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S. Cody Woll  
*Portland State University, scwoll@pdx.edu*

Jason E. Podrabsky  
*Portland State University, podrabsj@pdx.edu*

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Insulin-like growth factor signaling regulates developmental trajectory associated with diapause in embryos of the annual killifish *Austrofundulus limnaeus*

S. Cody Woll and Jason E. Podrabsky

Department of Biology, Portland State University, P.O. Box 751, Portland, OR 97207

Corresponding author:
S. Cody Woll
Department of Biology
Portland State University
P.O. Box 751
Portland, OR 97207
scwoll@pdx.edu

**Key Words:** diapause, life history evolution, insulin-like growth factor, metabolic depression,

**Summary Statement:** Insulin-like growth factor signaling plays a critical role in regulating entrance into embryonic diapause and may determine many of the complex life history characteristics unique to annual killifishes.
Abstract

Annual killifishes exhibit a number of unique life history characters including the occurrence of embryonic diapause, unique cell movements associated with dispersion and subsequent reaggregation of the embryonic blastomeres, and a short post-embryonic life span. Insulin-like growth factor (IGF) signaling is known to play a role in the regulation of metabolic dormancy in a number of animals but has not been explored in annual killifishes. The abundance of IGF proteins during development, and the developmental effects of blocking IGF signaling by pharmacological inhibition of the insulin-like growth factor I receptor (IGF1R) were explored in embryos of the annual killfish *Austrofundulus limnaeus*. Blocking of IGF signaling in embryos that would normally escape entrance into diapause resulted in a phenotype that was remarkably similar to embryos entering diapause. IGF-I protein abundance spikes during early development in embryos that will not enter diapause. In contrast, IGF-I levels remain low during early development in embryos that will enter diapause II. IGF-II protein levels are packaged at higher levels in escape-bound embryos compared to diapause-bound embryos. However, IGF-II levels quickly decrease and remain low during early development and only increase substantially during late development in both developmental trajectories. Developmental patterns of IGF-I and IGF-II protein abundance under conditions that would either induce or bypass entrance into diapause are consistent with a role for IGF signaling in the regulation of developmental trajectory and entrance into diapause in this species. We propose that IGF signaling may be a unifying regulatory pathway that explains the larger suite of characters that are associated with the complex life history of annual killifishes.
Introduction

The cellular mechanisms that support entrance into reversible states of metabolic and developmental arrest have been studied across a wide range of organisms. There is considerable evidence accumulating that decreases in insulin-like growth factor (IGF) signaling pathways are of central importance for induction of dormancy and expression of multiple aspects of the dormant phenotype including shifts in metabolic rate and substrate utilization, reduced cell proliferation, and protection from environmental stresses such as anoxia or starvation (Lin et al., 2001; Puig et al., 2003; Sim and Denlinger, 2008; Sim et al., 2015). The critically important and highly conserved role of IGFs in supporting cellular growth and proliferation suggests that downregulation of IGF signaling may be a critical and perhaps universal aspect of entrance into metabolic and developmental arrest. This paper explores the role of IGF signaling in the regulation of developmental and metabolic arrest associated with embryonic diapause in embryos of the annual killifish *Austrofundulus limnaeus*.

The annual killifish, *Austrofundulus limnaeus*, inhabits ephemeral ponds in Venezuela that dry on a seasonal basis (Hrbek et al., 2005; Podrabsky and Hand, 1999; Podrabsky et al., 1998). This harsh and often unpredictable environment has driven the evolution of multiple adaptations for survival in temporary aquatic habitats, the most crucial of which is the ability to arrest development in diapause to support survival through the dry season (Myers, 1952; Peters, 1963; Wourms, 1972a; Wourms, 1972b). Embryos may arrest development in up to three stages of diapause, termed diapause I, II, and III (Wourms, 1972a; Wourms, 1972b). Entrance into diapause is associated with decreased metabolic rate, inhibition of the cell cycle and cell growth, cessation of morphological development, and increased tolerance of environmental stress (Meller et al., 2012; Podrabsky et al., 2016a; Podrabsky et al., 2016b; Podrabsky and Hand, 1999). In *A. limnaeus*, survival of the dry season is likely due to arrest in diapause II, which occurs about midway through development after completion of somitogenesis but just prior to the major phases of organogenesis (Podrabsky et al., 2001; Podrabsky and Hand, 1999).

Under laboratory conditions, typically more than 80% of *A. limnaeus* embryos enter into diapause II (Podrabsky et al., 2010), and early studies considered entrance into diapause as the default developmental pathway (Wourms, 1972a; Wourms, 1972b). Embryos that do not enter diapause II develop directly to diapause III, and were considered to “escape”
diapause by Wourms; thus embryos that do not enter diapause II are termed escape embryos (Wourms, 1972b). Subsequent studies have detailed that entrance into diapause II is an alternative pathway that differs morphologically and physiologically from direct developing escape embryos in all annual killifishes (Furness et al., 2015; Podrabsky et al., 2010). Under laboratory conditions and incubation of embryos at 25°C, young females are known to produce a higher proportion of escape embryos, while older females produce almost exclusively diapausing embryos (Podrabsky et al., 2010). However, the incubation temperatures experienced by the embryos can override maternal programming. Regardless of female age, incubation at 20°C results in 100% diapausing embryos, while incubation at 30°C results in 100% escape embryos (Podrabsky et al., 2010). Temperature transfer experiments have established a “critical window” for this effect of temperature during somitogenesis in embryos possessing between 10 and 20 pairs of somites (Podrabsky et al., 2010). Escape embryos have the potential to complete their entire lifespan within a single rainy season, rather than enduring the harsh conditions imposed by the dry season. This ecological trade-off has clear implications for survival of an individual and survival of the species. Thus, the pathways that regulate these developmental decisions are likely to be under intense selection pressure.

In vertebrates, insulin-like growth factors are small protein hormones (~7.6 kDa) excreted via exocytosis into the extracellular space where they act as autocrine, paracrine, or endocrine signals (Lowe, 1991). Growth hormone signaling in adult tissues typically stimulates IGF transcription, translation, and excretion (Carter et al., 2002; Salvatori, 2004). In larvae and adults, hepatic (liver) cells are the primary producers of IGFs while a small amount is produced in a variety of other tissues. However, in embryonic tissues, IGFs are produced globally—meaning various cells with different lineages will produce IGFs throughout the entire embryo (Allan et al., 2001; Dupont and Holzenberger, 2003; Greene and Chen, 1997; Lowe, 1991).

IGF proteins and their associated signaling pathways are highly conserved across vertebrates (Kenyon, 2001). The IGF family includes IGF-I and IGF-II proteins, and both of these proteins have been shown to regulate various aspects of development in invertebrate and vertebrate embryos (Dupont and Holzenberger, 2003). Increased IGF signaling is almost always associated with increased cell growth, proliferation, and differentiation in a variety of vertebrates including humans, birds and fish (de Pablo et al., 1993; Eivers et al., 2004; Furstenberger and Senn, 2002; Kajimura et al., 2005; Kamei et al., 2011; Oldham and Hafen,
2003; Salvatori, 2004). In mice, IGF-II null mutants grew to only 60% of normal birth weight and IGF-II(-/-)IGF-I(-/-) double mutants suffered further depression of growth reaching only 30% of normal birth weight (Liu et al., 1993). In addition to their crucial role in regulating developmental growth, IGFs are also important differentiation factors and have been shown to induce differentiation in mice skeletal muscle and myoblast cell lines via autocrine and paracrine signaling (Tollefsen et al., 1989a; Tollefsen et al., 1989b).

IGF activity is mitigated through three classes of receptors in vertebrates: insulin receptors, insulin-like growth factor I receptor (IGF1R), and insulin-like growth factor II receptor (IGF2R, or cation-independent mannose-6-phosphate receptor) (LeRoith et al., 1995; Nissley et al., 1991). IGF1R is the main receptor for IGF-I and IGF-II proteins that activates IGF signaling in response to binding. Insulin receptor (IR) has a much higher affinity for insulin than for IGF-II and IGF-I doesn’t react with IR homodimers, and thus IR is likely not physiologically active in terms of IGF signaling (Kim and Accili, 2002; Massague and Czech, 1982). However, there is evidence of hybrid heterodimer formation by IR and IGF1R subunits (Kasuya et al., 1993; Soos et al., 1993) and these hybrid IR/IGF1R complexes have a high affinity for IGF-I rather than insulin, and behave like IGF1R receptors rather than IR receptors (Belfiore et al., 1999). The activation of IGF1R results in a context dependent regulation of a diversity of cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis (Jones and Clemmons, 2013; Vincent and Feldman, 2002). Loss of IGF receptor signaling during development leads to reduced growth. For example, Liu et al. (1993) showed that mutant mice that lacked IGF1R (IGF1R -/-), grew to only 45% of normal birth weight compared to wildtype. In contrast to the other receptors, IGF2R is a negative regulator of the IGF pathway and acts to attenuate IGF-II signals by stimulating endocytotic lysosomal degradation of IGF-II protein (Griffiths et al., 1988; Lau et al., 1994).

Cellular metabolism, growth, and proliferation are severely downregulated in most organisms that arrest development (Hand et al., 2016). The mechanisms responsible for developmental arrest are not completely understood in any species, but a wealth of evidence points to the central importance of IGF signaling in regulating entrance into metabolic dormancy. For example, it has been shown in Caenorhabditis elegans nematodes that downregulation of DAF-2 (the C. elegans homolog of IGF1R) plays a crucial role in regulating entrance into dormancy as “dauer” larvae (Kimura et al., 1997). The IGF signaling pathway in C. elegans shares many similarities with vertebrate IGF signaling. Activation of DAF-2 leads to
phosphorylation of multiple substrates; one major target of activation is AKT1/2, which can affect many subsequent pathways. Active AKT suppresses the action of proteins such as DAF-16 (FOXO homolog), which leads to increased protein synthesis and cell growth (Fielenbach and Antebi, 2008; Kimura et al., 1997; Pierce et al., 2001). Additionally, AKT activates pathways that lead to increased glucose metabolism, cell growth and survival, mobility, and blocks pathways that activate apoptosis. However, when larvae sense reduced food availability, IGF signaling is downregulated, which allows for increased activity of DAF-16 due to reduced activity of AKT and other regulatory proteins in the pathway (Lin et al., 2001; Ogg and Ruvkun, 1998). Thus, DAF-16 appears to be the major effector protein that induces the physiological phenotype associated with dauer formation (Hesp et al., 2015; Lin et al., 2001).

In addition, FOXO has been shown to be a regulator of diapause in the mosquito *Culex pipiens* and *Drosophila melanogaster* (Puig et al., 2003; Sim and Denlinger, 2008). A number of lines of evidence suggest that downregulation of IGF signaling and the resulting upregulation of FOXO activity can induce conditions that reduce metabolic rate and protect an organism during times of environmental stress such as anoxia or starvation (Sim et al., 2015).

Insulin-like growth factors are known to play major roles in the regulation of developmental growth and differentiation and have been implicated in the regulation of diapause-like states in other organisms. This study reports on the relative abundance of IGF-I and -II proteins during development and differentiation of two alternative developmental trajectories in *A. limnaeus*, escape embryos and embryos arresting in diapause II. Additionally, pharmacological inhibition of IGF1R mediated signaling during development is explored to test for a functional role for IGF signaling in the induction of diapause. Evidence is presented that implicates blockage of IGF signaling as an essential part of the program that induces diapause in embryos of *A. limnaeus*. 
Materials and Methods

Animal Care and Embryo Collection

Detailed methods for the husbandry of adults and collection of embryos for *Austrofundulus limnaeus* (Quisiro strain) have been previously established (Podrabsky, 1999). This work was conducted under protocols reviewed and approved by the PSU Institutional Animal Care and Use Committee (PSU Protocol numbers 16 & 33). Briefly, spawning pairs of fish were housed in 10 l glass aquaria connected to a common sump system with biological, chemical and mechanical filtration. Ten percent of the system water (Portland City tapwater with 1 ppt Coralife sea salt) was changed twice daily. Temperature of the water was regulated at 26 ± 1 °C and photoperiod was set at 14L:10D. Fish were fed twice daily with frozen chironomid larvae (Hikari USA) or chopped earthworms.

Embryos (collected twice weekly) were incubated in plastic culture dishes containing embryo medium as described in Podrabsky (1999). For temperature incubation experiments, embryos were transferred to 25°C or 30°C immediately after collection. Embryos were inspected and staged (WS = Wourms’ Stage) every 1-2 d according to Wourms (1972a) using an inverted microscope. Embryos used for protein quantification were flash-frozen individually in liquid nitrogen and stored at -80°C. A total of 880 embryos were observed from two spawning events.

To compare potential differences in maternal provisioning of IGFs in embryos bound to develop along the escape or diapause trajectories, 1-2 cell-stage embryos were sampled from females proven to be producing 100% diapausing or escape embryos as evidenced by following the developmental trajectory of a subset of each clutch of embryos. Only clutches of embryos that resulted in 100% diapause or 100% escape embryos were used to represent embryos maternally programmed for the diapause or escape trajectories.

Protein extraction and quantification

Individual embryos were homogenized on ice with an acrylic pellet pestle in 1.7 ml microcentrifuge tubes in 50 µl of 1X phosphate buffered saline (PBS) containing 1 µl of protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, Missouri, USA). The homogenate was subjected to centrifugation at 100 x g for 30 s at 4°C to pellet large chunks of chorion. Homogenates were stored at -20°C prior to protein analysis. On the day the ELISAs were run,
protein concentrations were determined using the Pierce MicroBCA protein assay kit according to the manufacturer’s instructions.

**Quantification of insulin-like growth factor I**

Relative concentrations of IGF-I were determined using an indirect enzyme-linked immunosorbent assay (ELISA). The validity of the assay was established by illustrating IGF-I protein standard and sample serial dilution collinearity and by confirming that the primary antibody only recognizes a single protein band of the correct molecular mass on a Western Blot (Figure S1). Samples and standards were run in triplicate. Standard curves for the assays were generated by serial dilution of a 1 mg ml\(^{-1}\) stock solution of IGF-I protein (*Oncorhynchus mykiss*, Lot FJI-AE01, Gropep, Adelaide, Australia) in carbonate buffer (15 mmol l\(^{-1}\) Na\(_2\)CO\(_3\), 35 mmol l\(^{-1}\) NaHCO\(_3\), pH 9.6) to a range of 1000 – 1 µg ml\(^{-1}\). Embryo homogenates were diluted to a total protein concentration of 5000 µg ml\(^{-1}\) in carbonate buffer. All washes and incubations were performed on a rocker with gentle rotation at room temperature unless otherwise noted. Solutions were removed from wells by pounding the microplate onto clean paper towels until the wells were dry unless otherwise noted. Wells of 96 well plates (flat bottom, polystyrene, Microlon® 200, med. binding, Greiner Bio-one, Germany) were coated with 100 µl of standards or samples for 24 h at 4˚C followed by 5 washes with 300 µl of 0.05% Tween-20 in 1X PBS for 5 min each. Wells were then blocked with 200 µl of 2% bovine serum albumin (BSA) in 1X PBS for 1 h at RT followed by 5 washes as described above. Wells were then incubated with 100 µl of IGF-I rabbit polyclonal antibody (anti-human IGF-I, Lot CJI-PAB-GA, 1:1000 diluted, Gropep, Adelaide, Australia) for 1 h followed by 5 washes. Wells were then incubated with 100 µl of goat anti-rabbit IgG antibody conjugated with HRP (Lot ab97051, diluted 1:500, Abcam, Eugene, USA) for 1.5 h. Wells were again washed 5 times. Color development was initiated by the addition of 100 µl of 1-Step Ultra TMB-ELISA substrate (Thermofisher Scientific) followed by 15 min incubation at RT in the dark. Color development was stopped by the addition of 100 µl of 500 mmol l\(^{-1}\) sulfuric acid. Absorbance at 450 nm (A\(_{450}\)) was measured using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). For each sample the average of three replicate values was used in subsequent calculations. Replicates were only used if they were within 20% of the sample mean. If a sample had less than 3 replicates within 20% of the mean, then the sample was re-run on a fresh plate from start to finish.
Quantification of insulin-like growth factor II

The relative concentrations of IGF-II proteins were determined using a competitive binding enzyme-linked immunosorbent assay (ELISA) modified from (Wilkinson et al., 2004). The validity of the assay was established by illustrating IGF-II protein standard and sample serial dilution resulted in parallel changes in absorbance and by confirming that the primary antibody only recognizes a single protein band of the correct molecular mass on a Western Blot (Figure S1). Standards and samples were prepared in triplicate in 1.5 ml centrifuge tubes (Natural Micro Centrifuge Tube, Sealrite, USA Scientific). Standard solutions of purified salmon IGF-II (Lot CAG-AT01, GroPep, Adelaide, Australia) were diluted to final concentrations ranging from 1000 – 1 µg ml\(^{-1}\) in 1X PBS containing 0.1% BSA in a final volume of 400 µl. For embryo homogenates, 125 µg of total protein was diluted in 1X PBS containing 0.1% BSA to a final concentration of 5000 µg ml\(^{-1}\) in a final volume of 400 µl. Samples and standards were acidified by the addition of 70 µl 100 mmol l\(^{-1}\) Glycine-HCl (pH 2.0) followed by a 30 min incubation. The samples/standards were neutralized by the addition of 30 µl 200 mmol l\(^{-1}\) Tris (pH 10.0) followed by a 2 h incubation with IGF-II antibody solution (rabbit polyclonal anti-trout IGF-II, Lot CA6-PAAA1, 1:3500 dilution, GroPep, Adelaide, Australia).

Wells of 96 well microplates (flat bottom, polystyrene, Microlon® 200, med. Binding, Greiner Bio-one, Germany) were coated with 100 µl of a 50 µg ml\(^{-1}\) IGF-II protein solution diluted in carbonate buffer (see above) for 24 h at 4°C. Coating buffer was removed and wells were then washed and blocked as described above for the IGF-I ELISA. After blocking, wells were washed again as described above followed by a 30 min incubation with 100 µl of IGF-II standards or embryo homogenates (prepared as described in the above paragraph). ELISA color development was essentially identical to the methods described above for the IGF-I assay except that the anti-rabbit IgG antibody conjugated with HRP was diluted 1:1000 and incubation time in this antibody was reduced to 1 hr.

Pharmacological inhibition of insulin-like growth factor signaling

All pharmacological agents were purchased from Selleck Chemicals (Houston, Texas, USA). Two inhibitors of IGF receptors were used. BMS-754807 is a specific inhibitor for IGF1R (Bid et al., 2013; Carboni et al., 2009), while NVP-AEW541 is a more general inhibitor of the insulin and IGF pathways due to inhibition of the kinase activity of those receptors (García-
Echeverría et al., 2004). In addition, inhibitors of signaling molecules that are often downstream of IGF1R activation were used as well. LY294002 is an inhibitor of phosphoinositide 3-kinase (PI3K) activity, a common enzyme implicated in growth factor signal transduction (Vlahos et al., 1994). U0126 inhibits the activity of Mek1/2 proteins, which are key components of the MAP kinase signaling cascade (Duncia et al., 1998). Embryos of A. limnaeus are known to be highly impermeable to exogenous substances, and thus higher than expected external concentrations and dimethyl sulfoxide (DMSO) in the medium are required to achieve biologically active concentrations within the embryos (Podrabsky and Hand, 2000; Pri-Tal et al., 2011). Lyophilized pharmacological agents were reconstituted in 100% DMSO stock solutions that were diluted into embryo medium (Podrabsky, 1999) without antibiotics. For each inhibitor, control embryos were exposed to the same amount of DMSO as embryos treated with the pharmacological agents; the final concentration of DMSO was always less than 1%. Final concentrations used in the experiments were: NVP-AEW541 (100, 50, 20, 10, and 5 µmol l\(^{-1}\)); BMS-754807 (5, 4, 3, 2.5, and 1 µmol l\(^{-1}\)); Ly294002 (100, 50, 25, 5, and 2.5 µmol l\(^{-1}\)); U0126 (200, 100, and 50 µmol l\(^{-1}\)). Preliminary range-finding experiments were used to determine these concentrations that are about 10 times the effective concentration used in similar experiments with BMS-754807 in embryos of the zebrafish, Danio rerio (Kamei et al., 2011).

At 24 h post-fertilization, individual embryos were transferred to wells of a 96 well plate, each well containing 300 µl of embryo medium containing one of the four pharmacological inhibitors. Embryos were incubated continuously in the inhibitors at either 25 or 30°C, and were observed daily and staged according to (Wourms, 1972a) using an inverted microscope. Heart rate in beats min\(^{-1}\) (as soon as heart contractions were visible) was monitored at RT (23-25°C) for embryos exposed to three concentrations of BMS-754807 (2.5, 3 and 4 µmol l\(^{-1}\)). In addition, the number of somite pairs was counted for each embryo.

**Statistical Analysis**

Graphical and statistical analyses were performed using Prism 7.0 software (GraphPad, La Jolla, CA, USA) and R version 3.2.1 (R Studio, https://www.rstudio.com). Where appropriate, analysis of variance (ANOVA), t-tests, or linear regression analyses were used. Tukey’s honest significant difference (HSD) or Dunnett’s test were used for post-hoc comparisons where appropriate. Statistical significance was always determined at a level of
P-value < 0.05. Relative abundance was calculated based on protein levels in 1-2 cell-stage embryos (WS 4).

Results

Pharmacological Inhibitors

Embryos incubated in low concentrations of the Insulin and IGF1R receptors inhibitor (NVP-AEW541), and inhibitors of downstream signaling proteins involved in growth factor signaling (LY294002, U0126) had developmental rates that were indistinguishable from control embryos incubated at 30°C (data not shown). In contrast, medium and high dosages of these pharmacological agents resulted in 80-100% death (data not shown). Those embryos that did survive exhibited severe developmental malformation. These data are interpreted to be the result of broad and non-specific effects caused by blocking signaling pathways that are indispensable for normal development and are discussed no further.

Inhibition of IGF1R

BMS-754807, a potent inhibitor of IGF1R, exhibited specific effects on development that were dosage-dependent. The rate at which embryos progress through development was significantly retarded by increasing concentrations of inhibitor (Figure 1). Compared to embryos developing at 30°C along the escape trajectory, embryos incubated at 30°C in inhibitor concentrations of 2.5 µmol l⁻¹ and greater were delayed significantly (ANOVA, Tukey’s HSD, P < 0.01). Embryos developing in intermediate concentrations of inhibitor (2.5, 3 and 4 µmol l⁻¹) that were incubated at 30°C developed at rates indistinguishable from control embryos developing at 25°C (ANOVA and Tukey’s HSD, P = 1.0, 1.0, 0.07, respectively). The 5 µmol l⁻¹ treatment group was significantly delayed compared to all other treatments, and embryos never progressed past the dispersed cell phases of development to establish a neural keel (Figure 1). Rates of somitogenesis (Figure 2) were significantly reduced by incubation in the IGF1R inhibitor. Linear regression analysis (of the linear portion of the curve) indicates that control embryos developing at 30°C added somites at a rate of 7.5 ± 0.7 (mean ± SD) somite pairs per day, while control embryos at 25°C and embryos treated with
inhibitor at 30°C all had significantly lower rates (regression analysis, comparison of slopes; P < 0.001) of somitogenesis that ranged from 3.1-3.6 somite pairs per day (25°C = 3.1 ± 0.1, 2.5 µmol l⁻¹ = 3.1 ±0.2, 3 µmol l⁻¹ = 3.6 ± 0.1, 4 µmol l⁻¹ = 3.3 ± 0.2). Regression analysis comparing the slopes for 25°C control embryos and those treated with inhibitor indicated no statistically significant differences in the slopes of these treatment groups (P = 0.3). Further, heart rate was significantly decreased in embryos incubated at 30°C in the presence of the IGF1R inhibitor (Figure 3). In fact, for embryos incubated at 2.5, 3, and 4 µmol l⁻¹ BMS-754807, heart rates were similar to embryos incubated at 25°C along the diapause trajectory (ANOVA and Tukey’s HSD, P = 0.3, 1.0, and 1.0, respectively).

Embryos incubated in 5 µmol l⁻¹ BMS-754807 arrested development in the dispersion and reaggregation (D/R) phases of development. Embryos treated with 2.5, 3, and 4 µmol l⁻¹ BMS-754807 developed a morphology very similar to those of diapause II embryos with some significant differences (Figure 4). First, melanocytes differentiate and migrate in embryos exposed to inhibitor concentrations up to 3 µmol l⁻¹, however the 4 µmol l⁻¹ treatment group did not develop melanocytes. Melanocytes never differentiate until after diapause II in normal embryos. Second, despite the low heart rates, circulating blood cells that express hemoglobin were observed in the embryos treated with all levels of inhibitor that allowed development past the D/R stages. Again, expression of hemoglobin does not normally develop until post-diapause II development. Otherwise, the general morphology and number of somite pairs are quite similar to those of diapause II embryos (Figure 4).

**Abundance of IGF-I**

When similar stage embryos are compared, there is a clear spike in IGF-I protein abundance between WS 20 and WS 30 in embryos developing at 30°C along the escape trajectory (Figure 5). Abundance increases almost 6-fold by WS 23 (reaggregating blastomeres), remains high through WS 28 (solid neural keel), and then returns to levels similar to those at fertilization at WS 30 (10 somite embryo) (ANOVA, Dunnett’s MCT, P < 0.001). Abundance of IGF-I in embryos developing at 25°C does not increase above levels present at fertilization until after embryos exit diapause II at WS 40 and later (ANOVA, Dunnett’s MCT, P < 0.0001). Thus, embryos incubated at 30°C experience a spike in IGF-I protein abundance early in development that is completely absent in embryos developing at 25°C (Figure 5). The pattern of IGF-I protein abundance during late development (WS 32 – 43)
is also very different in the two developmental trajectories. Escape embryo (30°C) IGF-I levels peak at WS 37, long before growth and development are complete, and then decrease to levels that are indistinguishable from WS 4 embryos at WS 43. In contrast, post-diapause II embryos (25°C) reach peak abundance of IGF-I at WS 43, essentially at the completion of development. These different patterns of abundance are supported by statistical differences in mean IGF-I protein levels at numerous developmental stages (Figure 5).

**Abundance of IGF-II**

The pattern of IGF-II abundance is quite different from that of IGF-I, for both developmental trajectories. First, in contrast to IGF-I levels embryos collected from females that were confirmed to be producing 100% escape embryos produced embryos with significantly higher levels of IGF-II protein at fertilization (Figure 5, WS 4 comparison). Second, there was a significant decrease in IGF-II protein levels during early development in both developmental trajectories. Statistically significant increases in IGF-II protein abundance are not observed until late embryonic development in both trajectories.
Discussion

Insulin-like growth factors are known to be critical for proper cell growth and differentiation during vertebrate development (de Pablo et al., 1993; Dupont and Holzenberger, 2003; Eivers et al., 2004; Richard-Parpaillon et al., 2002). In addition, IGFs have been shown to play a critical role in the regulation of metabolic dormancy associated with diapause or diapause-like states in a number of organisms (Fielenbach and Antebi, 2008; Hondo and Stewart, 2004; Kimura et al., 1997; Ogg and Ruvkun, 1998; Sim and Denlinger, 2013; Sim et al., 2015). This is the first study to evaluate the potential role of IGFs in development and diapause in embryos of the annual killifish Austrofundulus limnaeus.

Maternal provisioning and promotion of the escape trajectory

Maternal provisioning or influences are known to affect the developmental trajectory of A. limnaeus (Podrabsky et al., 2010). IGF-I protein levels were not differentially provisioned into embryos bound for either developmental trajectory. However, the higher abundance of IGF-II protein in 1-2 cell-stage embryos of A. limnaeus that are presumed to be developing along the escape trajectory may indicate a role for IGF-II in the regulation of maternal control over developmental trajectory. Indeed, the paternal IGF-II gene is imprinted in many species (DeChiara et al., 1991; O’Dell and Day, 1998; Vu and Hoffman, 1994) and thus there is precedent for regulation of IGF-II levels being important for normal development. The data presented in this study suggest a possible role for this differential packaging in determining developmental trajectory, but many future studies would be needed to test this hypothesis.

IGF signaling and entrance into diapause II

Inhibition of IGF1R signaling resulted in developmental and physiological phenotypes that approximate the diapause trajectory even under environmental conditions that should promote development along the escape trajectory. These similarities between embryos incubated at 30°C in the presence of inhibitors and those incubated at 25°C are striking and suggest a critical role for IGF signaling in promoting the escape trajectory in annual killifish. The lack of a significant increase in IGF-I or IGF-II protein levels until post-diapause II development in embryos incubated at 25°C compared to the early and sustained expression
of IGF-I in embryos at 30°C both support a role for reduced IGF-I signaling in the promotion of the diapause II phenotype.

Somitogenesis is a highly regulated patterning of the paraxial (somatic) mesoderm next to the developing neural tube. Once initiated, this process proceeds according to a highly stereotyped addition of somite pairs in an anterior to posterior pattern (Pourquie, 2001). The highly similar pattern and rate of somite development in embryos treated with IGF1R inhibitors compared to control embryos destined to enter diapause II suggests that the rate of this process is likely regulated by muted IGF signaling under developmental conditions that favor the diapause II trajectory. In fact, for embryos developing at 25°C levels of IGF-I and IGF-II protein do not increase above amounts present at fertilization until after diapause II is broken further supporting a role for muted IGF signaling in the promotion of diapause in this species.

Insulin-like growth factor signaling is crucial for normal cardiac and circulatory development in vertebrates. Zebrafish, Danio rerio, embryos with reduced IGF binding protein-2, which increases the half-life of IGFs and has been shown to mediate binding of IGF1R (Jones and Clemmons, 2013), had reduced amounts of IGF-I mRNA, reduced body growth, and disruptions to cardiovascular development (Wood et al., 2005). More recently IGF-II expression was found to be crucial for supporting increased cellular proliferation in primary mouse embryonic epicardial cells (Li et al., 2011). Thus, the delay in the development of cardiac activity observed in this study is also consistent with a role for reduced IGF signaling in determining the diapause phenotype.

Despite the above observations that suggest a critical role for IGF signaling in determining the diapause II phenotype, there were some key differences in phenotype between inhibited embryos and those that naturally enter diapause II. The pre-diapause II development of melanocytes and expression of hemoglobin are two notable differences. It is important to note that these differences are likely due to the difficulty of completely inhibiting IGF signaling through the use of pharmacological inhibitors, and indeed the results presented here suggest that different processes may require different thresholds of IGF activity, or may work through alternative signaling routes.

Under normal conditions, melanocytes do not develop in embryos of A. limnaeus until after diapause II. In contrast, embryos developing in the presence of inhibitor exhibited differentiation and migration of melanocytes in all but the highest two concentrations of
inhibitor. Melanocytes are derived from neural crest cells that migrate away from the developing neural tube and differentiate into a variety of embryonic structures – many or perhaps all of which are critical features of the vertebrate body plan. IGF-II is one of the signals that can rapidly induce neural crest cells to undergo an epithelium to mesenchyme transition, the first step in migrating away from the neural tube (Morali et al., 2001). Thus, there is reason to believe that IGF signaling may have an effect on melanocyte development, and indeed embryos exposed to the highest level of inhibitor that allowed development past D/R failed to develop melanocytes prior to arresting development in a diapause II-like stage.

Blood cell differentiation and proliferation is known to be regulated by IGF signaling, and many blood cancers exhibit increased levels of IGF signaling (Bertrand et al., 2006). Thus, it is not surprising that conditions that increase levels of IGF-I protein (incubation at 30°C) would accelerate development of differentiated red blood cells expressing hemoglobin. The inability to block this process with the inhibitor may indicate an especially strong effect of IGF signaling on blood cell differentiation, or it may indicate the use of alternative receptors that are not as sensitive to the inhibitor (e.g. insulin-IGF1R heterodimers).

**IGF signaling as a potential link between dispersion/reaggregation and diapause**

Previous studies indicate that incubation of embryos at 30°C leads to 100% of the embryos developing along the escape trajectory (Podrabsky et al., 2010). In addition, a “critical window” of developmental time in embryos between 10 and 20 somites was identified when temperature appears to exert irreversible effects on developmental trajectory. Interestingly, the spike in IGF-I protein levels in escape embryos occurs just prior to the critical developmental window. This spike in protein levels is likely important for supporting the completion of the D/R of the blastomeres, as embryos with the highest levels of IGF1R inhibitor were unable to develop past this point in development, and a small (not statistically significant) increase in IGF-I protein was observed during this phase of development in control embryos developing at 25°C. Thus, it is possible that reduced IGF signaling is critical for determining developmental patterns associated with both D/R and diapause II. In fact, others have suggested that the D/R process is simply an extensive prolongation of a normally very short part of teleost development (Berois et al., 2012). If this is the case, the data presented here are consistent with reduced IGF signaling playing a key
role in this unique aspect of annual killifish development as well. The ability to enter into
diapause and the developmental pattern that includes D/R always co-occur in annual
killifishes, and this paper represents the first potential mechanism for the co-evolution of
these two traits through a common mechanism.

Catch-up growth during post-diapause II development

Compensatory acceleration in growth and development is a common reaction in
animals following release from stressed states or adverse environments that have slowed or
arrested growth. This phenomenon is called “catch-up” growth and is found in embryos from
a variety of animals, and in response to a number of human diseases (Fielenbach and Antebi,
2008; Gillooly et al., 2002; Hales and Ozanne, 2003; Kajimura et al., 2005; Saenger et al.,
2007). In D. rerio embryos, compensatory growth after release from hypoxia-induced growth
arrest was confirmed to be fueled by over-expression of IGF-I (Kamei et al., 2011). The broad
phylogenetic representation of catch-up growth suggests that the phenomenon is likely an
important and evolutionarily conserved mechanism with adaptive value. Patterns of
metabolic enzyme activity measured during post-diapause II development in A. limnaeus
suggest catch-up growth is a normal part of development for this species. This observation is
based on the rate of increase in lactate dehydrogenase (LDH) and citrate synthase (CS) activity
in embryos developing post-diapause II at 25°C and those developing at 25 and 30°C along
the escape trajectory (Chennault and Podrabsky, 2010). The slope of the increase in activity
for both of these enzymes associated with post-diapause II development at 25°C was
equivalent to the embryos developing at 30°C, suggesting an acceleration in the rate that
overcame the expected decrease associated with the differences in temperature (Chennault
and Podrabsky, 2010). Patterns of IGF-I and IGF-II abundance in the present study support a
role for increased abundance of IGFs fueling this compensatory acceleration in the rate of
development. For example, both IGF-I and IGF-II increase dramatically late in development in
post-diapause II embryos. In contrast, escape embryos reach a peak in abundance for both
proteins much earlier in development. Thus, post-diapause II embryos complete development
with higher levels of IGF-I and IGF-II. The long term consequences of this pattern are
unknown, but the documented differences in adult physiology and reproductive fitness that
are associated with these two developmental trajectories in other species of annual killifishes
suggest that these developmental differences in IGF expression may have profound consequences on the biology of these fish (Polačik et al., 2014). In other systems, long-term effects have been noted. For example, in rats, *Rattus rattus*, male longevity was reduced in individuals that experienced developmental retardation and subsequent “catch-up” growth compared to those developing along a more continuous trajectory (Hales and Ozanne, 2003).

**Conclusions**

This study provides evidence that blocking of IGF signaling plays a critical role in the arrest of growth and differentiation observed as *A. limnaeus* embryos enter into diapause. There are striking similarities between embryos entering into diapause naturally, and the patterns of growth arrest and physiological development that are induced by treatment with a potent inhibitor of IGF1R. Further, patterns of IGF protein abundance during development suggest a relationship between IGF signaling and many aspects of the unique life history of annual killifishes including the occurrence of the dispersion and reaggregation phases of development, and differences in aging characteristics of adults that follow distinct developmental trajectories. This study is the first to present experimental evidence that blocking IGF signaling is important for promotion of diapause in annual killifishes, and that this mechanism may explain the many unique life history characteristics of annual killifishes.
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Competing Interests
The authors declare no competing interests.

Author Contributions
The experimental design was co-created by SCW and JEP. The experiments were conducted by SCW. Both SCW and JEB contributed significantly to the analysis and presentation of the data. The manuscript was drafted by SCW and edited by JEP. The work was funded by JEP.

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Figure 1. Developmental progression of embryos incubated at 30°C exposed to a range of concentrations of the IGF1R inhibitor BMS-754807. Increasing concentrations of the inhibitor significantly retard rate of development. At inhibitor levels of 2.5, 3, and 4 µmol l⁻¹, developmental progression in embryos at 30°C indistinguishable from those developing at 25°C (ANOVA and Tukey’s HSD, p-value = 1.0, 1.0, 0.07, respectively). Symbols are means ± sem (n = 10 individual embryos).
Figure 2. Rates of somitogenesis in embryos incubated at 30°C and treated with the IGF1R inhibitor BMS-754807. Linear regression analysis indicates that embryos treated with inhibitor and incubated at 30°C had rates of somitogenesis that were comparable to embryos incubated at 25°C (see text for details). Data for the DMSO controls and 30°C escape embryos were combined, because regression analysis indicated no significant differences (p = 1.0). Symbols are means ± sem (n = 5-13 individual embryos).
Figure 3. Heart rate in embryos incubated at 30°C in the presence of the IGF1R inhibitor BMS-754807. Treated embryos had heart rates comparable to embryos developing at 25°C along the diapause trajectory. (ANOVA and Tukey’s HSD, p = 0.3, 1.0, and 1.0, respectively). Control embryos incubated at 30°C have heart rates significantly higher than all other treatments (ANOVA, Tukey’s HSD p < 0.0001). Symbols are means ± sem (n = 1-10 individual embryos).
Figure 4. Images of embryos incubated at 30°C and treated with the IGF1R inhibitor BMS-754807. Embryos were observed over 32 days post-treatment (dpt). All groups were initially exposed to inhibitor at WS 10-12 (blastula stage) at 1 d post-fertilization and were continuously exposed for the duration of development. Embryos incubated at 2.5, 3, and 4 µmol l⁻¹ inhibitor developed in a manner very similar to embryos incubated at 25°C along the diapause trajectory.
Figure 5. Relative abundance of IGF-I and IGF-II protein in embryos incubated at 25°C (diapause II trajectory) and 30°C (escape trajectory) as a function of developmental stage. Protein levels are expressed relative to the mean of protein levels for embryos at Wourms’ stage (WS) 4 (0 days post-fertilization) incubated at 25°C. Symbols are means ± sem (n=3-10 individual embryos). Black dotted lines at y=1 indicate relative expression level of WS 4 embryos. Gray vertical dashed lines indicate various developmental stages as indicated in the top panel. The gray shaded area represents the critical window for temperature to effect developmental trajectory (Podrabsky et al., 2010). Statistical comparisons were made within each treatment group only using ANOVA and Dunnett’s multiple comparison test (see text for
details). Symbols with an asterisk are statistically different from levels at WS 4 for each treatment group (P < 0.01). Pound symbols indicate statistical differences between the two developmental trajectories, at those developmental stages where data were available for both (two-way ANOVA, Sidak’s Multiple Comparison test, P < 0.001). For IGF-I protein levels at WS 4, there were no differences found between embryos developing on either trajectory, and thus these data were combined. For IGF-II there were significant differences at WS 4 for the two trajectories and the escape embryos are represented by the filled purple circles.
**Figure S1. Validation of ELISA assays for detection and quantification of relative levels of IGF proteins in *Austrofundulus limnaeus*.** (A) Serial dilutions of an IGF-I protein standard and an *A. limnaeus* liver sample exhibit colinearity in the IGF-I indirect ELISA assay. The slopes of the lines are not statistically different (Regression analysis, \( P = 0.32 \)). (B) Western blot analysis indicates the IGF-I antibody used detects only a single band at just under 10 kDa in *A. limnaeus* liver samples that co-migrates with a purified IGF-I protein standard. (C) Serial dilutions of an IGF-II protein standard and an *A. limnaeus* liver sample change in parallel in the IGF-II competitive binding ELISA assay. Nonlinear regression analysis supported a single regression equation to best describe both sigmoidal curves (\( r^2 = 0.99 \)). (D) Western blot analysis indicates the IGF-II antibody used detects only a single band at just under 10 kDa in *A. limnaeus* liver samples that co-migrates with a purified IGF-II protein standard. MW = molecular mass markers, Con = IGF protein control, BL = blank lane, AL = *A. limnaeus* liver sample.

**Western Blot Methods**

Liver was extracted from a young adult male and the sample was prepared as described for the ELISA assays. Lanes were loaded with 20 µg of liver protein and 5 µg of IGF protein (see Materials and Methods for specifics on the standards). Proteins were separated on a 12 % SDS-PAGE gel (150 V for 90 min). Proteins were transferred to a PVDF membrane (130 V for 90 min). Membranes were washed 3 times with TNT buffer (0.1 M Tris, 150 mM NaCl, 0.1 % (v/v) Tween-20) for 5 min each. Membranes were blocked with 10 % nonfat milk in TNT buffer for 30 min, followed by 3 washes in TNT buffer for 5 min each. Membranes were exposed to the same primary antibodies used in the ELISA assays for 12 h at RT. (anti-human IGF-I, 1:1000 diluted, Lot CJ1-PAB-GA; anti-trout IGF-II, 1:500 diluted, Lot CA6-PAAA1. Both antibodies from GroPep, Adelaide, Australia). The membranes were washed 3 times with TNT buffer for 5 min prior to exposure to a 90 min incubation in secondary antibody (Goat, anti-rabbit-HRP, diluted 1:10,000, Lot ab97051, Abcam, Eugene, USA). The membranes were then washed with TNT buffer 3 times for 5 min. Antigen binding was determined by enhanced chemiluminescence (Pierce™ ECL Western Blotting Substrate, Catalog #32106, Lot # RJ239671A, Thermo Fisher Scientific, Waltham, USA) and luminescence was detected using a digital imager (Fluorochrome SP system, Alpha Innotech, San Leandro, USA).