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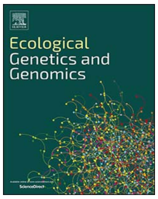

Gene expression during delayed hatching in fish-out-of-water

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ABSTRACT

"Fish-out-of-water" offer ecologists and evolutionary biologists ideal opportunities to study the evolution of stress resistance in vertebrate species. Annual killifishes (Cyprinodontiformes: Aplocheiloidei) constitute one of the most intriguing systems to study fish-out-of-water. "Annual" fishes possess a suite of complex and key features that include diapause stages and desiccation resistance, allowing them to complete their life cycle in seasonal bodies of water. Embryos of some non-annual killifishes have been shown to exhibit pre-hatching delays or symptoms of dormancy (similar to diapause), therefore, these species may represent an intermediate phenotype in the evolution of an annual lifestyle. The non-annual killifish *Aplocheilus lineatus* undergoes such hatching delay during aerial incubation. We use this species to study gene expression differences among water-incubated and aerially-incubated embryos. Differentially expressed genes are characterized and compared with expression patterns during diapause in annual species and with other developmental stages in non-annual killifishes. The annotation of 560 differentially expressed transcripts provides insight into how delayed-hatching embryos of *Aplocheilus lineatus* react to aerial incubation and suggest that delayed hatching is a phenomenon distinct from the diapause stages of related annual species. Similar patterns of gene expression are shared among *Aplocheilus* and other egg stranding and amphibious fishes.

1. Introduction

Organisms from all domains of life are known to survive under extreme conditions, enduring life-threatening levels of temperature, salinity, pH, pressure, or radiation [1], most likely because they possess unique adaptive traits. Understanding the origin of such traits is of central interest to evolutionary biologists because these traits are tightly linked to habitat use and organismal fitness. In aquatic environments throughout the world, from the deep ocean trenches to high mountain streams, many fish species are able to live in extreme habitats with acidic, hypoxic, or hypersaline water, as well as in underground water in complete darkness, in Antarctic sub-zero temperatures, and in temporary pools [2,3]. The expression "fish-out-of-water" is often used to describe a stressful situation, a hostile environment, or reduced chances to survive. However, a considerable number of fish species have adapted to desiccation and are able survive out of water for extended periods of time [4–7]. Fish-out-of-water provide unique opportunities for scientists to study the evolution and molecular mechanisms of stress response and adaptation to harsh environments.

Annual killifishes (Cyprinodontiformes: Aplocheiloidei) constitute one of the most intriguing systems to study special adaptations in vertebrates. The term "annualism" is used to describe the life history of these fishes that are uniquely endowed with a suite of complex and key features. Critical components of their life history include stress-tolerant embryos that can survive encased in dry substrate protected by thick, structurally diverse egg envelopes [8], a short adult lifespan that rarely exceeds one year, and a distinctive ability to enter up to three embryonic diapause stages (abbreviated DI, DII, and DIII), [9–11]. Annual killifishes thrive in seasonal habitats and can complete their life cycle in ephemeral pools. When their habitat dries, all adults die, but fertilized eggs buried in the substrate survive out-of-water until the next rainy season floods the habitat, and the larvae hatch to set off the next generation.

The evolution of annualism and associated dormant stages from non-annual killifish ancestors occurred independently in several lineages of aplocheiloid fishes [12–16]. However, delayed hatching and variable degrees of desiccation tolerance also are traits commonly found in non-annual killifish and many more distantly-related atherinomorph fishes, most of which regularly breed in unstable habitats that are subject to desiccation. The ubiquity of desiccation tolerance, and delayed hatching among atherinomorphs (Table 1) may represent an ancient, deeply homologous trait that could have facilitated repeated

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Table 1
Longest recorded pre-hatching delays in atherinomorph species. These values are not necessarily indicative of an upper limit, and species show a range of non-exact values. Time spent delaying hatching is measured from Wourms pre-hatching stage [9], to time of hatching. Comparative studies on the genetics of delayed hatching and desiccation avoidance in these species along with results from this experiment with *A. lineatus* will provide insight into the convergent evolution of fish-out-of-water.

Order	Family	Genera or species	Life history	Time of delay	Source
Beloniformes	Adrianichthyidae	*Oryzias latipes*	non-annual	20 days	[55]
Beloniformes	Adrianichthyidae	*Oryzias* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Aplocheilidae	*Aplocheilus* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Aplocheilidae	*Pachypanchax playfairii*	non-annual	24 days	[17]
Cyprinodontiformes	Aplocheilidae	*Pachypanchax* sp.	non-annual	4 weeks	[11]
Atheriniformes	Atherinopsidae	*Leuresthes tenuis*	tidal spawner	26 days	[23,56]
Cyprinodontiformes	Cyprinodontidae	*Cyprinodon* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Cyprinodontidae	*Jordanella* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Fundulidae	*Adinia xenica*	non-annual	10 days	[57]
Cyprinodontiformes	Fundulidae	*Fundulus confluentus*	non-annual	95 days	[58]
Cyprinodontiformes	Fundulidae	*Fundulus heteroclitus*	tidal spawner	28 days	[59]
Cyprinodontiformes	Fundulidae	*Fundulus* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Nothobranchiidae	*Aphyosemion australe*	non-annual	2 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Aphyosemion bivittatum*	non-annual	2 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Aphyosemion cognatum*	non-annual	2 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Aphyosemion labarrei*	non-annual	2 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Aphyosemion* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Nothobranchiidae	*Epiplatys* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax arnoldi*	*annual*	3 months	[11]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax cinnamomeus*	non-annual	2 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax fallax*	*annual*	3 months	[11]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax gardneri*	non-annual	84 days	[60]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax scheeli*	non-annual	39 days	[17]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax sjoestedti*	*non-annual*	3 months	[11]
Cyprinodontiformes	Nothobranchiidae	*Fundulopanchax walkeri*	annual	3 months	[11]
Cyprinodontiformes	Nothobranchiidae	*Nothobranchius guentheri*	annual	120 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Nothobranchius melanospilus*	annual	120 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Nothobranchius palmqvisti*	annual	120 days	[11]
Cyprinodontiformes	Nothobranchiidae	*Nothobranchius* sp.	annual	120 days	[11]
Cyprinodontiformes	Poeciliidae	*Aplocheilichthys* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Poeciliidae	*Micropanchax* sp.	non-annual	4 weeks	[11]
Cyprinodontiformes	Rivulidae	*Anablepsoides hartii*	non-annual	59 days	[17]
Cyprinodontiformes	Rivulidae	*Austrofundulus myersi*	annual	117 days	[11]
Cyprinodontiformes	Rivulidae	*Callopanchax occidentalis*	annual	200 days	[11]
Cyprinodontiformes	Rivulidae	*Cynodonichthys magdalenae*	non-annual	60 + days	[25]
Cyprinodontiformes	Rivulidae	*Cynodonichthys brunneus*	non-annual	40 days	[25]
Cyprinodontiformes	Rivulidae	*Cynopoecilia melanotaenia*	annual	“prolonged and of variable duration”	[11]
Cyprinodontiformes	Rivulidae	*Kryptolebias marmoratus*	non-annual	2.5 months	[26]
Cyprinodontiformes	Rivulidae	*Kryptolebias sepia*	non-annual	7 days	[61]
Cyprinodontiformes	Rivulidae	*Nematolebias whitei*	annual	“prolonged and of variable duration”	[11]
Cyprinodontiformes	Rivulidae	*Pterolebias longipinnis*	annual	“similar to *Rachovia brevis”*	[11]
Cyprinodontiformes	Rivulidae	*Rachovia brevis*	annual	103 days	[11]
Cyprinodontiformes	Rivulidae	*“Rivulus” sp.*	non-annual	4 weeks	[11]

colonization of ephemeral or tidal habitats and that eventually led to the evolution of obligatory diapause stages in annual fishes [17,18]. Embryos of atherinomorph fishes (a group that includes aplocheiloids) such as those in the genera *Oryzias* (Beloniformes: Adrianichthyidae), *Leuresthes* (Atheriniformes: Atherinopsidae)*, Aplocheilichthys, Procatopus*, and *Micropanchax* (Cyprinodontiformes: Poeciliidae)*, Orestias* and *Jordanella* (Cyprinodontiformes: Cyprinodontidae)*, Adinia* (Cyprinodontiformes: Fundulidae), and non-annual killifishes *Pachypanchax, Aphyosemion*, *Epiplatys,* and *Aplocheilus* (Cyprinodontiformes: Aplocheiloidei) have been shown to exhibit pre-hatching delays [11,19–22]. It is unclear whether delayed hatching in any of these species represents true dormancy because unequivocal evidence for depression of growth or metabolic function during delayed hatching is not available. In fact, evidence in a variety of other species that utilize delayed hatching suggests otherwise [23,24].

Wourms [11] showed that although most non-annual Cyprinodontiformes can hatch within two weeks after fertilization (14 dpf), some species are able to delay hatching, similar to annual species that enter DIII. However, according to Wourms and in contrast to annuals these species undergo “continued growth and differentiation … utilizing their yolk reserve.” More recently, Varela-Lasheras and Van Dooren [25] found that two closely related species of non-annual killifishes (*Cynodonichthys brunneus* and *Cynodonichthys magdalenae*) have very different capabilities to delay hatching as a reaction to desiccation. While *C. brunneus* extends the pre-hatching developmental period to ~40 dpf, *C. magdalenae* can delay hatching for more than 80 dpf [25]. These findings suggest that hatching time and desiccation tolerance among killifishes may be plastic phenotypic traits that allowed the extreme condition of annualism (with associated diapause stages) to evolve via “transitional forms.” Species exposed to increasingly extreme seasonality or ephemeral habitats may experience stronger positive selection for longer pre-hatching delays and lower metabolism than other non-annual species, leading eventually to the evolution of a diapause phenotype and an annual lifestyle.

Furness [17] suggested that delayed hatching and diapause represent ends of a continuum, while Martin and Carter [19] categorized different types of delays in teleost fishes and amphibians; however, little is known about the genetic changes involved to explain differences among species. It is conceivable that differences across these phenotypes hinge on the linkage between environmental factors and genetic processes that regulate developmental rate and metabolism. Different types of dormancy, on the other hand, may involve expression of significantly different genetic repertoires and cellular functions. Transcriptomics has become a promising tool to characterize the expression of genes and metabolic pathways that are most important during these critical embryonic stages. Recent studies reported transcriptomic