an established, high-fat-diet obese mouse model, which mimics the characteristic symptoms of diabetes and metabolic syndrome such as hyperinsulinemia, hyperlipidemia and hyperglycemia. Such an approach is challenging but feasible [34].

Mechanistic analysis using transgenic lines. While the functional validation will help us to link genotype to phenotype, we may not fully understand the mechanisms behind the observed phenotypes. For example in a related study that is not part of this grant (bioRxiv 647255 https://doi.org/10.1101/647255), we have found that cavefish display lower inflammation in fat tissues despite large amounts of hypertrophic adipocytes. We detect less expression of pro-inflammatory cytokines (e.g. IL-1β, TNF-α, and IL-6) and an almost total absence of crown-like structures (macrophages surrounding dying or dead adipocytes). In contrast, surface fish that were fed a high fat diet develop elevated levels of both pro-inflammatory cytokines and high numbers of crown-like structures in their visceral adipose tissue. These results shed light on some of the underlying mechanisms of the cavefish resilience against large hypertrophic fat depots [35]. However they require the development of new transgenic lines to visualize macrophages and other proinflammatory eliciting cells. Similarly, the study of the insulin resistance phenotypes would benefit from lineage tracing experiments and in vivo imaging approaches using transgenic lines. In order to link the observed metabolic phenotypes with mechanistic understanding, we plan to generate a battery of new transgenic lines for: insulin labeling, liver specific expression, and marking adipose precursors. In addition we propose to take advantage of a novel transgenic line that we have recently generated [19], which expresses GFP ubiquitously in all cells and tissues, including the fat and liver tissues. We will use the line to transplant GFP-positive cells into cavefish recipients to test for cell-autonomous vs. non-autonomous effects. Similar experiments have been successfully performed in zebrafish [36] allowing for detailed mechanistic analysis. It is for example possible to transplant surface fish cells into cavefish and monitor how the cell clones will behave in the context of the host. Taken together, these results will allows us to link genotype to phenotype from a detailed mechanistic perspective. We expect many more tools to emerge in the next three years, as my lab together with three other cavefish labs has recently been awarded the NSF EDGE grant, which is a novel grant mechanism to develop tools for emerging model organisms. In this we proposed to establish attP integration sites and the generation of an inducible GAL4 system for tissue and temporal specific manipulation. All these tools will facilitate a mechanistic understanding of the impressive metabolic adaptations in cavefish and further our knowledge of many basic processes in human physiology.

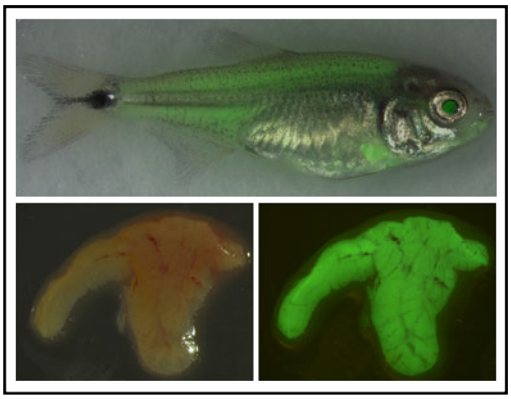

Fig. 4. Transgenic lines will facilitate mechanistic understanding
Example of a transgenic line recently generated in *A. mexicanus* surface fish ubiquitously expressing GFP.

Experimental planning, sex and circadian rhythm as a variable. For all previous studies and for future results, care has been taken in the planning of the experiments to assure robust and unbiased results. This includes strict adherence to the scientific method and application of validated and appropriate statistical tests for significance of all results obtained. It is particularly important in this context to account for potential sex-based physiological differences in analyzing the metabolic aspects of the cavefish so that unintended skewing of sex ratios does not falsely affect the statistical tests. This is particularly important, as it is known that blood sugar levels can be influenced by gender differences in mice and humans. Even though the situation is less clear in fish, and zebrafish do not display gender differences in glucose homeostasis [37], we have taken particular care in addressing this variable. For experiments that utilize juvenile fish, this concern is minimized, as at that stage, sex is still undetermined biologically. In experiments that use adults, however, careful monitoring of the different sexes is performed. In this regard, we have observed no sex-specific differences in preliminary studies for most of the phenotypes being analyzed (e.g. both male and female fish from cave populations develop fatty livers, while both males and females of surface populations do not). There are differences in some of the phenotypes, such as total fat content and starvation resistance. Hence, it is important to keep the sexes separate for analysis. For the "omics" approaches it is critical to keep biological variables to a minimum, so sexes will be kept strictly separated. For the QTL analyses the more fish that can be entered into analysis, the greater the statistical power. For this reason we will pool male and female data and keep track of the sex of each F2 fish, and the computational genetic analysis will also be carried out individually on each sex. Our expectation is that the same QTL will be identified in each sex, but with higher LOD scores and (importantly) narrower critical intervals using the pooled data. Should this turn out to be incorrect, we will focus on the loci identified in sex-specific analyses in subsequent work. An additional biological variable in the context of the proposed studies is the potential impact of the circadian rhythm on metabolism. To address this, each of the assays will be tested in parallel on surface fish raised in the dark as well as at several random time points for cavefish raised either in the dark or in the light. Although cavefish do not show a circadian rhythm in the cave environment, in the laboratory (under regular feeding and lighting conditions) cavefish retain a food-entrainable clock that oscillates with an infradian period [38]. I therefore speculate that the effects will be marginal, and we will be able to control for it using the additional sampling. If, however, this variable proves problematic, we can conduct the entire set of proposed experiments raising the fish entirely under dark conditions using commercially available fish tank encasements.

Innovativeness. This project is innovative, as it not only aims to understand the genetic and molecular mechanisms underlying metabolic adaptations such as starvation resistance in a natural species but also will focus on the specific resilience mechanisms these animals have evolved as a response to extreme metabolic states such as fatty liver, high blood sugar, and hypertrophic adipocytes. There are many examples in nature where animals have to withstand long starvation periods and, as a consequence, increase their body fat levels and develop diabetes-like conditions. However, exploiting this variation has been problematic in the past, mainly due the lack of a closely related species with a more normative phenotype and lack of established genetic vertebrate models. This work is only now possible because I have established Mexican cavefish as a model to investigate resilience to obesity and diabetes[28]. This system has the advantage of utilizing two different populations of the same species with radically different phenotypes, thereby allowing us to combine the power of genetics with more traditional molecular, cellular, and metabolic analysis. By comparing these two populations on a genome-wide level, we will be able to identify genetic changes the cavefish have acquired to circumvent negative health consequences that are usually associated with such extreme metabolic alterations. The central innovation of this proposal lies in the recognition that the specialized physiological adaptations of the cavefish are an extraordinary resource for gaining new insight into metabolic regulation and preservation of homeostasis in the face of fluctuating environmental conditions. This innovation consists of two areas. *1) Conceptual innovation*: the realization that cavefish display extreme differences in their lipid and glucose responses from their river counterparts. *2) Technical innovation*: the system is extraordinarily well suited to genetic analyses and gene discovery. Thus, our approach provides an exciting opportunity for achieving new knowledge regarding the regulation of metabolism.

Investigator qualifications. I am uniquely poised to undertake the proposed research. I am an early career investigator with a strong track record in developmental biology, evolutionary genetics and genomics. I have published high-impact papers as a graduate student [redacted], as a postdoc with [redacted], and as senior author of my own lab. I have substantial experience in the model systems that will be

used for the research proposal (>9 years with cavefish, >14 years with zebrafish, and >8 years with mouse if necessary). I have substantially contributed to establishing the emerging model system ***A.mexicanus*** as a widely recognized organism, for which genetic and genomic tools are available. I am actively promoting cavefish as an important vertebrate model system for the study of developmental- and evolutionary-related questions, including its resilience phenotypes. These efforts include the establishment and maintenance of a community website (cavefin.org), the organization of the International Cavefish Meeting in 2017 in Mexico and the upcoming Meeting in 2021, and the publication of review articles and book chapters promoting comparative approaches. Since my start as an independent investigator, I have given 12 invited plenary presentations at major international meetings and 18 departmental seminars. As part of being an active member in the community, I maintain strong collaborative ties (currently around 15 labs distributed over the US, Europe and Latin America), and I am part of several collaborative grants using the cavefish system to study the interaction of metabolism and behavior, funded both by NSF and NIH. I have substantial experience in the necessary techniques and applications, which will facilitate the proposed research. I can also fully utilize the unique environment at the Stowers Institute, which has exemplary resources for accomplishing the proposed project. I have the necessary lab management skills to execute the proposed project by leading, managing, motivating and supervising my team members. This skill set has been enhanced by taking a lab managing course before starting my lab and has since been successfully emplo ed in a rowin and diverse lab of currentl nine members ran in from under raduates to postdocs . [redacted]. In addition to regular meetings and interactions, [redacted] is also a standing member of the thesis committee o e gra uate studen my lab. T the expertise, training, motivation, and support necessary to successfully carry out the proposed research project.

Suitability for the New Innovator Award program. The current proposal is an ideal fit for the New Innovator Award program. My overall research program and the experiments described within this proposal are truly pioneering and unique to my group. Although my work is certainly shaped by the expert mentorship that I received as a postdoc under Cliff Tabin, my research is not derivative of past or ongoing work in his lab. I am confident that, given my dual training in genetics and evolutionary and developmental biology, my group is uniquely poised to make groundbreaking discoveries about metabolic adaptation. However, I am still relatively early in my independent carreer and as such have not yet accumulated extensive unpublished data required by more traditional grants. In addition, the high-risk nature of some of the experiments, (e.g. the "omics" analysis), are generally not suited to more conservative grants. Finally, the large impact that studying the genetic basis of metabolic adaptation can have in contributing to our understanding of resilience in human populations is not currently widely appreciated. Innovative research, like that proposed in this grant, will change this.

Statement of research effort commitment. I am committed to dedicate a minimum of 25% of my research effort to be the PI of the proposed project.

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PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001 and 0925-0002

Expiration Date: 03/31/2020

Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Does the proposed research involve human specimens and/or data	❍ Yes ● No
Other Requested information	

Vertebrate Animals

This work utilizes several distinct populations of the Mexican cave tetra, *Astyanax mexicanus*, as a model system. Work with this species and work on zebrafish (*Danio rerio*) and mouse (*Mus musculus*) has been approved by the Stowers IACUC (Protocol Number: 2019-084, 2019-096 and 2019-080.)

1. Description of procedures

The fish are housed in a specialized satellite aquatics facility that meets all the standards of the American Veterinary Medical Association (AVMA). Cave fish populations (*Astyanax mexicanus*) are derived from wild-caught populations and are bred and maintained in the laboratory facility. Zebrafish (*Danio rerio*) and mice (*Mus musculus*) are derived from common laboratory stocks. Experimental procedures carried out in fish include, overfeeding, food deprivation, injection of nutrients such as glucose, and blood draws for metabolic assays. Fasting fish will have food withheld for 2 months. During this time, fish typically lose ~30% body mass. While most mammals would be unable to tolerate 30% loss of mass, these fish are cold blooded and have inherently different metabolic requirements (i.e. do not need food to maintain body temperature). They are expected to completely tolerate their weight loss. Indeed, in the wild it has been documented that these fish survive at least 9 months without food. Blood collection is by cardiac puncture (or dorsal aorta puncture). Both methods are accepted methods for repeated blood draws in zebrafish. In total, we anticipate using 2500 juvenile and adult fish in the course of the proposed studies, the majority of these being required for genetic mapping studies, which need large numbers to attain statistical power. Experimental procedures carried out on mice include: High Fat Diet, Drug Treatment, and Blood Collection. On the High Fat Diet, mice will be given a variety of high-fat diets: 10%, 45% and 60% fat. The mice will be maintained on these diets for a maximum of 6 months. Blood Collection will be by cardiac puncture, or dorsal aorta puncture. Both methods have been used previously for repeated blood draws in mice. In total we anticipate using less than 500 mice.

2. Justification

The goal of this work is to understand the genetic underpinnings of metabolic regulation in response to changes in nutrient availability. This is a physiological response that cannot be studied in tissue culture or cell free systems. Among vertebrate animals, cavefish have a unique response to starvation and their study promises to lead to the novel discovery of potential importance to treatment of human metabolic diseases. Moreover, the cavefish are also unique in having conspecific surface populations which lack their specialized metabolic traits, which is critical for comparison and genetic analyses. This work cannot be done in another system. The work on mice could become necessary to validate candidate genes we find in cavefish, in a mammal system to test the potential relevance in humans. This work cannot be done in alternate systems.

3. Minimization of pain and distress

Fish are removed from the experiment when they have lost more than 30% of their original body mass. Fish are anesthetized with tricaine methansesulfanate for 30 seconds, or until disoriented, prior to injections and blood draws. Blood collection is by cardiac puncture (or dorsal aorta puncture). The duration of discomfort is minimal and the injury will be minor. Fish are monitored immediately after blood draws for abnormal swimming patterns. Fish that fail to recover within an hour after blood sampling are euthanized. In all cases, every effort will be made to minimize stress on the animals, including social housing whenever possible. In some cases, to control the amount of food individual fish receive, it will be necessary to house fish separately for the duration of the experiment. Environmental enrichment will be provided in the form of plastic plants and tubes in which the fish can hide. We expect the mice to become obese. They are expected to become hyperinsulinemic, hyperglycemic and hyperlipidemic. If this affects their ability to stand on their hind legs to obtain food or water, advice will be sought from the attending veterinarian and appropriate accommodations will be made if necessary. Mice will be monitored regularly for obvious signs of distress, which could potentially arise due to increase in body mass, and euthanized if necessary.

4. Euthanasia

Euthanization will be carried out following protocols consistent with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia of Animals.

Authentication of Key Biological and/or Chemical Resources

The key biological resource being used in the proposed studies is fish of the species *Astyanax mexicanus*. There are multiple populations of *A. mexicanus* employed in this work, including both surface (river) fish and several distinct cave populations. The surface fish can be easily distinguished morphologically (have eyes, pigment). Authentication of the different cave populations is not as easy, but their identity can be ascertained by more subtle morphological traits (head shape, fin length, etc.) as well as through verification using established molecular markers. Fish are carefully recorded and housed in small groups in tagged tanks to keep accurate track of their population of origin, breeding history, and age. In addition we use visible implant elastomer tags to color tag and identify individual fish in the tanks. The zebrafish line that will be used is AB, which is the main zebrafish line used at Stowers Institute. The line is maintained and its identity regularly controlled by the Aquatics Facility.

Antibodies will be validated and experiments with antibodies performed according the guidelines set forth by FASEB in their 2016 Enhancing Research Reproducibility recommendations. All other biological and chemical reagents are standard laboratory reagents that are routinely purchased from major reputable biological companies.