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Insulin-like Growth Factor Pathway Described in *Austrofundulus limnaeus* Diapause and Escape Embryos

Steven Cody Woll
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Insulin-like Growth Factor Pathway Described in
Austrofundulus limnaeus Diapause and Escape Embryos

by

Steven Cody Woll

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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Portland State University
2016

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Abstract

Development in the annual killifish *Austrofundulus limnaeus* can follow two distinct developmental trajectories. Typical development includes the entrance of embryos into a state of metabolic and developmental arrest termed diapause. Alternately, embryos can escape diapause and develop directly without pause. These two trajectories are characterized by differences in the rate and timing of developmental, morphological, and physiological traits. Insulin and Insulin-like growth factor (IGF) signaling (IIS) is known to regulate entrance into diapause in a variety of invertebrates. In this Thesis I explore the possible role of IGFs in the regulation of development and diapause in embryos of *A. limnaeus*. Here I report stage-specific expression of IGF-I and II proteins and their associated mRNA transcripts. Patterns of IGF-I protein expression are consistent with IGF signaling playing a major role in supporting the escape trajectory. In addition, treatment of embryos with a potent inhibitor of the IGF-I receptor (IGF1R) mimics the diapause developmental pattern even under conditions that should favor direct development. Evaluation of mRNA gene expression patterns in the two developmental trajectories suggests a role for IGF-I signaling through the RAS-MAPK-ERK pathway, which may be promoting the escape phenotype. Additionally, IGF-I activity may be enhanced in escape trajectory embryos through upregulation of IGF binding protein 2 (IGFBP-2) mRNA. These data suggest a major role for IGF signaling in the promotion of the escape trajectory, and thus we predict that specific mechanisms are in place in diapause-bound embryos that block IGF signaling and thus promote entrance into

diapause. The data presented here suggest that blocking IGF signaling is critical for induction of diapause, but also suggests that other signaling pathways are likely also at play. Other pathways such as the TGF-beta signaling molecules and SMAD pathway, may also be involved in the direct regulation of the diapause phenotype, as has been shown for other animal models of developmental arrest.

For my parents, T. Scott and Barbara M. Woll, and siblings who supported me through every endeavor I have ever embarked on. Without you I would not be the person I am.

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Chapter 1: Introduction

Development is a highly regulated process of cellular proliferation and differentiation. Proper regulation of cellular proliferation relies on the precise timing and level of expression of a variety of signaling factors (Bondy, Werner et al. 1990, Dillin, Crawford et al. 2002). In addition, target cells must be competent to receive and transduce the signals. In almost every case the outcome of these signaling pathways is context dependent and may result in growth, differentiation, apoptosis, and even developmental arrest (Lowe 1991, Baxter 2000). Developmental arrest, or diapause, has been seen in various taxa ranging from nematodes and insects, to fish and mammals (Ozanne and Hales 2004, Fielenbach and Antebi 2008). Metabolic dormancy is often associated with increased tolerance of environmental stress, and allows for a species to persist during times that it would otherwise perish (Vecchio, Soldani et al. 2006, Podrabsky, Tingaud-Sequeira et al. 2010). Diapause is initiated through endogenous cues, and precedes the onset of unfavorable environmental conditions, and thus embryos enter into dormancy under conditions that are conducive to normal development. The mechanisms responsible for developmental arrest and the associated increase in environmental tolerance are not completely understood in any species. However, the insulin-like growth factor (IGF) signaling pathway appears to be involved in the regulation of metabolic dormancy in a variety of animals (de Pablo, Perez-Villamil et al. 1993, Dorman, Albinder et al. 1995, Kimura, Tissenbaum et al. 1997, Cheng 2005). In

this thesis I explore the potential role of IGF signaling in the regulation of entrance into diapause in embryos of the annual killifish, *Austrofundulus limnaeus*.

***Austrofundulus limnaeus* Development**

Austrofundulus limnaeus, an annual killifish, inhabits ephemeral ponds in Venezuela (Podrabsky, Hrbek et al. 1998, Podrabsky 1999). These ponds exist for a few months (approximately 1-4 months) during the rainy season, and are dry for the duration of the year. Due to the nature of this extreme environment, embryos must arrest their development in diapause to extend the time of development to coincide with the return of the next rainy season (Podrabsky and Hand 1999) (Figure 1.1). Diapausing embryos likely endure severe conditions while embedded in the soil, and have been illustrated in the lab to survive large fluctuations in temperature and exposure to UV radiation, anoxia, and extreme pHs (Podrabsky, Hrbek et al. 1998, Podrabsky and Somero 2004, Podrabsky, Garrett et al. 2010, Podrabsky, Riggs et al. 2015).

Diapause in embryos of *Austrofundulus limnaeus*

Diapause in *A. limnaeus* is a state of developmental and metabolic arrest that precedes on the onset of unfavorable environmental conditions (Podrabsky, Riggs et al. 2015, Podrabsky, Romney et al. 2015). Embryos may arrest development at up to three distinct developmental phases termed diapause I, II, and III (Wourms 1972, Wourms 1972). Diapause I occurs early in development during a specialized developmental period in embryos of annual killifish known as the dispersed cell phases (Wourms 1972,

Wourms 1972, Wourms 1972). Diapause II may occur midway through development after neurulation, but prior to the major phases of organogenesis. Diapause III occurs in the late pre-hatching embryo in an essentially fully formed precocious fish larvae.

Diapause is controlled through endogenous cues, and thus dormancy may occur even under conditions that are conducive to development. A great deal is known about the biochemical and physiological properties of diapause II in embryos of *A. limnaeus*. During diapause II, *A. limnaeus* embryos halt growth at Wourms' Stage (WS) 32-33, which is near the end of somitogenesis with 38-40 pairs of somites and just prior to the major phases of organogenesis (Thomerson 1987, Podrabsky 1999, Berois, Arezo et al. 2015). There is a substantial change in cell numbers, complexity, and metabolic activity in diapausing embryos (Podrabsky and Hand 1999). Diapausing embryos have a drastically decreased metabolism (>90% reduction) that begins up to 12 days prior to developmental arrest (Podrabsky and Hand 1999, Hand and Podrabsky 2000). Oddly, there is a significant increase in total DNA content and embryonic complexity during this time when metabolism is decreasing. The cell cycle appears to arrest in the G₁ phase of the cell cycle (Meller, Meller et al. 2012). Embryos exhibit a drastic reduction in heart rate relative to non-diapausing embryos at the same stages (Podrabsky, Garrett et al. 2010).

Alternative developmental trajectories in embryos of *A. limnaeus*

Entrance into diapause II is an alternative developmental trajectory that can be regulated by both maternal inputs and the developmental environment experienced by the

embryo (Podrabsky, Garrett et al. 2010, Podrabsky, Romney et al. 2015). Embryos can also develop directly to hatching, thus allowing the completion of their entire lifecycle during a single rainy season (Wourms 1972, Podrabsky, Garrett et al. 2010, Podrabsky, Romney et al. 2015). These direct-developing embryos were termed “escape” embryos because they escape dormancy in diapause II (Wourms 1972). These two trajectories represent a bet hedging strategy (King and Masel 2007) that has been observed in many species that inhabit extreme or unpredictable environments, because it decreases risk of extinction by producing offspring of two different phenotypes. Escape embryos provide the potential for multiple spawning generations during a single rainy season, while diapausing embryos ensure survival of the population through to the next rainy season.

Evidence exists that maternal influences can control development along the diapause or escape trajectory (Podrabsky, Garrett et al. 2010). Young females tend to produce embryos that develop along the escape trajectory, while older females tend to produce a large proportion of embryos that enter diapause II. However, conditions experienced by the embryos can override this maternal input. Temperature appears to have a major effect on development trajectory, with temperature of 25°C and lower favoring diapause II. In contrast, an incubation temperature of 30°C favors development along the escape trajectory (Podrabsky, Garrett et al. 2010) (Figure 1.2).

Development along the two trajectories may result in different biochemical and physiological capacities at the completion of development. Chennault and Podrabsky (2010) found that *A. limnaeus* embryos in diapause II and escape embryos develop at different rates and complete development with different enzymatic capacities in both

anaerobic and aerobic metabolic pathways. Embryos that enter and exit from diapause II have an increased capacity for citrate synthase and lactate dehydrogenase activity relative to the escape embryos of the same developmental stage.

IGF signaling in metabolic dormancy

Cellular metabolism, growth, and proliferation are severely down-regulated in organisms that arrest development (Rahman and Thomas 2014). 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 down-regulation of DAF-2 (the *C. elegans* homolog of IGF1R) plays a crucial role in regulating entrance into dormancy as a “dauer” larvae (Kimura, Tissenbaum et al. 1997, Guo, Rena et al. 1999, Bonafe, Barbieri et al. 2003). In addition, IGF levels are decreased in red snapper, *Lutjanus campechanus*, in response to metabolic depression associated with hypoxic stress (Rahman and Thomas 2014). *I hypothesize that IGF expression and signaling plays a crucial role in the regulation of dormancy in vertebrates, and specifically in diapausing embryos of A. limnaeus.*

The IGF signaling pathway in *C. elegans* (Figure 1.3) shares many similarities with vertebrate IGF signaling (Figure 1.4), but is also unique in many ways. The *C. elegans* pathway is simplified compared to vertebrates, and thus may be useful as a construct from which to build a more coherent picture of the important aspects of IGF signaling. In *C. elegans*, extracellular IGF binds to a transmembrane bound receptor

protein called DAF-2. Ligand binding to DAF-2, induces autophosphorylation which activates the receptor and leads to phosphorylation of multiple substrates, such as the subunits of Age-1 (the PI3K equivalent) (Dorman, Albinder et al. 1995, Kimura, Tissenbaum et al. 1997). IGF signaling in *C. elegans* works mainly through activation of 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 (Kimura, Tissenbaum et al. 1997, Pierce, Costa et al. 2001, Fielenbach and Antebi 2008). 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. DAF-18, (a PTEN homolog), is also activated by low IGF signaling which leads to lower activity of PDK-1 and AKT (Ogg and Ruvkun 1998). DAF-18 thus facilitates an increase in DAF-16 activity. DAF-16 appears to be the major effector protein that induces the physiological phenotype associated with dauer formation (Lin, Hsin et al. 2001, Hesp, Smant et al. 2015).

Insulin-like growth factors in vertebrates

IGFs are powerful regulators of growth and development in vertebrate and non-vertebrate organisms. There are two basic IGFs that are found in all vertebrates, and both regulate cellular growth, division, differentiation, and migration (Lowe 1991). IGF-II activity has been associated with early embryonic development, but it is also known to

persist at much lower levels through adulthood (Scott, Cowell et al. 1985, Lowe 1991, Chastant 1994). In contrast, IGF-I is associated with later embryonic development and adulthood (O'Dell and Day 1998). In adults, IGFs are produced in the liver, muscle, brain, and bone and are delivered to all cells through the circulatory system. Regulation of IGF activity and IGF signaling is complex and includes a variety of transcriptional, and post-translational levels of control.

IGFs are transported in the blood as free hormone, or bound to IGF binding proteins (IGFBPs) that extend their half-life and typically lead to increased action (Clemmons 1991). Free IGFs bind to dimers of insulin-like growth factor I receptor (IGF1R), insulin receptor (IR), or heterodimers of the two. Binding to the receptor causes autophosphorylation that activates kinase activity of the cytoplasmic portion of the receptor (Felder, Miller et al. 1990). Phosphorylation of the receptor leads to the recruiting of IR adaptor proteins and other downstream proteins, that ultimately lead to the activation of several important intracellular signaling cascades (e.g. mTOR, AKT, MAPK) (White and Yenush 1998, Oldham and Hafen 2003). The activation of the AKT signaling pathway results in increased cell survival, metabolism, protein synthesis, and growth. Additionally, IGF1R activates the MAPK pathway that results in the activation of ERK. ERK activates a number of different proteins such as FOXM1, AP-1, ELK1 and others that typically lead to the activation of genes that lead to increased cell survival, metabolism, protein synthesis, and growth (Kim and Chung 2002, Liang and Slingerland 2003, Milton, J Dirk et al. 2008, Hers, Vincent et al. 2011). The exact effects of IGF signaling are context-dependent and can be altered by previously received signals.

Many of the processes that are regulated by a single IGF pathway in *C. elegans*, are regulated by other means in vertebrates. For example, in *C. elegans* the IGF pathway is responsible for regulation of the activity of genes involved in glucose metabolism (Ruvkun 1998). In vertebrates this activity is regulated mainly by insulin through binding to insulin receptor (Saltiel and Kahn 2001, Chang, Chiang et al. 2004). IGFs in vertebrates do have some control over the activity of genes involved in glucose metabolism, but these signaling cascades are activated much more weakly through IGF1R signaling compared to that observed through insulin receptors. There is cross reactivity in vertebrates between IGFs and IR, but insulin has a much higher affinity for IR than IGFs (Werner, Weinstein et al. 2008). Additionally, there is a third receptor in vertebrates, IGF2R (also called cation-independent mannose-6-phosphate receptor), which is not found in *C. elegans*. Unlike the other insulin family receptors, IGF-2 is the only insulin/IGF ligand that has been found to bind to IGF2R. IGF2R appears not to transduce a signal, but rather leads to IGF-II degradation and thus attenuation of IGF signaling through IGF-II. Binding of IGF-II to IGF2R leads to endocytosis and formation of an endosome where IGF-II is released and degraded, while IGF2R is then recycled and transported back to the plasma membrane (Ghosh and Kornfeld 2004) (See Figure 1.5).

In vertebrates, production and secretion of IGFs is stimulated by growth hormone (GH). In adult vertebrates, IGFs are produced primarily by hepatic tissue, but during embryogenesis, prior to liver formation, IGFs are produced globally (Lowe 1991, Nissley, Kiess et al. 1991). GH binds to growth hormone receptor (GHR), which phosphorylates Janus kinase 2 (Jak2). Activation of Jak2 ultimately results in the

activation of the genomic effects of GH signaling by inducing transcription through the phosphorylation of signal transducer and activator of transcription factor proteins 1, 3, and 5 (STAT 1, 3, and 5). STAT proteins dimerize and activate transcription of numerous genes that inhibit apoptosis, activate the cell cycle and proliferation, and induce differentiation. Increased transcription of mRNAs for IGFs is one major effect of GH signaling (Figure 1.6). GH will also affect the MAPK signaling and PI3K-AKT pathway, as does IGF (Cassoni, Papotti et al. 2001). GH also has non-genomic effects on cell physiology including the stimulation of IGF secretion.

IGF expression is essential to normal development in many organisms. In *Xenopus* embryos, it was found that expression of IGFs in the anterior region leads to head development through inactivation of Wnt signaling, while IGF1R diminution with antisense oligonucleotides radically reduces cranial structures (Pera, Wessely et al. 2001, Richard-Parpaillon, Heligon et al. 2002). Furthermore, IGF-I and II are capable of stimulating cellular proliferation and differentiation in a variety of tissues such as mouse skeletal muscles and myoblast cells (Tollefsen, Lajara et al. 1989).

In addition to their normal function as regulators of cell growth and proliferation throughout life, IGFs are also recognized as mitogens promoting the growth of various cancerous tumors (Froesch, Schmid et al. 1985, Macaulay 1992, Renehan, Zwahlen et al. 2004). There are vast amounts of data showing a link between cancerous cellular proliferations in different types of cancer and IGF dysregulation. Renehan (2004) found in a meta-analysis, which included 3609 cases and 7137 control individuals, that high concentrations of IGF-I were associated with an increase in prostate and premenopausal

breast cancer in humans. In 1998, Ma found that IGF-I levels correlated with an increased risk of colorectal cancer in humans (Ma, Pollak et al. 1999). Samani (2007) reports an association with increased levels of IGFs in various cancers in humans including: ovarian, endometrial, prostate, seminoma, osteosarcoma, gallbladder, renal cell carcinoma, glioma, astrocytoma, meningioma, medulloblastoma, colon, gastric, gastrinomas, pancreatic, hepatoma, liver, hepatocellular carcinoma, lung cancer, thyroid, and breast cancers. Increases in levels of IGF-I, II, or both have been reported in various cancers compared to normal tissue (Samani, Yakar et al. 2007). A study of age-associated proteotoxicity and Alzheimer Disease in mice has shown that IGF-I signaling plays an important role in this disease as well. Transgenic mice with reduced IGF receptor signaling and Alzheimer Disease lived longer and had reduced neuroinflammation compared to age-matched Alzheimer Disease mice (Cohen, Paulsson et al. 2009).

IGF signaling has been shown to play a critical role in the aging process in vertebrates and invertebrates, and has received a great deal of attention in *C. elegans* (Daniel and Krishnan 1969, de Pablo, Perez-Villamil et al. 1993, Kimura, Tissenbaum et al. 1997, Tissenbaum and Guarente 2001). Originally, aging was considered to be a passive process—meaning deterioration occurred in an entropic random way (Kenyon 2010). It was chaotic. However, modern theory suggests that aging can be placed into two categories: programmed and damage related. Damage related factors stem from environmental stresses that require coping mechanisms to prevent or repair damage. From yeast to primates, caloric restriction has been shown to extend lifespan and reduce the aging process (Colman, Anderson et al. 2009). Programmed aging implies that cells

are following a timetable that perhaps is just a prolonged aspect of development and growth. There are several subcategories within these two theories, and in reality both likely work together to determine the longevity of an organism.

There are many classical signaling pathways that are associated with aging, such as TOR, AMP kinase, sirtuins, and IGFs (Rogina and Helfand 2004, Greer, Dowlatshahi et al. 2007, Greer and Brunet 2009). Because of their critical roles in regulating cell growth and metabolism in all life stages, IGFs are excellent candidates for regulating the programmed aspects of organismal aging. Transgenic male mice which expressed increased Sirt6 had reduced IGF-I serum levels with increased IGFBP, but also increased lifespan compared to male mice with lower Sirt6 expression and higher IGF-I expression (Kanfi, Naiman et al. 2012) suggesting that high expression of IGFs and/or activation of the IGF pathway causes an acceleration in the rate of the aging process

IGFs are an important group of molecular signals that if understood could help lead to better cancer treatments (Denduluri, Idowu et al. 2015) and potentially lead to an extension of life or an increase in the quality of life in humans through decreasing diseases associated with aging (chronic kidney disease, diabetes mellitus, chronic obstructive pulmonary, and cardiovascular disease) and other features of aging (muscle wasting, hypogonadism, osteoporosis, and arteriosclerosis) (Stenvinkel, Kooman et al. 2016).

Insulin-like growth factors and regulation of development in *A. limnaeus*

IGF-I and -II ligands and the IGF signaling pathway are an interesting target for research on the regulation of diapause in *A. limnaeus* due to their importance in regulating cellular growth, proliferation, and aging. Insights gained from studying IGF expression and regulation in *A. limnaeus* can inform how IGFs and IGF signaling pathways change with regard to rapid as compared to arrested development.

The annual killifish, *Austrofundulus limnaeus*, offers a unique model system for addressing the role of IGFs in the control of arrested development (Wourms 1972). The presence of alternative developmental trajectories within a single species that can be experimentally manipulated allows for controlled experiments to be conducted within the same genomic background. We hypothesize that IGF expression and activity will regulate the cellular events associated with entrance into developmental arrest in this species.

In this thesis, I detail the developmental pattern of IGF protein expression using enzyme-linked immunosorbent assays (Wilkinson, Elliott et al. 2004) in both diapause and escape embryos at various developmental stages (staging determined using Wourms' Stages for killifish) (Wourms 1972, Wourms 1972). I predict that there will be differential expression of IGFs in the two trajectories: the diapausing embryos will have lower amounts of IGF proteins, while higher amounts of IGF proteins will be expressed in escape embryos. I attempt to override the predicted developmental trajectory of an embryo through pharmacological manipulation of IGF signaling. Additionally, I attempt to create a more global image of IGF signaling and how it may be important in the regulation of developmental trajectory in this species using transcriptomic data obtained

from embryos developing along the escape and diapause trajectories. This thesis illustrates that: (1) there are quantifiable differences in IGF expression between diapause and escape embryos, (2) inhibition of IGF1R activity can mimic development along the diapause trajectory even under conditions that should favor development along the escape trajectory, and (3) differential expression of genes involved in the IGF signaling pathway can explain many of the features observed in escape and diapause trajectory embryos.

Lifecycle of Austrofundulus limnaeus

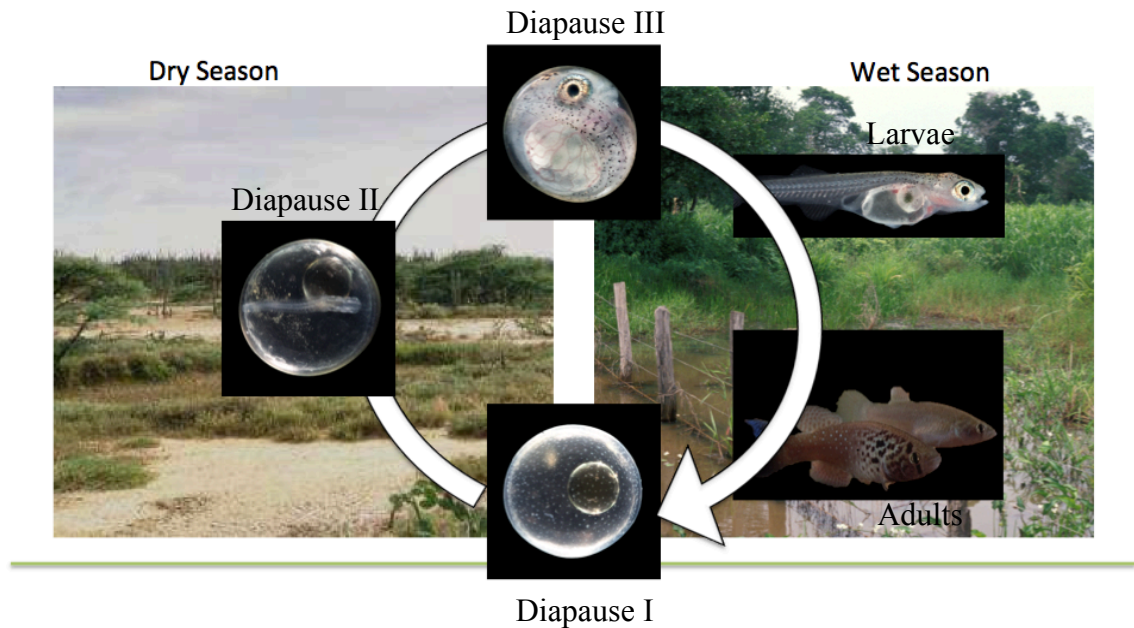


Figure 1.1. The Lifecycle of the *Austrofundulus limnaeus* Killifish.

At the onset of the rainy season, which lasts for 3-4 months, larvae hatch and develop rapidly to sexual maturity. After reaching sexual maturity, adults breed daily and produce embryos that are embedded in the sediment. At this point embryos can proceed down two trajectories: diapause or escape. Continuous embryonic development may be interrupted by diapause at each of three distinct stages of diapause, termed diapause I, II, and II: Diapause I can occur at WS 22 during dispersion of cells after full epiboly, diapause II can occur at WS 32-33 when the embryo develops 38-40 somites, and diapause III can occur at WS 43 in a prehatching embryo that is fully formed and waiting for the right signals to hatch. The most common form of diapause found in *A. limnaeus* is diapause II.

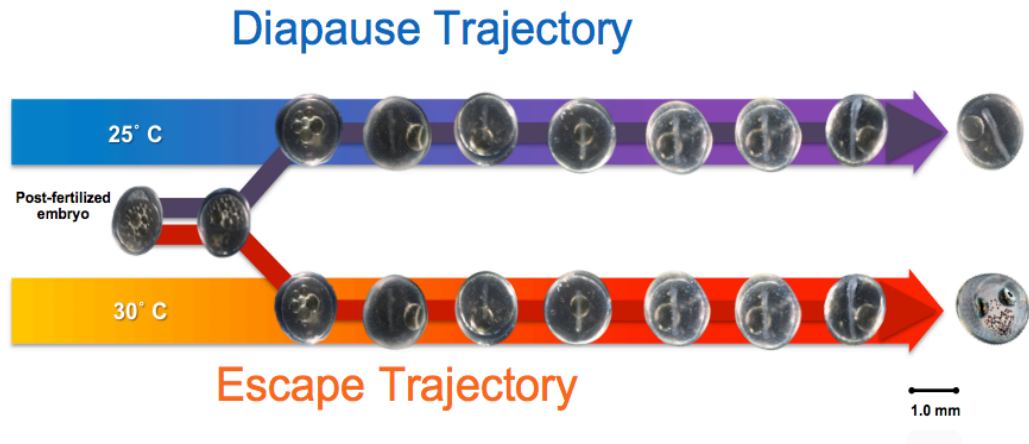


Figure 1.2. Temperature effects on developmental trajectory.

Austrofundulus limnaeus embryos reared at different temperatures will develop along two different developmental trajectories. Embryos incubated at 25°C will develop mainly along the diapause trajectory (80-90%). When incubated at 20°C, 100% of embryos develop along the diapause trajectory, while 100% of those reared at 30°C develop as escape embryos (Podrabsky, Garrett et al. 2010).

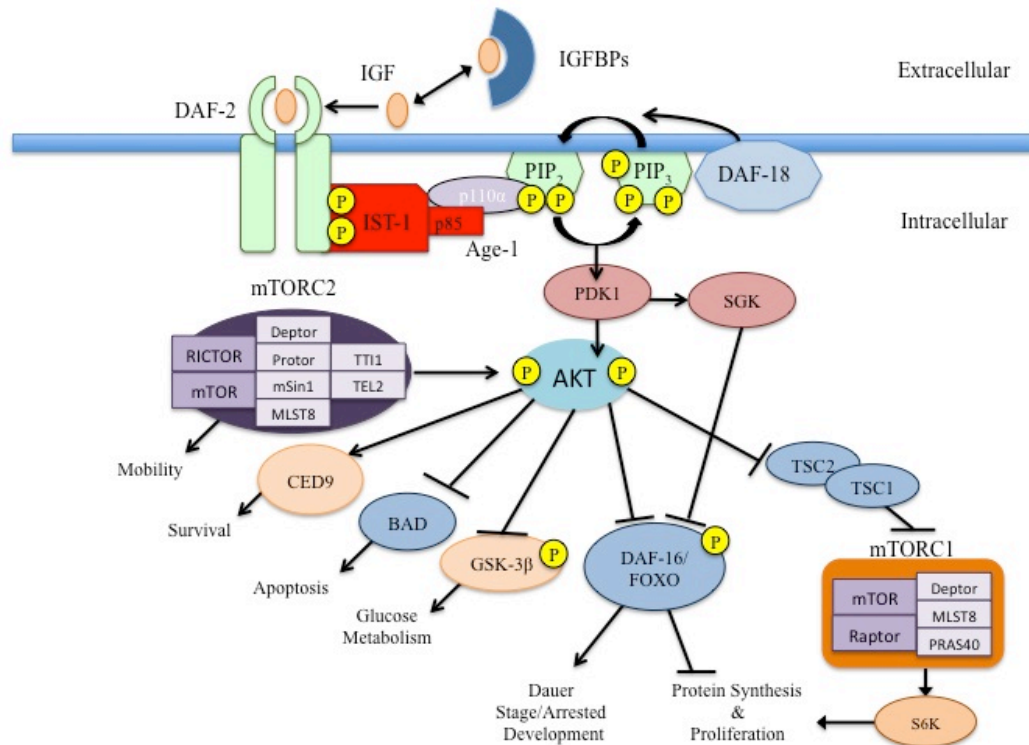


Figure 1.3. Simplified IGF signaling pathway in *Caenorhabditis elegans*.

Insulin-like protein signaling results in the activation of AKT, which acts to regulate a variety of factors and pathways to promote cell growth and proliferation. The main effector of dormancy in *C. elegans* appears to be release of inhibition of DAF-16 (FOXO homolog) in the absence of strong IGF signaling. Active DAF-16 results in many of the physiological phenotypes associated with dauer dormancy. Arrows depict activation, while lines depict inhibition. IGF – insulin-like growth factor; DAF-2 – insulin-like growth factor 1 receptor/insulin receptor homolog; IGFBP – insulin-like growth factor binding protein; IST-1 – insulin receptor substrate homolog; p85 and p110homolog – regulatory and catalytic subunits of AGE-1, respectively; PIP₂ and PIP₃ - Phosphatidylinositol 4,5-bisphosphate and Phosphatidylinositol (3,4,5)-trisphosphate ; DAF-18 - Phosphatase and tensin homolog (PTEN) tumor suppressor homolog; PDK1 - Phosphoinositide-dependent kinase-1; AKT – Protein Kinase B; SGK – Serine/threonine-protein kinase; TSC1/2 – Tuberous sclerosis 1 and 2; mTOR – mechanistic target of rapamycin; RICTOR – Rapamycin-insensitive companion of mTOR; Deptor – DEP domain-containing mTOR-interacting protein; PROTOR – Proline-rich protein 5-like; mSin1 – Target of rapamycin complex 2 subunit MAPKAP1; MLST8 – Target of rapamycin complex subunit LST8; TTI1 – Telo2-interacting protein 1 homolog; TEL2 – Telomere length regulation protein; RAPTOR – Regulatory-associated protein of mTOR; PRAS40 – Proline-rich AKT1 substrate 1; CED9 – Apoptosis regulator ced-9; BAD – Bcl2-associated agonist of cell death; GSK-3Beta – Glycogen synthase

kinase-3 beta; DAF-16 – Forkhead Box O homolog; and S6K – Ribosomal protein S6 kinase (UniProt 2015, Kanehisa, Sato et al. 2016).

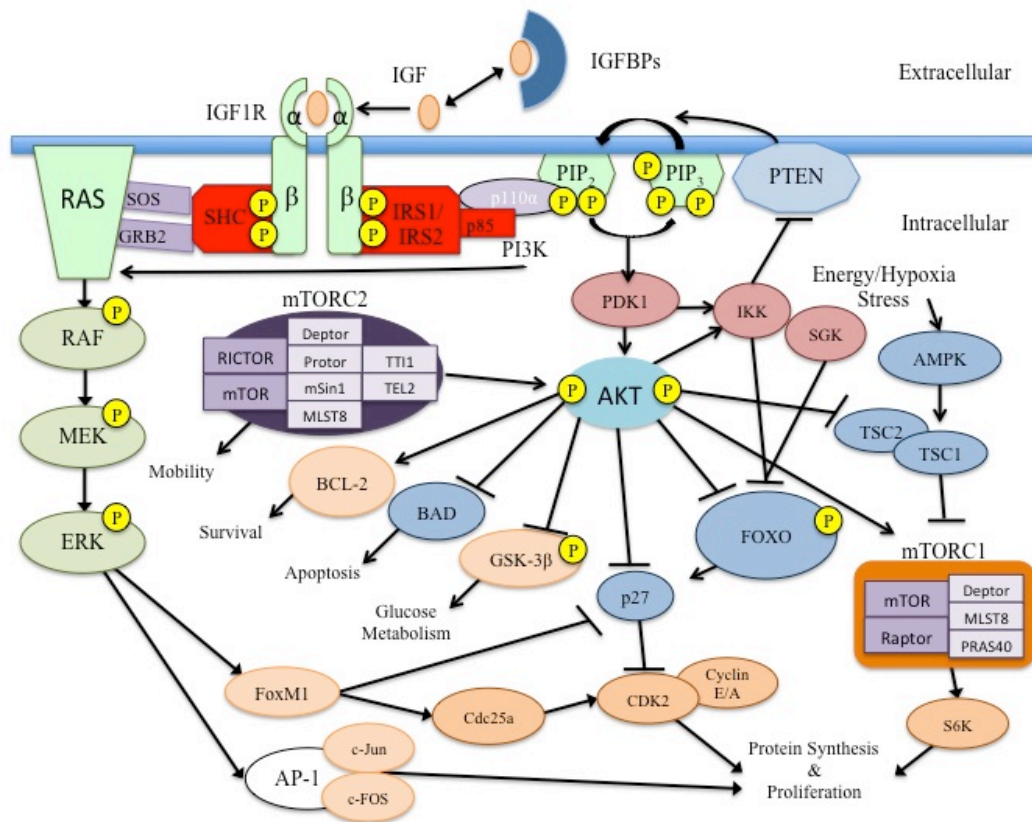


Figure 1.4. Simplified insulin-like growth factor signaling pathway in vertebrates.

Insulin-like growth factor signaling can be initiated through two different receptors, IGF1R and IR, as well as heterodimers of these two receptors. IGF-II can also be degraded through binding to the IGF2R. Activation of IGF signaling can activate at least two known pathways in vertebrates. One pathway activates AKT, in a fashion similar to that known to occur in *C. elegans*. In addition, IGF signaling can act through the ERK pathway and activation of AP-1 (c-Jun/c-Fos) transcriptional activation. The outcome of IGF signaling is similar in *C. elegans* and vertebrates. IGF – insulin-like growth factor; IGF1R – insulin-like growth factor 1 receptor/insulin receptor homolog; IGF1BP – insulin-like growth factor binding protein; IRS – insulin receptor substrate; p85 and p110 homolog – regulatory and catalytic subunits of PI3K, respectively; PIP2 and PIP3 - Phosphatidylinositol 4,5-bisphosphate and Phosphatidylinositol (3,4,5)-trisphosphate ; PTEN - Phosphatase and tensin homolog tumor suppressor; PDK1 - Phosphoinositide-dependent kinase-1; AKT – Protein Kinase B; IKK – Inhibitor of nuclear factor kappa-B kinase; SGK – Serine/threonine-protein kinase; AMPK – 5'-AMP-activated protein kinase; TSC1/2 – Tuberous sclerosis 1 and 2; mTOR – mechanistic target of rapamycin; RICTOR – Rapamycin-insensitive companion of mTOR; Deptor – DEP domain-containing mTOR-interacting protein; PROTOR – Proline-rich protein 5-like; mSin1 – Target of rapamycin complex 2 subunit MAPKAP1; MLST8 – Target of rapamycin complex subunit LST8; TTI1 – Telo2-interacting protein 1 homolog; TEL2 – Telomere length regulation protein; RAPTOR – Regulatory-associated protein of mTOR; pras40 – Proline-rich AKT1 substrate 1; BCL-2 –

Apoptosis regulator; BAD – Bcl2-associated agonist of cell death; GSK-3Beta – Glycogen synthase kinase-3 beta; FOXO – Forkhead Box O; and S6K – Ribosomal protein S6 kinase; p27 – Cyclin-dependent kinase inhibitor; SHC – ; GRB2 – Growth factor receptor-bound protein 2; SOS – Son of seven; RAS – GTPase; RAF – RAF proto-oncogene serine/threonine-protein kinase; MEK 1/2 - Dual specificity mitogen-activated protein kinase; ERK – Mitogen-activated protein kinase; - FOXM1 – Forkhead Box M1; CDC25A – M-phase inducer phosphatase; CDK2 – Cyclin-dependent Kinase 2; AP-1 - AP-1 transcription factor; c-Jun and c-Fos – c-Jun and c-Fos transcription factors (UniProt 2015, Kanehisa, Sato et al. 2016).

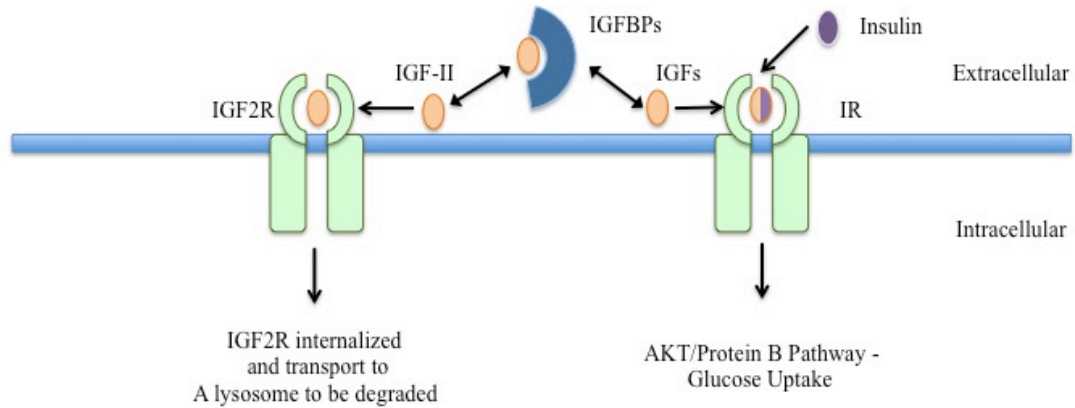


Figure 1.5. Simplified insulin-like growth factor signaling through the insulin and insulin-like growth factor 2 receptors.

Insulin-like growth factors can bind to two other possible receptors, the insulin receptor (IR) and the insulin-like growth factor 2 receptor (IGF2R). IR can bind insulin, IGF-I, or -II. IGF2R only binds with IGF2. IR activates a similar pathway as IGF1R, but it does not appear to have the same capacity for growth, differentiation, and proliferation, and has more of an effect on metabolism than IGF1R (Werner, Weinstein et al. 2008). IR also has a much higher affinity for insulin than either IGF-I or -II. IGF2R only binds IGF-II, to induce IGF-II degradation (Nissley, Kiess et al. 1991).

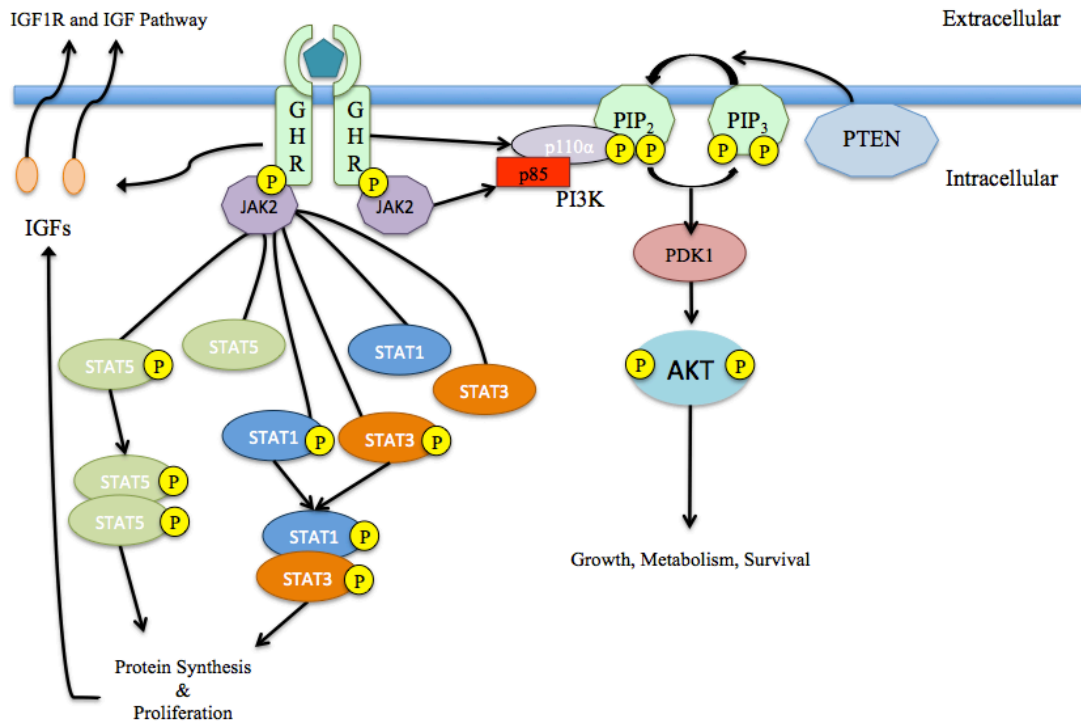


Figure 1.6. Simplified growth hormone signaling pathway.

Growth hormone (GH) signaling results in the activation of STAT-dependent transcription through the JAK-STAT signaling pathway as well as the AKT signaling pathway. Activation of STAT proteins leads to increased transcription of mRNAs for insulin-like growth factors I and II (IGFs). IGFs are secreted if GH signaling continues or are sequestered until GH signaling occurs again (Kanehisa, Sato et al. 2016).

Chapter 2: Insulin-like Growth Factor Protein Expression Profiles of Diapause II and Escape Embryos of *Austrofundulus limnaeus*

Abstract

In nearly all vertebrates, embryogenesis is a continuous process, however, some species have evolved mechanisms to reversibly halt development. One such species is *Austrofundulus limnaeus*, a killifish endemic to ephemeral ponds in Venezuela. *A. limnaeus* embryos enter diapause II (DII), a state of metabolic and developmental arrest promoted by endogenous cues, midway through development at approximately 24 days post-fertilization when incubated at 25°C (Wourms' Stage (WS) 32-33). As embryos enter diapause, heart rate declines causing blood to pool in the vasculature and morphogenesis is put on hold. Additionally, *A. limnaeus* embryos can develop down an alternative pathway where embryos “escape” from diapause and develop directly. To investigate developmental signaling factors associated with escape embryogenesis, I examined expression of insulin-like growth factor (IGF) proteins (I and II) in embryos developing along the escape and diapause trajectories. Embryos were incubated at two temperatures, one that favors entrance into diapause II (25°C) and one that favors escape embryos (30°C). Embryos were sampled throughout development, and relative expression of IGF proteins was determined by enzyme linked immunosorbent assay (ELISA). IGF-I expression peaks early in development in embryos developing along the escape trajectory, while in embryos on the diapause pathway IGF-I expression is muted until post-diapause II development. In addition, IGF-II protein levels are higher in newly

fertilized embryos that are destined to develop along the escape trajectory. These data suggest a role for increased IGF signaling in the promotion of the escape developmental trajectory. Both IGF-I and IGF-II expression is higher at the end of development in post-diapause II embryos suggesting a role for increased IGF signaling in the support of compensatory developmental acceleration that is observed in post-diapause II development for this species. These suggest a critical role for IGF signaling in the development of *A. limnaeus* and in critical trade-offs associated with entrance into diapause.

Introduction

The Annual killifish Austrofundulus limnaeus

The killifish, *Austrofundulus limnaeus*, is an annual species – a group of fishes that spend their entire life in temporary bodies of water. These bodies of water are filled during the local rainy season, but desiccate afterwards, existing as dry, depressions of cracked mud for most of the year (Myers 1952, Peters 1963, Wourms 1972, Wourms 1972). During the dry season the juvenile and adult fish die due to a variety of factors including increased predation and natural causes (Nico and Thomerson 1989). Post-embryonic life in this species appears adapted for survival and reproduction in the temporally limited environment, which includes rapid growth, very early sexual maturation, and high fecundity (Podrabsky 1999). In contrast to post-embryonic development, embryonic development in the species is relatively slow and prolonged compared to similar species with similar sized eggs; This is presumably an adaptation for survival of the dry season. The species persists through the dry season as embryos deposited into the substrate of the pond beds. Individuals may spend the majority of their lifetime as an embryo encased in drying mud, and only a few short months in post-embryonic life stages.

Embryos of *A. limnaeus* prolong development in two distinct ways. First, they develop slowly, taking almost a month to complete developmental processes that are completed within a few days in similar closely related species (Wourms 1972, Podrabsky 1999). Second, they can enter into a state of arrested development termed diapause in up to three embryonic stages. Entrance into diapause is associated with a decreased

metabolic rate, inhibition of the cell cycle and cell growth, and cessation of morphological development (Podrabsky and Hand 1999, Podrabsky and Hand 2000). Entrance into diapause II is common in *A. limnaeus*, and is the focus of this study (Hand and Podrabsky 2000). Importantly, there is an alternative pathway of development in *A. limnaeus*, where embryos “escape” from diapause II (Wourms 1972, Wourms 1972). Escape embryos develop without pause and complete their entire embryonic development without arresting. It is possible that escape embryos could complete their entire lifespan within one 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 critical developmental decisions are likely to be under intense selection pressure. Insulin-like growth factors are known to play major roles in the regulation of developmental growth and differentiation (Furstenberger and Senn 2002, Vincent and Feldman 2002, Oldham and Hafen 2003) and have been implicated in the regulation of diapause-like states in other organisms (Kimura, Tissenbaum et al. 1997, Kenyon 2001, Kenyon 2010). Thus, here I detail the expression of insulin-like growth factor proteins during development and diapause in embryos of *A. limnaeus* to assess a possible role for these critical signaling hormones in regulating developmental trajectories associated with diapause.

Insulin-like Growth Factors in Development and Diapause

Insulin-like growth factors are protein hormones excreted via exocytosis into the extracellular space (Nissley, Kiess et al. 1991). When no circulatory system is present,

these proteins act as autocrine or paracrine signals. However, in embryos and adults with a circulatory system the proteins largely act via endocrine signaling (Lowe 1991). IGF transcription, translation and excretion is typically stimulated by growth hormone signaling in adult tissues (Carter, Ramsey 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 the brain for local signaling. However, in embryonic tissues, IGFs are produced globally—meaning various cells with different lineages will produce IGFs throughout the entire embryo (Lowe 1991, Nissley, Kiess et al. 1991, Rosen and Pollak 1999).

IGF proteins and their associated signaling pathways are highly conserved across vertebrates (Greene and Chen 1997, Kenyon 2001). The IGF family includes IGF-I and IGF-II proteins, which bind to and signal through the IGF 1 receptor (IGF1R) (Hsu, Chiu et al. 2008, Hsu and Chiu 2009). IGFs have been shown to regulate various aspects of development in invertebrate and vertebrate embryos. IGF signaling is almost always associated with increased growth and differentiation. Upregulation of this pathway is found in growth in vertebrates from humans (Furstenberger and Senn 2002, Oldham and Hafen 2003, Salvatori 2004) to chickens (de Pablo, Perez-Villamil et al. 1993) to zebrafish (Eivers, McCarthy et al. 2004, Kajimura, Aida et al. 2005, Kamei, Ding et al. 2011). Liu et al. (1993) showed that mutant mice that lacked IGF1R (*IGF1R* $-/-$), grew to only 45% of normal birth weight compared to wildtype. Additionally, IGF-II null mutants grew to only 60% of normal birth weight. Double mutants IGF-II($-/-$)IGF-I($-/-$) suffered further depression of growth reaching only 30% of normal birth weight (Liu, Baker et al.

1993). In addition to their crucial role in regulating developmental growth, IGFs are also important differentiation factors, such as the case with skeletal muscle satellite cells. IGF-I and -II have been shown to induce differentiation in mice skeletal muscle and myoblast cell lines via autocrine and paracrine signaling (Tollefsen, Lajara et al. 1989, Tollefsen, Sadow et al. 1989).

The strong action and highly conserved role of IGFs in supporting cellular growth and proliferation suggests that downregulation of IGF signaling must be a critical aspect of entrance into metabolic and developmental arrest associated with embryonic diapause. There is evidence already for IGF regulation being a crucial factor in dormancy in *C. elegans* through regulation of the IGF1R (DAF-2). Entrance into dauer dormancy in *C. elegans*, a diapause-like state, is regulated by reduction of IGF signaling through IGF1R (DAF-2), and an associated increased activity of the transcription factor FOXO (DAF-16) (Lin, Hsin et al. 2001). In addition, FOXO has been shown to be a regulator of diapause in the mosquito *Culex pipiens* and *Drosophila melanogaster* (Puig, Marr et al. 2003, Sim and Denlinger 2008). Downregulation of IGF and upregulation of FOXO is thought to create conditions that protect an organism during times of environmental stress such as anoxia or low food (Sim, Kang et al. 2015).

IGFs and compensatory growth during development

Under natural conditions, developing embryos are likely to experience environmental or genetic stress that results in the retardation of growth or even a pause or arrest of development (Tamemoto, Kadowaki et al. 1994, Gillooly, Charnov et al. 2002,

Soto, Bazaes et al. 2003, Koide, Hayata et al. 2005, Saenger, Czernichow et al. 2007). When embryos then return to conditions that support growth and the continuation of development, they often show an acceleration in growth and developmental rate that has been referred to as “catch-up” or “compensatory” growth (Wilson and Osbourn 1960). This has been reported in animals as diverse as the nematode *C. elegans*, fish, and mammals (Ozanne and Hales 2004, Fielenbach and Antebi 2008, Kamei, Ding et al. 2011). The wide range of animals that exhibit this phenomenon suggests a highly conserved mechanism for embryonic growth in response to stress. There are two models that have been proposed to explain catch-up growth (Finkelstain, Lui et al. 2013). Systemic regulation theory proposes the central nervous system compares actual body size with developmental stage and adjusts growth and developmental rate according to what is age appropriate using the endocrine system (Tanner 1963, Kamei, Ding et al. 2011). The second theory proposes cell-autonomous regulation at each peripheral organ and tissue (Tanner 1963, Boersma, Houwen et al. 2002). However, it is also argued that a combination of local and systemic mechanisms working together that drive tissues towards catch-up growth (Finkelstain, Lui et al. 2013). While the exact model of action is still debated, there is concrete evidence that insulin-like growth factors I and II (IGF-I, IGF-II) and the IGF signaling pathway play a crucial role in catch-up growth (Kamei, Ding et al. 2011).

This project quantified the levels of IGF-I and -II protein using an enzyme-linked immunosorbent assay (ELISA), IGF levels were quantified during development and differentiation of two alternative developmental trajectories, direct-developing “escape”

embryos and embryos developing and arresting in diapause. *We hypothesize that escape embryos produce more IGFs throughout early development than diapausing embryos.*

Material and Methods

Animal Care and Embryo Collection

Embryos were harvested from a laboratory stock of *A. limnaeus*, originally collected in 1995 from the ephemeral ponds of Maracaibo basin located in Northwestern Venezuela (Podrabsky, Hrbek et al. 1998). Detailed methods for the husbandry of adults and collection of embryos have been previously established (Podrabsky 1999). Adults were housed in glass aquaria with a density of one spawning pair, one male and one female, in each 10 l tank. Individual tanks were connected to a common sump equipped with mechanical, chemical, and biological filtration. Ten percent of the system water was changed twice a day using dechlorinated, UV treated, 10 micron filtered City of Portland tap water (from the Bull Run Watershed) that was supplemented with 1 ppt Coral Life sea salt (Podrabsky 1999). The fish were fed twice a day with either bloodworms or earthworms. Water temperature was maintained at approximately 26°C, with photoperiod was maintained at 14hrs of light and 10hrs of dark.

Embryos were collected twice a week, Monday and Thursday, for approximately 2 hours. This interval was found to provide the highest embryo yield and quality (Podrabsky 1999). Embryos were collected through natural spawning activity into trays of glass beads (500 µm) as previously described.

Embryos were sampled from the combined output of 42 spawning pairs of fish. A total of 600 were taken from each spawning date, and 300 embryos were placed into two temperature treatment groups of 25°C or 30°C. Embryos were incubated in 100 mm x 20 mm plastic culture dishes at a density of 50 embryos or less per dish. Embryo medium (Podrabsky, 1999) containing methylene blue (0.0001%) was used for the first 4 days of development to hinder fungal growth. After four days, the embryos were bleached with sodium hypochlorite (2, 5 min washes with 0.02% with a 5 min rest between), and then transferred into embryo medium containing 10 mg/l gentamicin sulfate (Podrabsky 1999). Medium was changed and dead embryos were removed every 24 hr. Incubation temperature was controlled by placing the embryos in incubators at their corresponding temperatures in complete darkness (Podrabsky 1999). Every two days post-fertilization (dpf), embryos were inspected and staged according to Wourms (1972) using an inverted microscope (WS = Wourms' Stage). Embryos were sampled from the 25°C and the 30°C groups for each developmental stage at each temperature. Embryos were flash frozen individually in liquid nitrogen, and then stored in a -80°C freezer until protein extraction and quantification.

Identification and sampling of escape and diapause II trajectory embryos

Freshly fertilized embryos at the 1-2 cell stage were classified as diapause or escape trajectory through the monitoring of individual female reproductive output. Immediately after embryo collection, most of the embryos produced by a female (all but 10-20 embryos) were flash frozen in liquid nitrogen. A subset of each of these clutches of

embryos from each female were then monitored using an inverted compound microscope to determine the ratio of embryos that developed along the diapause and escape trajectories when incubated at 25°C. 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.

Enzyme-linked Immunosorbent Assays

Protein extraction and quantification

Individual embryos were homogenized on ice in microcentrifuge tubes with 50 μ l of 1X PBS containing 1 μ l of protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, Missouri, USA) using an acrylic pellet pestle tissue grinder. The sample was then centrifuged at 800 RPM at 4°C. Homogenates were stored at -20°C prior to protein quantification and ELISA 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. The kit provided bovine serum albumin (BSA) standard solution was used to prepare a standard curve. Linear regression analysis (minimum R^2 for the standard curve of 0.95) was used to generate standard curves (Prism v5, GraphPad) and embryo homogenate protein concentrations were interpolated from the standard curves.

Indirect ELISA for IGF-I

The relative concentrations of IGF-I were determined using an indirect enzyme-linked immunosorbent assay (ELISA) modified from (Wilkinson, Elliott et al. 2004). All samples and standards were run in triplicate. A 1 mg/ml stock solution of IGF-I protein (Salmon/Trout Insulin-like Growth Factor-I (*Oncorhynchus mykiss*, Gropep, Adelaide, Australia) was serially diluted in carbonate buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, pH 9.6) to a range of 1000 – 0.9 µg/ml. Samples were diluted to 5000 µg/ml. The wells of a 96 well plate (flat bottom, polystyrene, Microlon® 200, med. Binding, Greiner Bio-one, Germany) were coated with standards or samples for 24 hr at 4°C on a rocker with gentle rotation. Coating buffer was removed by pounding the microplate onto clean paper towels until the wells were dry. The microplate wells were then washed with 300 µl of wash solution (1 X PBS, 0.05% Tween-20) for 5 min on a rocker with gentle rotation at room temperature. Wash solution was removed by pounding the microplate onto clean paper towels until wells were dry. Washing was repeated 5 times. Following the washes, wells were incubated in 200 µl of blocking buffer (2% BSA in 1 X PBS) for 1 hr at room temperature. After blocking, wells were washed as described above. Wells were then incubated with 100 µl of IGF-I rabbit polyclonal antibody (raised against human IGF-I, Gropep, Adelaide, Australia, diluted 1:1000) for 1 hr at room temperature on a rocker with gentle rotation. The microplate wells were again washed 5 times as previously detailed. Wells were then incubated with 100 µl of anti-rabbit IgG antibody conjugated with HRP (Abcam, diluted 1:500) for 1.5 hr at room temperature on a rocker with gentle rotation. The microplate wells were again washed 5 times as previously detailed. ELISA

color development was initiated by the addition of 100 μ l of 1-Step Ultra TMB-ELISA (ThermoFisher Scientific) coloring substrate followed by a 15 min incubation at RT in the dark. Color development was stopped by the addition of 100 μ l of 0.5 M sulfuric acid. Absorbance at 450 nm (A_{450}) was measured for each well using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). For each sample the average of the three A_{450} 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.

Competitive ELISA for IGF-II

Standards and samples were prepared in 1.5 ml centrifuge tubes (Natural Micro Centrifuge Tube, Sealrite, USA Scientific). Standard solutions of purified salmon IGF-II (GroPep, Adelaide, Australia) were diluted in 1X PBS containing 0.1% BSA to final concentrations ranging from 1000 – 0.9 μ g/ml in a final volume of 400 μ l. For embryo homogenates, 125 μ g of total protein was diluted in 1X PBS containing 0.1% BSA to a final concentration of 5000 μ g/ml in a final volume of 400 μ l. Samples and standards were treated with acid to release IGF-II from IGF-binding proteins (IGFBP). The diluted standard and samples were acidified by the addition of 70 μ l 0.1 M Glycine-HCl (pH 2.0) to each tube followed by a 30 min incubation with gentle rocking at room temperature. The samples/standards were neutralized by the addition of 30 μ l 0.2 M Tris (pH 10.0). IGF-II antibody solution (200 μ l of 1:1000, diluted in 1X PBS with 0.1% BSA) was added to each standard or sample. The IGF-II antibody was a rabbit polyclonal antibody

raised against Salmon/Trout IGF-II (Gropep, Adelaide, Australia). The standards and samples were then incubated with gentle rotation for 2 hr at room temperature.

The relative concentrations of IGF- II proteins were determined using a competitive binding enzyme-linked immunosorbent assay (ELISA) modified from (Wilkinson, Elliott et al. 2004). All samples and standards were run in triplicate. A 1 mg/ml stock solution of IGF protein (Salmon/Trout Insulin-like Growth Factor-II (*Oncorhynchus kisutch* & *Oncorhynchus mykiss*, Gropep, Adelaide, Australia) was diluted in carbonate buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, pH 9.6) to a final concentration of of 50 µg/ml. Each well of a 96 well microplate (flat bottom, polystyrene, Microlon® 200, med. Binding, Greiner Bio-one, Germany) was coated with 100 µl of IGF-II for 24 hr at 4°C on a rocker with gentle rotation. Coating buffer was removed by pounding the microplate onto clean paper towels until the wells were dry. The microplate wells were then washed with 300 µl of wash solution (1 X PBS, 0.05% Tween-20) for 5 min on a rocker with gentle rotation at room temperature. Wash solution was removed by pounding the microplate onto clean paper towels until the wells were dry. Washing was repeated 5 times. Following the washes, wells were incubated in 200 µl of blocking buffer (2% BSA in 1 X PBS) for 1 hr at room temperature. After blocking, wells were washed as described above. IGF-II standards or embryo homogenates were prepared as described above and 100 µl of each preparation was added to a well and incubated for 30 min with gentle rocking. The microplate wells were then washed 5 times as described above. Wells were then incubated with 100 µl of anti-rabbit IgG antibody conjugated with HRP (Abcam, diluted 1:1000) for 1 hr at room temperature on a rocker with gentle

rotation. The microplate wells were again washed 5 times as previously detailed. ELISA color development was initiated by the addition of 100 μ l of 1-Step Ultra TMB-ELISA (ThermoFisher Scientific) coloring substrate followed by 15 min incubation at RT in the dark. Color development was stopped by the addition of 100 μ l of 0.5 M sulfuric acid. Absorbance at 450 nm (A_{450}) was measured for each well using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). For each sample the average of the three A_{450} 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.

Standard curves for each plate were generated using linear regression analysis. If the R^2 value of the standard curve was less than 0.90, the whole assay was discarded. Amounts of IGF proteins in embryo homogenates were calculated by interpolation using their A_{450} values and the standard curve. Relative concentrations of IGFs were then calculated relative to the amount of IGF-I or II present in a newly fertilized embryo sample that was run on each plate.

Statistical Analysis

Graphical and statistical analyses of the data were performed using Prism 5.0 software (GraphPad) and R version 3.2.1 (R Studio). 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.

Fold change was calculated by based on the either corresponding DPF or WS depending on what was appropriate (Mariani, Budhreja et al. 2003).

Results

Validation of the ELISA Protein Assay

The linear range of the IGF-I and IGF-II ELISAs was determined by serial dilution of a random sample from the data set for each protein. Linear regression analysis was used to determine if changes in concentration were detected in a linear fashion. For both the IGF-I and –II, the serial dilutions resulted in a linear relationship between protein concentration and color development as illustrated in Figure 2.1. For both proteins an R^2 value of 1.0 was calculated for the relationship between protein concentration and assay color development, illustrating that 100% of the variation in the assay signal was due to differences in the amount of IGF protein in the sample.

Expression of IGF-I

There are clear differences in the expression of IGF-I as a function of developmental time in embryos developing along the diapause II and escape trajectories (Figure 2.2). Because the two trajectories develop at very different rates, it is easier to compare similar developmental stages if the data are compared as a function of developmental stage, rather than age (Figure 2.3). When similar stage embryos are compared, there is a clear spike in IGF-I protein expression between WS 20 and WS 30

in embryos developing at 30°C along the escape trajectory. Expression 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, $p < 0.001$). Expression 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, it is clear that embryos incubated at 30°C experience a spike in IGF-I protein expression early in development that is completely absent in embryos developing at 25°C (Figure 2.3). The pattern of IGF-I protein expression 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 expression of IGF-I at WS 43, essentially at the completion of development. These different patterns of expression are supported by statistical differences in mean IGF-I protein expression at numerous developmental stages (Figure 2.3).

Expression of IGF-II

The pattern of IGF-II expression is quite different from that of IGF-I, for both developmental trajectories. First, 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 2.3, 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 expression 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 (Dupont and Holzenberger 2003). In addition, IGF's 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 (de Pablo, Perez-Villamil et al. 1993, Kimura, Tissenbaum et al. 1997, Richard-Parpaillon, Heligon et al. 2002, Eivers, McCarthy et al. 2004, Hondo and Stewart 2005, Simao, Camps et al. 2007, Fielenbach and Antebi 2008). This is the first study to evaluate the potential role of IGFs in development and diapause in embryos of the annual killifish *Austrofundulus limnaeus*.

Entrance into diapause II in *A. limnaeus* is an alternative developmental trajectory that can be programmed by maternal inputs and also triggered by incubation temperatures (Podrabsky et al., 2010). This study documents significant differences in the timing and magnitude of IGF protein expression in the two developmental trajectories. These differential expression patterns suggest a major role for IGF protein signaling in the promotion of the escape trajectory during early development, and in the regulation of the rate of embryonic growth during post-diapause II development.

Regulation of developmental trajectory

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, when embryos are incubated at 30°C, a spike of IGF-I protein is observed in the stages just leading up to this critical window. Thus, it is possible that IGF-I signaling initiates a gene expression program that favors direct development and prevents the embryos from entering into diapause II. This is the first evidence that implicates IGF signaling in the control of diapause in annual killifish.

Maternal provisioning or influences are known to affect the developmental trajectory of *A. limnaeus* (Podrabsky et al., 2010). The higher expression of IGF-II protein in 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, Robertson et al. 1991, Vu and Hoffman 1994, O'Dell and Day 1998). Analysis of downstream targets of IGF-II signaling may help to identify these targets.

Catch-up growth during post-diapause II development

Compensatory acceleration in growth and development is a typical 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 throughout the animal kingdom, and in response to a number of human diseases (Gillooly, Charnov et al. 2002, Hales and Ozanne 2003, Kajimura, Aida et al. 2005, Saenger, Czernichow et al. 2007, Fielenbach and Antebi 2008). Building off of Kajimura et al. (2005), Kamei (2011) shows that after hypoxia there is a “catch-up” pattern in *D. rerio* embryos whose IGF pathways were inhibited pharmacologically or by hypoxia. These studies confirmed that over-expression of IGF-I fueled the compensatory growth (Kamei, Ding 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. However, Hales and Ozanne (2003) point out that there are long-term consequences to this short-term fix that have potential drawbacks in relation to long-term survival. 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). Reproductive fitness has also been affected in some species such as skewed sex ratios in flesh flies destined for diapause (Denlinger 1981).

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. 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 expression in this study support a role for increased expression 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 expression 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 killifish suggest that these developmental differences in IGF expression may have profound consequences on the biology of these fish (Polačik, Blažek et al. 2014).

Conclusions

This study documents for the first time the expression patterns of IGF-I and IGF-II during development and diapause in annual killifishes. The patterns of expression suggest that IGF-I and IGF-II may both play distinct roles in regulating developmental trajectory in early embryos of *A. limnaeus*. In addition, patterns of IGF expression are vastly different during late development in these two developmental trajectories, and may lead to long-term differences in adult physiology and behavior. Further exploration of the IGF signaling pathway in this species is likely to shed light on the regulation of diapause

in this species, and perhaps help to elucidate the connection between diapause and longevity that has been observed in other species.

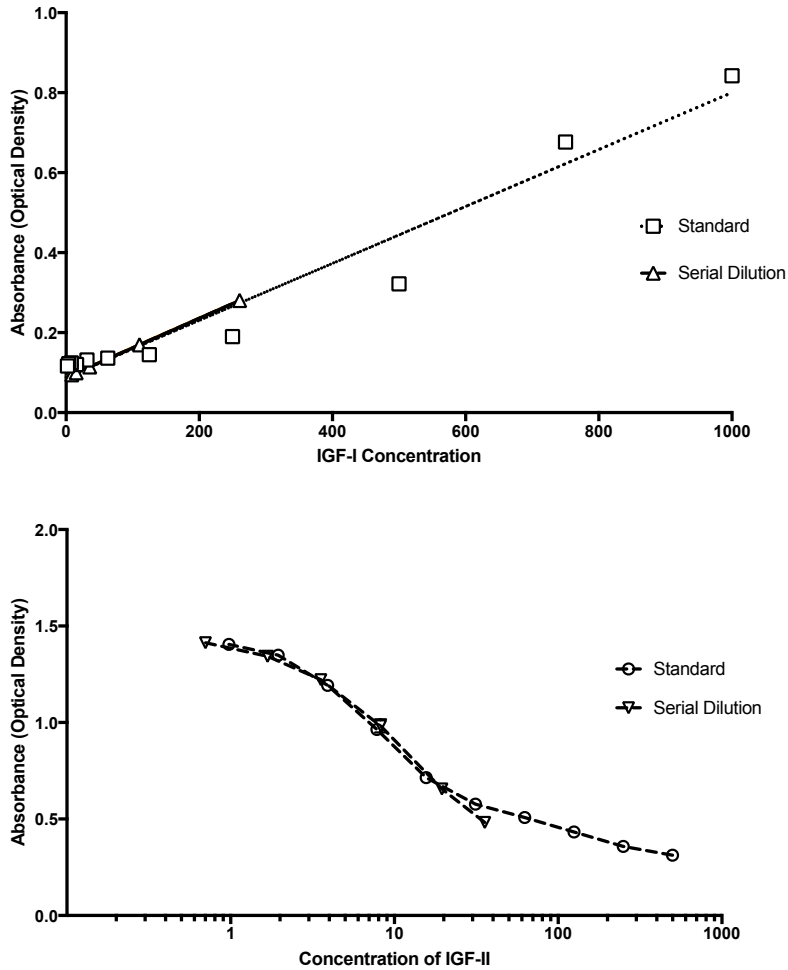


Figure 2.1. Absorbance is directly proportional to the concentration of IGF-I and IGF-II protein in the ELISA assays used for this study.

A.) Symbols are means \pm sem (n=3-5). For IGF-I, the data represent a serial dilution of a sample derived from 4 days post-fertilization embryos at 25°C ($R^2 = 1$, $p < 0.0001$). B.) For IGF-II, the data represent a serial dilution of a sample derived from 2 days post-diapause II embryos at 25°C ($R^2 = 0.99$, $p < 0.0001$).

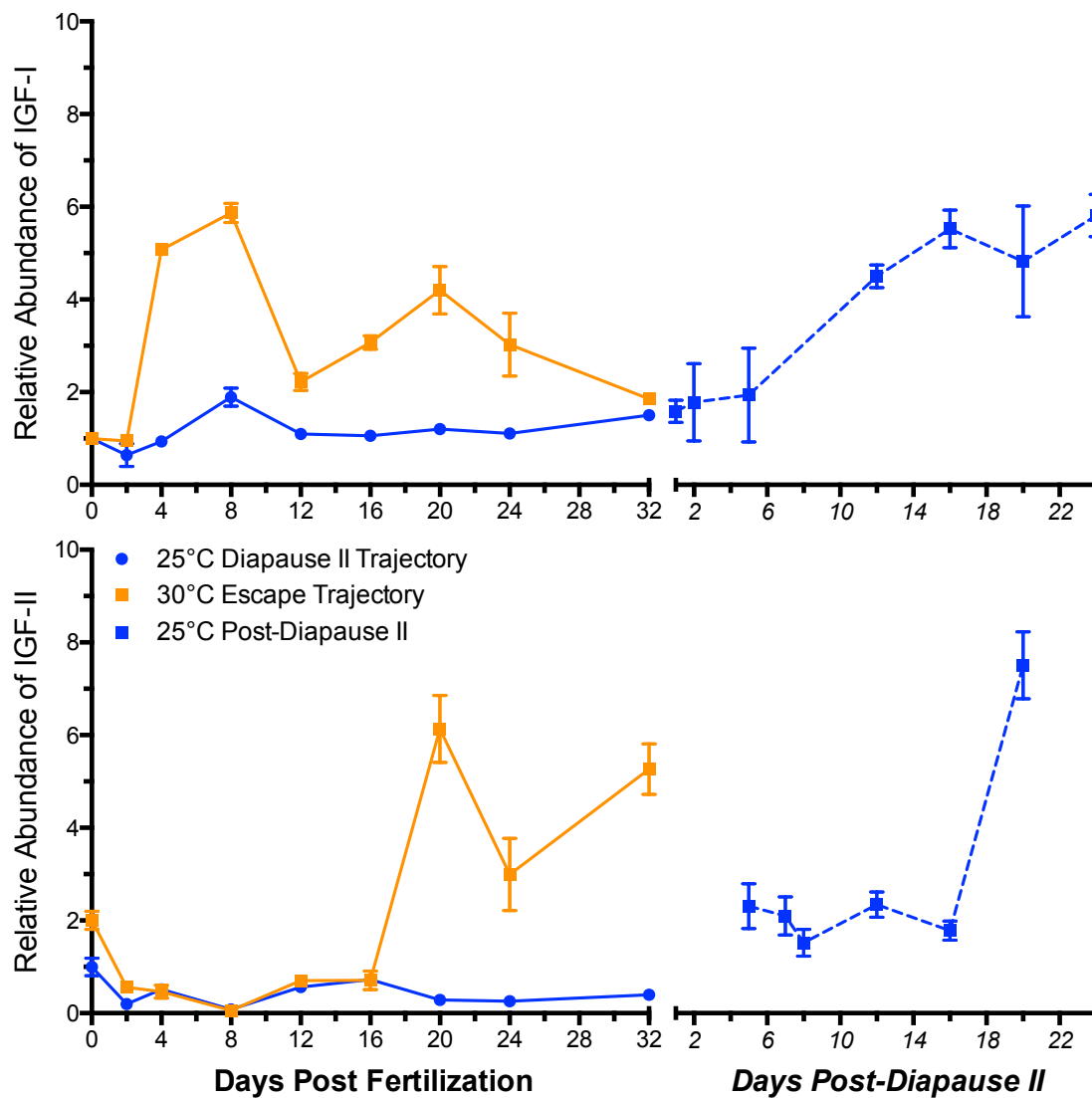


Figure 2.2. Relative abundance of IGF-I and IGF-II in embryos incubated at 25°C (diapause II trajectory) and 30°C (escape trajectory) as a function of developmental time.

Protein levels are expressed relative to the mean of protein levels for embryos at 0 days post-fertilization (Wourms' Stage 4) incubated at 25°C. Symbols are means \pm sem (n=3-10).

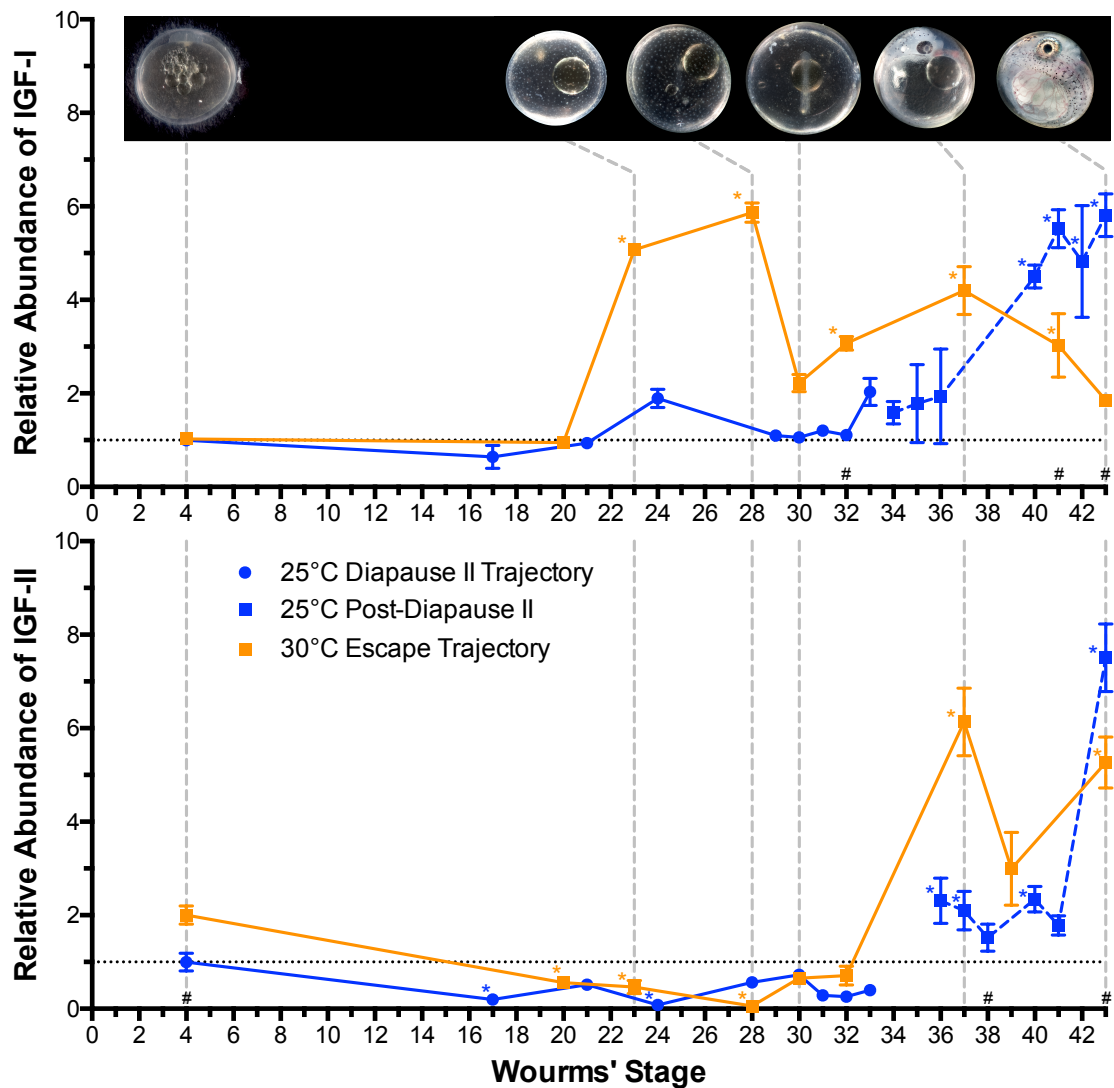


Figure 2.3. Relative abundance of IGF-I and IGF-II incubated at 25°C (diapause II trajectory) and 30°C (escape trajectory) as a function of developmental stage.

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 \pm sem (n=3-10). Black dotted lines at y=1 indicates relative expression level of WS 4 embryos. Gray vertical dashed lines indicate various developmental stages as indicated in the top panel. Statistical comparisons were made within each treatment group only using ANOVA and Dunnett's multiple comparison test. 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, $p < 0.001$).

Chapter 3: Development of *Austrofundulus limnaeus* Embryos Subjected to Pharmacological Inhibition of Insulin-like Growth Factor Pathway

Abstract

Many animals respond to environmental stress by slowing down or arresting growth and development. However, some animals, like *Austrofundulus limnaeus* have the ability to arrest development in diapause through endogenous mechanisms as a means to “escape in time” from unfavorable environmental conditions. Blockage of insulin-like growth factor signaling has been implicated in the regulation of entrance into diapause and diapause-like states in a number of animal groups. To determine if the IGF pathway plays a critical role in regulation of diapause in embryos of *A. limnaeus*, we inhibited the IGF pathway with pharmacological agents. NVP-AEW541, BMS-754807, LY294002 and U0126 were used to inhibit the Insulin receptor/IGF1R, IGF1R, PI3K and Mek1/2 activities, respectively. Embryos in LY294002 and U0126 were unaffected at non-lethal levels of inhibitors and had no significant difference in development from control embryos. Embryos placed in NVP-AEW541 and BMS-754807 experience reduced growth in both 25°C and 30°C, but embryos in NVP-AEW541 were able to compensate and catch-up in growth rapidly, and had no arrested development. Embryos treated with BMS-754807 experienced significant delays in morphological and physiological development that in many ways mimic entrance into diapause II. Activation of the IGF

signaling pathway appears to be part of a cohort of regulatory events that lead to active development in this species.

Introduction

The annual killifish, *Austrofundulus limnaeus*, inhabits the ephemeral ponds of Venezuela that dry on a seasonal basis. This harsh and often unpredictable environment has driven the evolution of embryonic development to include diapause as an adaptation for survival through the dry season. The ability to arrest development and endure the extreme stresses of this environment is not a simple pause in development, but an alternative developmental pathway that differs morphologically, physiologically, and biochemically from direct-developing “escape” embryos (Chennault and Podrabsky 2010, Podrabsky, Garrett et al. 2010, Furness, Reznick et al. 2015) (also see Chapter 2). In other animal groups Insulin-like growth factor signaling appears to play a critical role in regulating entrance into metabolic dormancy. Here I explore the possible role of IGF signaling in the regulation of diapause in embryos of *A. limnaeus*.

Insulin-like growth factors (IGFs) are a group of highly conserved protein hormones (Bonafe, Barbieri et al. 2003) that regulate various developmental processes such as cell division, growth, differentiation, and migration (Jones and Clemmons 2013). IGF activity is mitigated through three classes of receptors: insulin receptors, insulin-like growth factor 1 receptors (IGF1R), and insulin-like growth factor 2 receptor (IGF2R, or cation-independent mannose-6-phosphate receptor) (LeRoith, Werner et al. 1995). The main receptor for IGF-I and IGF-II that activates the IGF signaling pathway is the IGF1R. Insulin receptor (IR) has a much higher affinity for insulin than it does 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 (Massague and Czech 1982, Kim and Accili 2002). However, there is evidence of hybrid heterodimers formed by one IR α and β subunit and one IGF1R α and β subunit (Kasuya, Paz et al. 1993, Soos, Field 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, Pandini et al. 1999). In contrast to the other receptors, IGF2R is a negative regulator of the IGF pathway that is responsible for attenuating IGF-II signals by binding IGF-II and stimulating endocytotic lysosomal degradation (Griffiths, Hoflack et al. 1988, Lau, Stewart et al. 1994).

The IGF1 receptor is activated when either IGF-I or II binds with two extracellular α -subunits of IGF1R. Binding of the ligand stimulates autophosphorylation of the IGF1R intracellular domain, which activates intracellular signaling cascades including the MAPK/Erk (Ras-Raf-Mek-Erk) pathway, and the PI3K/AKT/mTOR pathway (phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein kinase B (PKB or AKT)/mammalian target of rapamycin) pathway (Furstenberger and Senn 2002, Kim and Accili 2002). The activation of these pathways results in a context dependent regulation of a diversity of cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis (Vincent and Feldman 2002, Jones and Clemmons 2013).

A number of pharmacological reagents have been developed that can inhibit the function of various proteins in the IGF pathway. NVP-AEW541 is one novel and potent inhibitor of IGF receptors, but it also has significant cross-reactivity with the insulin

receptor (Garcia-Echeverria, Pearson et al. 2004). BMS-754807 is another potent inhibitor that specifically targets IGF1R with less cross-reactivity with the insulin receptor (Carboni, Wittman et al. 2009, Kamei, Ding et al. 2011). Both of these inhibitors block receptor activity and thus will act to reduce or abolish IGF-I or II signaling through the IGF1R. LY294002 and U0126 are both inhibitors of signaling pathways downstream of the IGF1R receptor. LY294002 is a PI3K $\alpha/\delta/\beta$ inhibitor, which is involved in crucial cellular functions such as cellular growth, proliferation, differentiation, motility, survival, and intracellular trafficking and is the first step in the PI3K-AKT-mTOR pathway. U0126 is a MEK 1/2 inhibitor, which is involved in various crucial cellular functions as well and is part of the link in the Ras-Raf-Mek-Erk pathway. Both of these downstream signals are not solely activated by IGF-I and II through the IGF pathway. The PI3K-AKT-mTOR is also activated through prolactin (PRL) and epidermal growth factor (EGF) (Bjorge, Chan et al. 1990, Ratovondrahona, Fournier et al. 1998). While these inhibitors are likely to have more non-IGF-specific effects, they may also help to shed light on the signaling pathways that IGF receptors are acting through during development in *A. limnaeus*.

Animal embryogenesis is believed to be shielded against perturbation from genetic and environmental stressors to ensure correct and accurate development of form and function. This concept of development referred to as “canalization” was first put forth by Waddington (Waddington 1942). Waddington stated that, “developmental reactions, as they occur in organisms submitted to natural selection...are adjusted so as to bring about one definite end-result regardless of minor variations in conditions during the

course of the reaction”. Canalization and developmental stability work together to define the temporal and spatial constraints of the developmental processes that are commonly observed in developing embryos (Willmore, Young et al. 2007). Canalization is thought to be especially important during early development and the establishment and patterning of the primary body axes, which has been established for over a century by Von Baer and his three laws (Von Baer 1828, Duboule 1995) that suggest developmental plasticity and heterokairy (see below) are minimized during the development of most systems (Spicer and Burggren 2003, West-Eberhard 2005, Gomez-Mestre and Buchholz 2006).

Developmental plasticity comes in two forms: Developmental conversion and phenotypic modulation. The difference between phenotypic modulation and developmental conversion is that phenotypic modulation is a sliding scale that can have a variety of phenotypic outcomes, where conversion is a “switch” that is turned on or off (Smith-Gill 1983). Developmental conversion states that organisms use a specific environmental cue to activate alternative genetic programs controlling development. A classical example is temperature effects on fish embryogenesis, Stockard (1921) defined the concept of “critical periods,” in which it was noted that teratogenic effects are not agent-specific but are determined by the stage of development in which exposure occurs—meaning that at specific stages in development different organs or tissues will be more or less sensitive to environmental stressors. Such differential sensitivities can then alter the relative timing of growth and maturation of various tissues or organs (Stockard 1921). Environmentally induced acceleration or retardation of development of individual parts relative to other parts may lead to significant phenotypic alterations (Berven, Gill et al. 1979). Alternative

pathways may lead to different morphologies, rates of development, and even to developmental arrest as illustrated in *A. limnaeus* (Podrabsky, Garrett et al. 2010).

Heterokairy is defined as variability or plasticity in timing of various developmental events at the individual or population level (Spicer and Burggren 2003, Spicer and Rundle 2007). This can apply to both rates of development or the scheduling of developmental events. A classical example of heterokairy can be found in cultured brine shrimp *Artemia*. When *Artemia* are cultured under hypoxia (PO₂ 50% of normoxia), they precociously develop the adult phenotype of being oxyregulators at developmental stage 3 instead of the typical stage 6 (Spicer and El-Gamal 1999). Most documented cases of alternative phenotypes have been shown to occur late in the developmental sequence, and typically as rather minor alterations in morphology or physiology (Spicer and Burggren 2003, Gomez-Mestre and Buchholz 2006, Spicer and Rundle 2007, Storz and Travis 2007). In the case of *A. limnaeus*, two alternative developmental pathways, diapause II and escape, diverge early in development during formation of the primary embryonic axis (Podrabsky, Garrett et al. 2010). Alternative pathways are part of the normal developmental program in this species and can be regulated by maternal effects and by the incubation temperature experienced by the embryo (Podrabsky, Garrett et al. 2010).

The goal of this study is to determine if pharmacological blocking of IGF signaling will affect the developmental phenotypes in *A. limnaeus* embryos. Specifically, I hypothesize that blocking the IGF pathway during the temperature-sensitive critical window for determining developmental trajectory (Podrabsky, Garrett et al. 2010) will

result in embryos entering diapause II even at temperatures that should induce the escape pathway.

Materials and Methods

Animal Care and Facilities

Animal care and facilities are identical to section in chapter 2.

Sampling for Pharmacological Inhibition

Embryos were spawned on February 12th and March 4th, 2016 following the same protocols as seen in chapter 2. After 24 hours, embryos were staged according to Wourms' staging (WS) protocol (Wourms 1972) using an inverted microscope. Typical developmental timing for embryos developing at 25 and 30°C are presented in Table 4.1. A total of 880 embryos were observed across the two spawning events in this experiment. For pharmacological exposures, individual embryos were incubated in wells of a 96-well plate that contained 300 µl of media. All embryos were observed daily for the duration of the experiment using an inverted compound microscope (Leica, DMIRB).

Pharmacological Inhibition Protocol

Four inhibitors were chosen for this study: NVP-AEW541, BMS-754807, LY294002, and U0126 were used to inhibit the Insulin receptor/IGF1R, IGF1R, PI3K and Mek1/2 activities, respectively. All pharmacological inhibitors were purchased from

Selleck Chemicals (Houston, Texas, USA). BMS-754807 is a specific inhibitor for IGF1R (Bid, London et al. 2013), while NVP-AEW541 is a more general inhibitor of the insulin and IGF pathways. Lyophilized pharmacological agents were reconstituted in dimethyl sulfoxide (DMSO), as stock solutions. Stock solutions were diluted into embryo medium (Podrabsky 1999) without antibiotics and with a final concentration of less than 1% DMSO. For each inhibitor, control embryos were exposed to the same amount of DMSO as embryos treated with the pharmacological agents. Final concentrations used in the experiments were: NVP-AEW541 (100, 50, 20, 10, and 5 μM); BMS-754807 (5, 4, 3, 2.5, and 1 μM); Ly294002 (100, 50, 25, 5, and 2.5 μM); U0126 (200, 100, and 50 μM). These concentrations are based on similar experiments performed in *Danio rerio*, but taking into account known differences in the permeability of *A. limnaeus* embryos compared to *D. rerio* embryos for such compounds. Due to these known differences, the concentrations used here are about 10 times the effective concentration used in *D. rerio* (Brummett and Dumont 1981, Kim, Hwang et al. 2006, Kamei, Ding et al. 2011).

Heart Rate Monitoring

Heart rate was monitored for embryos exposed to three concentrations of BMS-754807 (2.5, 3 and 4 μM) as soon as heart contractions were visible. Heart rate is expressed in beats min^{-1} . The number of somite pairs was counted for each embryo.

Statistical Analysis

Graphical and statistical analyses of the data were performed using Prism 7.0 software (GraphPad). Where appropriate, analysis of variance (ANOVA), t-tests or linear regression analysis 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 $P < 0.05$. For percentage data, the statistics were applied to the arcsine transformation of the proportions (Zar 1996).

Results

Embryos incubated in low concentrations of NVP-AEW541, LY294002, and 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. 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.

Developmental progression

The inhibitor that is specific for IGF1R, BMS-754807, exhibited much more 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 3.1). Compared to embryos developing at 30°C along the escape trajectory, embryos incubated in inhibitor concentrations of 2.5 μ M and greater were delayed significantly (ANOVA, Tukey's HSD, $p < 0.01$). Embryos developing in intermediate concentrations of inhibitor (2.5, 3 and 4 μ M BMS-754807) that were incubated at 30°C were indistinguishable from control embryos developing at 25°C (ANOVA and Tukey's HSD, $p = 0.995, 0.9996, 0.0691$, respectively). The 5 μ M 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 3.1).

Rates of somitogenesis were significantly reduced by incubation in the IGF1R inhibitor. Linear regression analysis indicates that embryos developing at 30°C in the presence of inhibitor had rates of somitogenesis comparable to embryos developing at 25°C on the diapause trajectory (Figure 3.2).

Heart Rate

Heart rate was significantly decreased in embryos incubated at 30°C in the presence of the IGF1R inhibitor BMS-754807 (Figure 3.3). In fact, for embryos incubated at 2.5, 3, and 4 μ M BMS-754807, heart rates were similar to embryos incubated at 25°C along the diapause trajectory (ANOVA and Tukey's HSD, p -value = 0.2764, 0.9882, and 0.9998, respectively).

Morphological Development

Embryos incubated in 5 μ M BMS-754807 arrested development in the dispersion and reaggregation phases of development. Embryos treated with 2.5, 3, and 4 μ M BMS-754807 developed a morphology very similar to those of diapause II embryos with some significant differences. First, melanocytes differentiate and migrate in embryos exposed to inhibitor, while these are never expressed until after diapause II in normal embryos. Second, despite the low heart rates, circulating blood cells that express hemoglobin are present in the embryos treated with inhibitor, while this trait 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 3.4)

Discussion

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. However, inhibition of IGF1R does not appear to completely mimic entrance into diapause, and it appears that some processes that arrest in association with diapause may continue despite blocking of the IGF1R. These results may be interpreted in a number of different ways, but the two main conclusions are: (1) blockage of IGF-I signaling was incomplete in this study due to

continued IGF-I signaling through the IR/IGF-I heterodimers, and (2) IGF signaling is a critical regulator of cell growth and proliferation that is blocked in embryos that enter diapause, but it is not the ultimate signaling mechanism that induces the diapause or escape programs. Both of these explanations have merit and will be evaluated below.

The IGF1R inhibited phenotype

Treatment with BMS-754807 caused a significant delay in a number of developmental and physiological processes that approximate the diapause trajectory even under environmental conditions that should promote developmental along the escape trajectory. First, the pattern of heart development and physiological activity were almost indistinguishable from embryos developing along the diapause trajectory. However, one apparent difference is the appearance of pigment (presumably the expression of hemoglobin) in the circulating precursors of red blood cells. This is a trait that normally does not develop until after embryos have exited from diapause II. IGF-I and -II have been shown to affect the developing cardiovascular system in many different ways. However, there is no support for IGF signaling regulating the timing of hemoglobin expression in other developing fishes. In *Danio rerio*, IGF signaling is crucial for normal cardiac and circulatory development. Wood et al (2004) found that embryos with inhibited and reduced IGFBP-2, which increases the half-life of IGFs and has been shown to mediate binding of IGF1R (Shen and Singh 2004), had reduced amounts of IGF-I mRNA, reduced body growth, and disruptions to cardiovascular development (Wood, Schlueter et al. 2005). More recently, Li et al (2011) found that IGF-II expression was

crucial for supporting increased cellular proliferation through activation of the Ras-Raf-Mek-Erk pathway or simply MAPK/Erk pathway in primary mouse embryonic epicardial cells (Li, Cavallero et al. 2011). The data presented in this chapter are consistent with what is known about the effects of IGF signaling on cardiovascular development in fishes, and since hemoglobin expression was not blocked in these experiments, it is reasonable to conclude that other signaling pathways must be involved in the arrest of development associated with diapause II that are independent of IGF signaling.

Another major difference between normal entrance into diapause II and the phenotype of IGF1R embryos is the differentiation and migration of neural crest cells. In normal embryos, melanocytes are not observed in embryos until several days post-diapause II. 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 transition from epithelium to mesenchyme (the first step in migrating away from the neural tube) through changes in cell adhesion activated by degradation of E-cadherin (Morali, Delmas et al. 2001). Later in development, expression of E- and β -cadherins increases in neural crest cells as they differentiate into melanoblasts – the precursors for melanocytes (Nishimura, Yoshida et al. 1999). Thus, there is reason to believe that IGF signaling may have an effect on melanocyte development. Data presented here indicate that delay of somitogenesis and cardiac development are much more sensitive to inhibition of IGF signaling when compared to melanocyte development because those processes were significantly

inhibited at much lower levels of inhibitor. However, at the highest level of inhibitor that still allowed embryos to develop past the dispersion and reaggregation phases, melanocyte development was not observed. This suggests that there may be some threshold of IGF signaling activity required to induce neural crest migration and/or melanocyte differentiation (Erickson and Goins 1995).

The rate of somitogenesis appears to be very sensitive to the strength of IGF signaling in embryos of *A. limnaeus*. Somitogenesis is a highly regulated patterning of 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. The highly similar pattern and rate of somite development in embryos treated with IGF1R inhibitors suggests that this process is likely regulated by IGF signaling. Thus, these data suggest that regulation of somatic growth and differentiation associated with entrance into diapause II may very well be controlled by inhibition of IGF signaling. This is certainly consistent with the patterns of IGF expression observed in embryos developing along the diapause trajectory.

Conclusions

These data highly support a major role for IGF signaling, and specifically the blockage of IGF-I protein expression in mediating the growth arrest associated with diapause II in embryos of *A. limnaeus*. To better understand the relationship between IGF signaling and entrance into diapause, we must next investigate the mechanisms that

regulate IGF-I and -II expression in developing embryos. The next logical step is to investigate the expression and signaling capabilities for growth hormone (GH) during development, as GH is a known and potent stimulator of IGF-I protein expression (Davey, Xie et al. 2001, Herrington 2001). Expression and activity of the hormone insulin may also help to inform these studies because insulin can induce many of the same physiological effects of IGFs, and insulin is known to regulate expression of IGFBPs (Cotterill, Holly et al. 1993). Further, key regulators of IGF signaling such as IGFBPs must be evaluated to explore physiological regulation of this pathway throughout development. IGFBPs sequester and inactivate IGF-I and -II, but they also increase the half-life of IGFs drastically, which can alter the timing and strength of their action. Further exploration of IGF signaling and its role in the regulation of diapause will likely lead to a better understanding of how growth and differentiation are regulated during vertebrate development, and how these pathways can be manipulated to induce reversible arrest of development.

Table 3.1. Time of Development for *Austrofundulus limnaeus* at 25°C and 30°C

| Worm's Stage | Developmental Description | 25°C Timing | 30°C Timing |
|--------------|--|-------------|-------------|
| 1 | Unfertilized egg | 0 HPF | 0 HPF |
| 2 | Activated egg | - | - |
| 3 | One cell stage | 1 HPF | - |
| 4 | Two cell stage | 2 HPF | - |
| 5 | Four cell stage | 3 HPF | - |
| 6 | Eight cell stage | 4 HPF | - |
| 7 | Sixteen cell stage | 5 HPF | - |
| 8 | Thirty-two cell stage | 6 HPF | - |
| 9 | Sixty-four cell stage | 7 HPF | - |
| 10 | High solid blastula | 24 HPF | 1 DPF |
| 11 | Flat solid blastula | 36 HPF | - |
| 12 | Early flat hollow blastula | 40 HPF | - |
| 13 | Late flat hollow blastula | - | - |
| 14 | Expanding flat hollow blastula | 2 DPF | - |
| 15 | Early epiboly | 2 DPF | - |
| 16 | One-fourth epiboly | 2 DPF | - |
| 17 | One-half epiboly | 2.25 DPF | - |
| 18 | Three-fourths epiboly | 2.5 DPF | 2 DPF |
| 19 | Completion of epiboly | 3 DPF | 2.5 DPF |
| 20 | Dispersed phase 1 | 4-6 DPF | 3 DPF |
| 21 | Dispersed phase 2 | 4-6 DPF | 4.5 DPF |
| 22 | Reaggregation 1 | 6-10 DPF | 5 DPF |
| 23 | Reaggregation 2 | 6-10 DPF | - |
| 24 | Reaggregation 3 | 6-10 DPF | - |
| 25 | Reaggregation 4 | 6-10 DPF | - |
| 26 | Reaggregation 5 | 6-10 DPF | 6 DPF |
| 27 | Definitive axis | - | 6.5 |
| 28 | Solid neural keel | 10 DPF | 7 DPF |
| 29 | Early somite embryo | 11 DPF | 8 DPF |
| 30 | Ten somite embryo | 14 DPF | 9 DPF |
| 31 | Twenty-five somite embryo | 19 DPF | 10 DPF |
| 32 | Diapause II - 38 somite pairs | 24 DPF | - |
| 33 | Post diapause II - 1 | 1 DPD | - |
| 34 | Post diapause II - 2 | 1 DPD | - |
| 35 | Post diapause II - 3 gut formation | 2 DPD | - |
| 36 | One-half overgrowth - no eye pigments | 4 DPD | 11 DPF |
| 37 | Eye pigments, tail bud free | 6 DPD | 12 DPF |
| 38 | Reflecting material in eye, gall bladder | - | 13 DPF |
| 39 | Three-fourths overgrowth | 9 DPD | 16 DPF |
| 40 | Full overgrowth | 12 DPD | 17 DPF |
| 41 | Early pre-hatching | - | 18 DPF |
| 42 | Mid pre-hatching | - | 20 DPF |
| 43 | Late pre-hatching - diapause III | 24 DPD | 24 - 32 DPF |
| 44 | Hatching | 24 DPD + | 33 DPF + |
| 45 | Yolk absorbed | - | - |
| 46 | Larva | - | - |

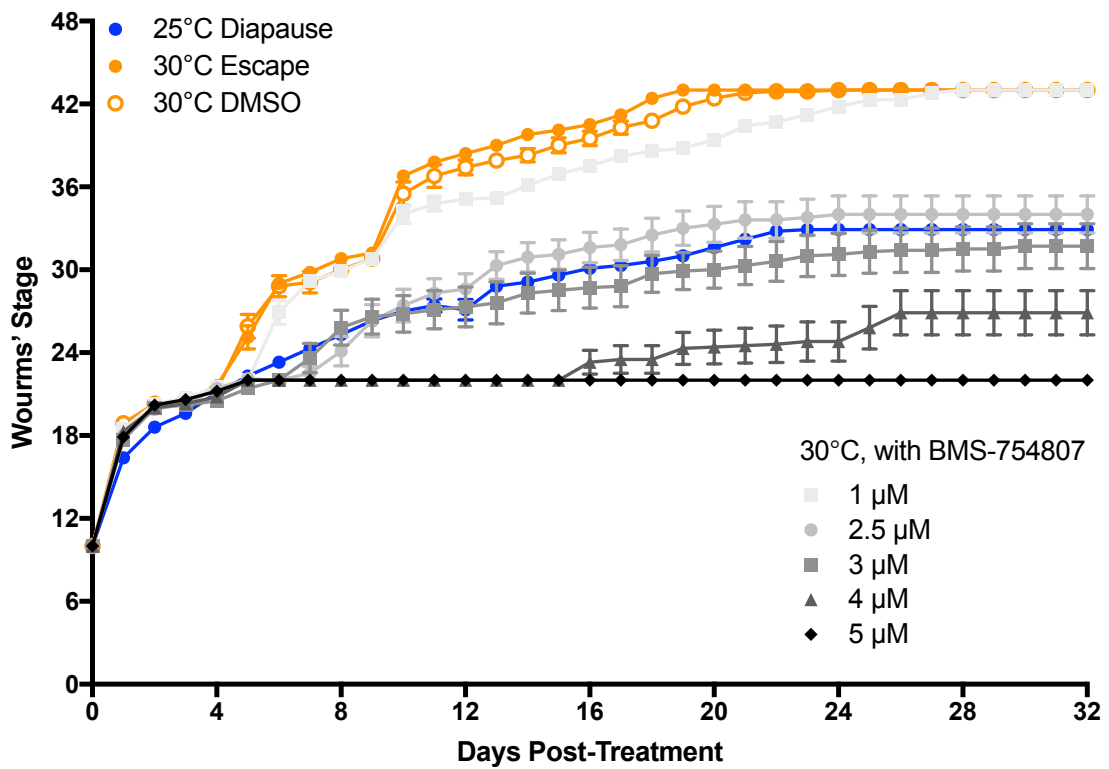


Figure 3.1. Development of embryos at 30°C exposed to a range of concentrations of the IGF1R inhibitor BMS-754807.

Increasing concentrations of the inhibitor significantly retards rate of development. At inhibitor levels of 2.5, 3, and 4 μM, developmental progression in embryos at 30°C indistinguishable from those developing at 25°C (ANOVA and Tukey's HSD, p-value = 0.995, 0.9996, 0.0691, respectively). Symbols are means ± sem (n = 10).

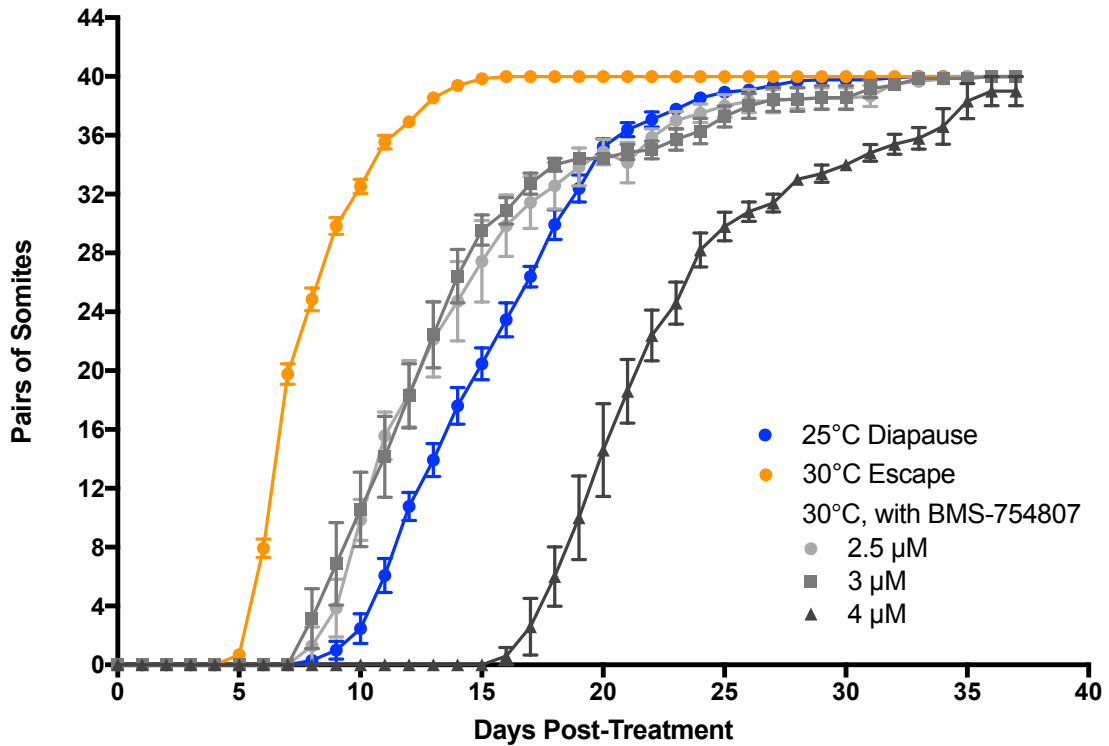


Figure 3.2. Rates of somitogenesis in embryos incubated at 30°C and treated with the IGF1R Inhibitor BMS-754807.

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 (t-test, $p < 0.0001$). Data for the DMSO controls and 30°C escape embryos were combined, because regression analysis indicated no significant differences (t-test, $p = 0.9915$). Symbols are means \pm sem ($n = 5-13$).

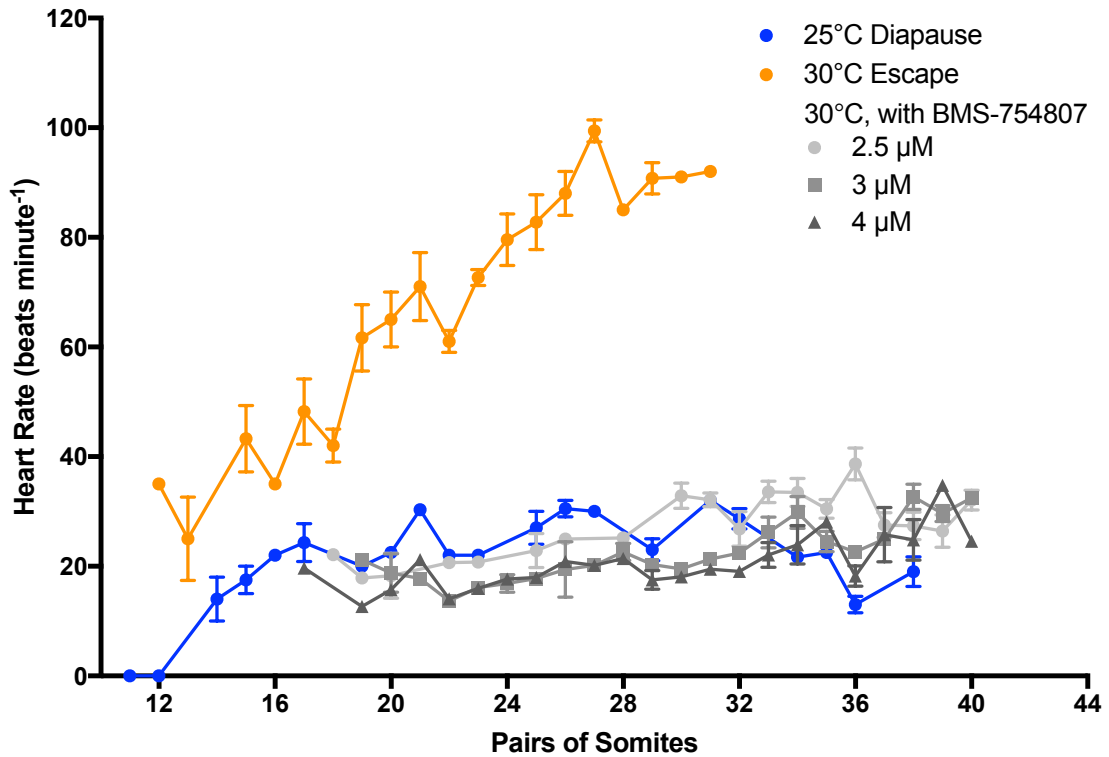


Figure 3.3. Heart rate in embryos incubated at 30°C in the presence of the IGF1R inhibitor BMS-754807.

Treated embryos had heart rates comparable to embryos developing at 25°C along the diapause trajectory. (ANOVA and Tukey's HSD, $p = 0.2764, 0.9882, \text{ and } 0.9998$, respectively). Control embryos incubated at 30°C have heart rates significantly higher than all other treatments (ANOVA, $p\text{-value} < 0.0001$, Tukey's HSD $p < 0.0001$). Symbols are means \pm sem ($n = 1\text{-}10$).

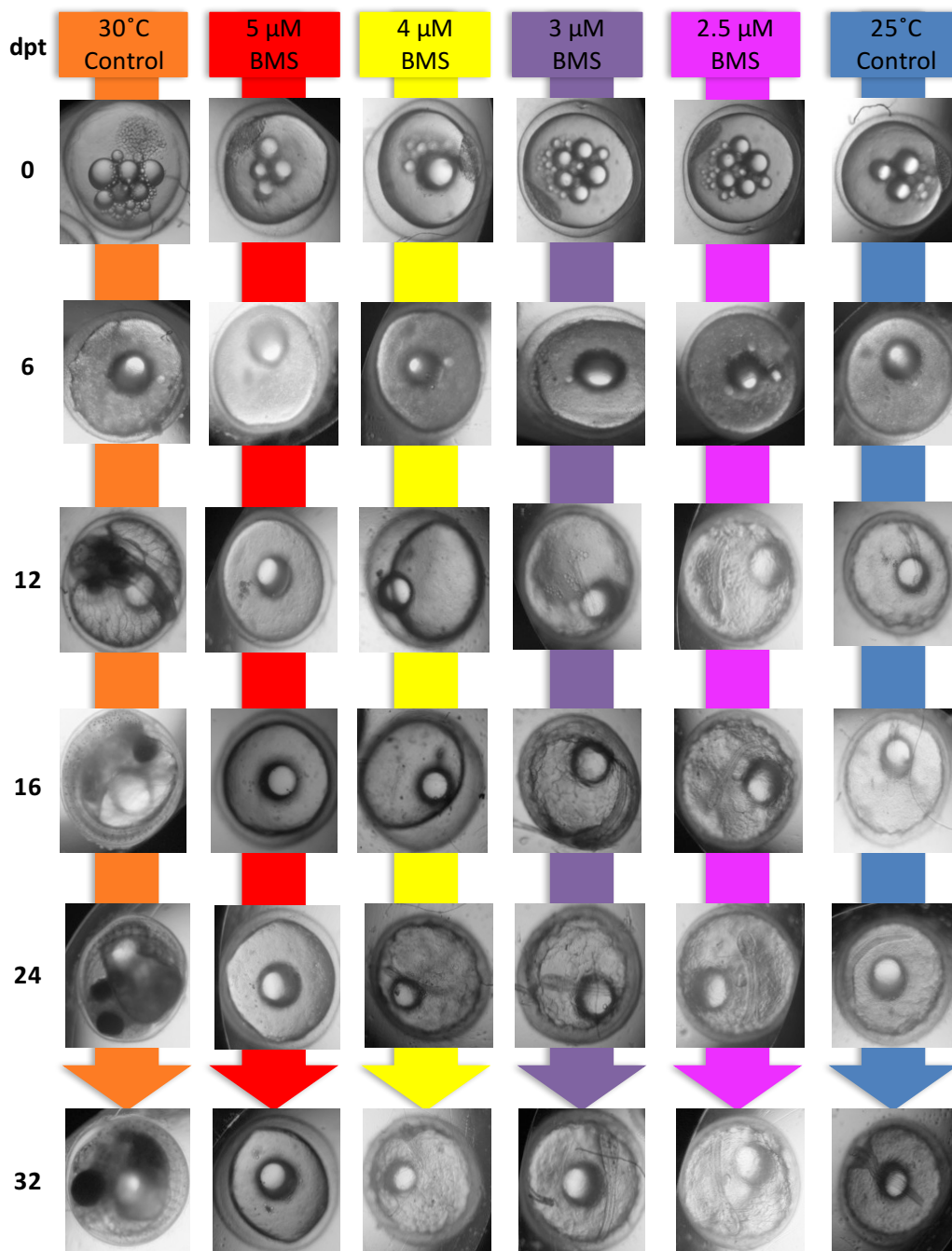


Figure 3.4. Images of embryos incubated at 30°C and treated with the IGF1R inhibitor BMS-754807.

Embryos were observed over 32 days post-treatment (dpt). All groups were initially exposed to inhibitor at WS 10-12 at 1 day post-fertilization and were continuously exposed for the duration of development. Embryos incubated at 2.5, 3, and 4 μM inhibitor developed in a manner very similar to embryos incubated at 25°C along the diapause trajectory.

Chapter 4: Profiling of mRNA Transcript Expression for Genes Involved in the Insulin-like Growth Factor Pathway in embryos of *Austrofundulus limnaeus* developing along the diapause and escape developmental trajectories

Abstract

The insulin-like growth factor (IGF) pathway is a crucial regulator of growth, proliferation, and differentiation in most cells. The biological actions of IGFs are mediated by the plasma membrane receptors that bind to an IGF and induce intracellular signaling cascades. IGF signaling ultimately leads to increases in cellular proliferation, differentiation, rates of protein synthesis, metabolism, and suppression of apoptosis. There are numerous genes responsible for receiving and transducing an IGF signal. RNAseq techniques were used to profile the expression of genes involved in known IGF signaling pathways in embryos of the annual killifish *Austrofundulus limnaeus* developing along two different developmental trajectories. Developmental trajectory was controlled by incubation temperature, with 20°C incubation leading to diapausing embryos, and 30°C incubation leading to embryos that escape dormancy in diapause. RNAseq analysis reveals differential expression of several key regulators of IGF activity, and IGF signaling that may have important implications for the regulation of entrance into diapause.

Introduction

Entrance into diapause in embryos of *Austrofundulus limnaeus* is part of an alternative developmental trajectory that appears to be regulated by differential expression of insulin-like growth factors early in development (see Chapters 2 and 3). Large-scale studies that explore gene expression in developing and diapausing embryos of *A. limnaeus* can provide important insight into the global relationship that exists between transcription, development, and diapause. Here I explore differential gene expression of signaling pathways known to be involved in IGF signaling in vertebrates in embryos developing along the two alternative developmental trajectories.

Suppression of growth is an important characteristic of diapause in various animals, including mammals, insects, nematodes, and fish (Bodenheimer and Shulov 1951, Carter and Wourms 1993, Antebi, Yeh et al. 2000, Denlinger 2002, Fordham, Georges et al. 2006, Furness 2015). The IGF/FOXO pathways are critical in the regulation of entrance into the dauer stage in *Caenorhabditis elegans*; Dauer dormancy is a state of arrested development found in *C. elegans* analogous to diapause (Kimura, Tissenbaum et al. 1997, Lin, Hsin et al. 2001). In *C. elegans*, the pathway for growth and dormancy involves DAF-2 and DAF-16, the cognates of vertebrate IGF 1 Receptor (IGF1R) and FOXOs, respectively (van Heemst 2010). Additionally, in *Drosophila melanogaster* it was found that transcripts for modulation of the IGF/FOXO pathway was

part of entrance into diapause (Baker and Russell 2009). *D. melanogaster* showed suppression of insulin and its receptor in diapause eggs. A picture is emerging that paints a reduction in TOR activity and an increase in FOXO as more generalized characteristics of diapause across invertebrate species (Sim and Denlinger 2008, Sim, Kang et al. 2015). However, very little is known about how these pathways act in vertebrate diapause. Here I surveyed the mRNA transcriptome for genes involved in IGF signaling during the critical window in *A. limnaeus* development where incubation temperature is known to affect developmental trajectory. Using the Kyoto Encyclopedia of Genes and Genomes's Pathway Database as a guide, I extract expression data for all IGF signaling-related genes from the transcriptome (Table 4.1). Exploring the differences in the timing of gene expression between these two pathways may lead us to understand what regulatory elements help define the escape and diapause developmental trajectories in *A. limnaeus*.

Methods and Materials

Animal Care and Facilities

Animal care and facilities are identical to section in chapter 2.

Sampling for Sequencing

Groups of twenty 1-2 cell stage embryos from 6 different females known to produce exclusively diapausing or escape embryos (see Methods for Chapter 2) were selected and flash-frozen in liquid nitrogen. For the rest of the time points, the total

spawning output from 42 females were pooled and incubated at 25°C until they reached mid-epiboly. At this point pooled embryos were split into two groups and placed into incubators at either 20 or 30°C. Temperature was maintained in each group by placing the embryos in 100 x 15 mm Petri dishes into temperature-controlled incubators. Embryo media was changed daily. Embryos were sampled at specific developmental time points from both temperature treatments: 100% epiboly (WS 19), neural keel (WS 28), 6 somites (WS 29), 10 somites (WS 30), 16 somites (WS 30.1), 20 somites (31) and 24 somites (WS 31.1). Embryos were sampled as above with 20 embryos making up a single replicate.

RNA Extraction

Groups of embryos were weighed, and immediately homogenized in Trizol reagent at a ratio of 50 mg of embryo tissue to 1 ml of Trizol reagent. Homogenates were incubated at room temperature (RT) for 15 min. to ensure dissociation of nucleoprotein complexes and then subjected to centrifugation at 10,000 x g for 30 min at 8°C to pellet insoluble material. The supernatant was decanted into a clean 2 ml centrifuge tube and 0.2 ml of chloroform was added per 1 ml of Trizol reagent used for homogenization. The sample tubes were inverted 15 times gently for 30 seconds to mix the organic and aqueous phases. The solution was then spun at 10,000 x g for 20 min at 8°C to separate the organic and aqueous phases. The aqueous phase (top, clear phase) was removed and transferred to a new 2 ml centrifuge tube and the RNA precipitated by addition of 0.25 ml of high salt solution (0.8 M sodium citrate and 1.2 M NaCl) and 0.25 ml of 100%

isopropanol per ml of Trizol reagent used. RNA was allowed to precipitate at -20°C overnight. The samples were then spun at 10,000 x g for 30 min at 4°C to pellet the RNA. The supernatant was decanted and discarded without disrupting the RNA pellet. The pellet was washed three times by vortexing with 2 ml of 60% ethanol followed each time by centrifugation at 10,000 x g for 30 min at 8°C to pellet the RNA. The RNA pellet was air dried in a chemical fume hood for 5-10 min to remove any remaining ethanol. The RNA was resuspended in 50 µl of 1 mM sodium citrate, pH 6.4. Samples were stored at -80°C.

cDNA library preparation and mRNA sequencing

Sequencing cDNA libraries were prepared with 1 µg of total RNA using the Illumina TruSeq RNA Sample Preparation v2 kit according to the manufacturer's instructions. The purified cDNA libraries were screened for quality on an Agilent Bioanalyzer 2100 using a DNA 1000 chip and quantified by RT-PCR prior to sequencing. DNA sequences were obtained through sequence-by-synthesis technology on an Illumina HiSeq 2000 (San Diego, CA) at the Oregon Health and Sciences University Massively Parallel Sequencing Core.

Analysis of mRNA sequence data

RNA sequence reads were analyzed using in house computational support and resources. All reads were initially characterized by FastQC to determine read length and

quality. Sequenced reads were filtered on quality scores and trimmed for the presence of adapter sequences using Trimmomatic (Bolger, Scossa et al. 2014). Quality sequence reads were then mapped to the *A. limnaeus* genome 1.0 using Bowtie2. Only aligned reads were used for further gene-level expression analysis. Gene counts were generated for all samples using the *summarize Overlaps* function of the Genomic Alignments package in Bioconductor that quantifies the number of reads per gene feature based on the *A. limnaeus* Genome Annotation (v100) available in GenBank (http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Austrofundulus_limnaeus/100/). Count matrices were filtered for genes with counts of 1 or more (normalized to library size) summed across all biological replicates to improve computational performance and reduce the data for subsequent expression analysis. Expression analysis was performed using the Bioconductor package, DESeq2, which determines differential expression of sequence count data based on the negative binomial distribution. Expression of a gene was calculated as the number of reads per kilobase of exon model per million mapped reads (FPKM). Differential expression was determined for genes between diapause and escape conditions within each developmental stage with a threshold of significance set at $p < 0.001$ and adjusting for multiple comparisons using the Benjamini-Hochberg method for false discovery rate (FDR).

Results

Overall patterns of expression

The *A. limnaeus* genome contains 26,712 genes and pseudogenes. The 26,712 genes are comprised of 23,844 protein-coding, 2,313 non-coding, and 55 pseudogenes. Of these genes 6,136 were found to have variants. A total of 24,334 mRNA transcripts were identified in the transcriptomes of *A. limnaeus* embryos used in this study. Of those 24,334 transcripts, 20,713-24,268 transcripts were identified in various developmental stages as summarized in Figure 4.1. About 43% of these transcripts are expressed in each stage at a threshold of 2 FPKM or greater. Of these transcripts between 420-472 (depending on stage) were identified as being involved in the IGF signaling pathway. Of these transcripts, between 0 and 53 were found to be differentially expressed when stage-specific comparisons were performed between the diapause and escape trajectories at an FDR-adjusted p-value of 0.001 (Figure 4.1). There is a clear pattern of increasing number of differentially expressed genes in later developmental stages.

IGF-I and IGF-II transcripts

IGF-I and IGF-II transcripts are not present in 1-2 cell stage embryos, and therefore are not maternally packaged in *A. limnaeus* based on the expression threshold of 2 FPKM set for this study (Figure 4.2). However, transcripts for IGF-I and IGF-II are expressed above the 2 FPKM threshold at all subsequent stages in both trajectories. IGF-II transcripts were not found to be differentially expressed in the stages sampled for this

study. In contrast, transcripts for IGF-I were significantly upregulated (4-fold) at neural keel stage of development in escape embryos (two-tailed T-test, FDR 0.001, p-value < 0.001). This spike in expression at neural keel stage in escape embryos is based on an average expression level of 58.58 ± 1.39 FPKM (mean \pm s.e.m., n=3) (Figure 4.2). IGF-I transcripts were found to be 8-fold upregulated in 24-somite embryos developing along the diapause trajectory with an average FPKM of 23.13 ± 1.36 mean \pm s.e.m., n=3) (Figure 4.2). Thus, this apparent increase in expression at this stage is not due to an increase in expression in embryos along the diapause trajectory, but rather due to relatively stable expression in the diapause-bound embryos and a major decrease in expression in the escape embryos.

IGF receptor transcripts

Transcripts for the IGF1R, IGF2R, and IR are all present in 1-2 cell stage embryos and are likely of maternal origin (Figure 4.3). IGF2R expression levels are high (around 12 FPKM) at fertilization and initially decrease, followed by a spike in expression (12-14 FPKM) at the 6 somite stage in both developmental trajectories. For both developmental trajectories, the highest expression of IGF1R is observed in 1-2 cell stage embryos and then decreases to relatively low and stable values until the 16 somite stage when levels increased again. IR transcript levels begin low in fertilized embryos (just above 2 FPKM) and steadily increased during development in both developmental trajectories.

IGF binding protein transcripts

Transcript levels for IGF binding proteins (IGFBP) are presented in Figure 4.4. There are clear developmental patterns of expression for IGFBPs that are independent of incubation temperature and developmental trajectory. However, there are some patterns of expression that suggest a role for IGFBPs in regulating IGF signaling. Transcripts for IGFBP2 are significantly upregulated (about 8-fold) in escape embryos starting at the 16 somite stage (Two Tailed T-test, FDR 0.001, p-value < 0.001). While transcripts for many other IGFBPs were identified, no others were significantly differentially expressed at the stringent threshold set for this study. For example, IGFBP4 transcripts are nearing statistically different upregulation in 24 somite embryos on the diapause trajectory (Two Tailed T-test, FDR 0.001, p-value =0.040) with about a 3-fold increase (Figure 4.4).

Growth hormone and GH receptor transcripts

Both growth hormone (GH) and GH receptor (GHR) are expressed (>6 FPKM) in 1-2 cell stage embryos, and thus there is at least some level of maternal packaging (Figure 4.5). GH transcript levels increase substantially between fertilization and the completion of epiboly and then remain relatively stable in subsequent stages of development. In contrast, GHR drops drastically (Escape 68% and Diapause 72%), from highs near 80 FPKM in 1-2 cell stage embryos to relatively stable levels in later developmental stages between 12-32 FPKM (Figure 4.5). Both the diapause and escape trajectories have similar expression patterns during development.

IGF signaling pathways

There are a number of canonical intracellular signaling pathways that are known to be activated through IGF and GH receptors. Differential expression analysis of genes known to be involved in these signaling pathways reveals several interesting patterns of expression in both developmental trajectories. For embryos developing along the escape trajectory, differential expression was first observed in embryos at completion of epiboly with an upregulation of a RAS-related protein, indicating a potential upregulation of the RAS-MAPK-ERK pathway (Figure 4.6). In subsequent developmental stages, even more genes involved in this pathway are upregulated, including two histone deacetylases in the neural keel stage and the immediate early genes Jun-B-like and c-Fos-like in 6 -16 somite embryos, directly in the critical window when temperature is known to affect developmental trajectory. Transcripts for IGF-I are significantly upregulated in the neural keel stage, and a known positive regulator of IGF-I activity, IGFBP2 is upregulated starting at the 16 somite stage. The AKT pathway is not transcriptionally upregulated until the 16 somite stage and later in escape trajectory embryos. For embryos developing along the diapause trajectory, the transcripts involved in RAS-MAPK-ERK signaling were also the first to be upregulated. However, a transcript important for regulation of the AMPK pathway is also activated early (neural keel stage) and during the critical window of development where temperature exerts its effects on developmental trajectory. Late in the developmental progression, transcripts associated with AKT signaling are upregulated, as are two STAT proteins and finally the transcript for IGF-I. The

upregulation of these transcripts as developmental rate is decreasing and embryos are preparing to arrest in diapause II appears paradoxical.

Discussion

General Caveats to the Following Discussion

Before beginning a discussion of the gene expression patterns detailed in this thesis, it is critical to point out that these data, and all the data presented in this Thesis, are derived from whole embryos. This level of analysis is powerful because it provides a global picture of what may be happening in the embryo across tissues. However, it is also a severe limitation because spatial and tissue-specific patterns of expression remain unknown. Thus, it is important to point out that what appear to be very small levels of expression in whole embryos, could represent a massive amount of expression in a small number of cells or an individual tissue. In addition, a similar *level* of expression in the two trajectories cannot be equated with equivalent potency of action because the exact location and context of expression are as critical, perhaps even more critical, in determining the outcome of gene expression or cell signaling. Thus, much of the discussion below must be viewed with a great deal of caution. However, these gene expression data are useful and will certainly inform future studies on the regulation of diapause in this species.

Insulin-like Growth Factor Transcript Expression

There is a consistent pattern between IGF-I mRNA transcript levels reported in this Chapter and IGF-I protein levels presented in Chapter 2 (Figure 2.3). This spike in IGF-I gene expression that results in increase protein expression suggests a role for IGF-I in regulating the increases in developmental rate and progression that are observed in embryos developing along the escape trajectory. However, it appears that this expression is not solely linked to increased rates of growth or cellular proliferation because this spike in expression is seen far in advance of the most rapid periods of growth and development associated with the escape trajectory. In fact, the spike occurs in embryos that are otherwise morphologically and physiologically indistinguishable from diapause-bound embryos. The high levels reached may represent a threshold of IGF-I expression that must be achieved in order to move past a critical point in development that supports continuous development. This pattern suggests a role for IGF-I in regulating the program that bypasses diapause II, and not just growth or metabolic rate. Following this same line of reasoning, it is also possible that the levels of IGF-I observed in the diapause-bound embryos never reach a critical threshold that activates the escape trajectory. If a critical threshold exists for IGF-I expression, it is likely highly influenced by the expression and activity of the IGFBPs. In this model we would expect the critical threshold to be met when the IGF-I levels oversaturate the binding and storage capacity of the IGFBPs (Baxter 2000). This threshold model would be similar to the counteracting activities of juvenile hormone (JH) and diapause hormone (DH) in insect diapause (LINGER and L 2013). In silkmoths, JH acts as an antagonist to DH, and high JH/DH ratios lead to the

non-diapause developmental program (Morohoshi 1959). Alternatively, the pattern of IGF-I expression reported here for *A. limnaeus* could be a temporal issue, where the signal of IGF-I must occur at a specific time in development. For example, in *C. elegans*, IGF signaling is age- and developmental stage-dependent with specific windows of increased IGF sensitivity (Dillin, Crawford et al. 2002). In mammals, there is a strong temporal component to IGF-I signaling during brain development achieved through a complex interaction of IGF-I expression and the expression levels of various inhibitory and stimulatory IGFBPs (Walter, Berry et al. 1997). While the complex nature of the regulation of IGF signaling makes it difficult to draw clear conclusions from this study, it is clear that future investigations of IGF-I signaling are warranted and likely to yield interesting insights into the regulation of diapause in *A. limnaeus*.

After the early peak in IGF-I expression in escape embryos, there is a rather dramatic drop of IGF-I protein and transcript levels. This drop eventually leads to an apparent upregulation of IGF-I transcripts in diapause-bound embryos. Interestingly, there is also an associated increase in IGF-I protein levels in diapause II embryos (Figure 2.3). This same pattern is observed in a number of transcripts for signaling proteins that lead to increases in rates of cellular growth and proliferation (Figure 4.6). While this pattern may seem counter-intuitive, one possible explanation is that the diapause-bound embryos are initiating a gene expression program during pre-diapause II development that is needed to support post-diapause II development. This hypothesis is based on the fact that protein synthesis is known to be arrested in diapausing embryos of *A. limnaeus* (Podrabsky and Hand 2000) and it is common in dormant animals for rates of

transcription to be severely attenuated as well (Storey and Storey 1990, Guppy, Fuery et al. 1994, Kwast and Hand 1996). Thus, it may be critical for embryos to prepare for the early events of post-diapause II development, and stress tolerance during diapause, prior to entry into diapause. A more detailed look at the entire transcriptomes of these two developmental trajectories, and profiling of gene expression during exit from diapause II may help to shed light on this hypothesis.

IGF Receptor Transcript Expression Patterns

In general, the expression of transcripts that code for the three main IGF receptors exhibit similar expression patterns in both developmental trajectories. Thus, it appears that changes in IGF signaling that may be associated with regulation of developmental trajectory are likely due to variations in the IGF proteins and their associated IGFBPs, rather than a lack of receptor expression. While these patterns may not be trajectory-specific, they may shed light on when various IGF and insulin signals are likely to be important during development. Transcript levels for IGF1R have the highest expression level of all the receptors. Further, their peak in expression is observed just after fertilization, suggesting high levels of maternal provisioning. Transcript levels for IGF1R then fall dramatically by the completion of epiboly and remain low for the duration of early development. This pattern of expression suggests an important role for IGF1R during early development, and we would predict a large increase in IGF1R protein expression to occur by the completion of epiboly. Interestingly, this would prime the embryonic cells to receive the spike in IGF-I protein that is observed starting at the

completion of epiboly. Thus, while we do not have data on IGF1R protein expression at this time, these transcript data are consistent with the production of cells that are IGF-I competent prior to the peak in IGF-I protein observed.

The second most abundant receptor was IGF2R, a receptor that is responsible for specifically reducing the signaling activity of IGF-II (Nissley, Kiess et al. 1991). Indeed, levels of IGF-II are low in early embryos of *A. limnaeus* and do not increase significantly until post-diapause II developmental stages. Also of interest is the large spike in IGF2R transcripts observed in the 6-somite stage embryos. While the exact nature of this spike is not understood, it appears to be important developmentally because it is observed in both developmental trajectories. Blocking the action of IGF-II may be an important part of the early development in this species and may help to explain the relatively slow rates of development in pre-diapause II embryos of *A. limnaeus*, and all annual killifish, compared to other species with similar sized embryos.

At fertilization the insulin receptor (IR) has low, but clearly detectable expression levels. This level of maternal provisioning suggests an important role for insulin signaling or IGF signaling through the IR receptor during early development. At this point insulin levels during early development have not been determined in embryos of *A. limnaeus*, but these data suggest this may be an important hormone to explore.

Insulin-like Growth Factor Binding Proteins (IGFBPs) Transcripts

There are many steps at which IGF activity is post-translationally regulated. IGFBPs are extracellular proteins with high affinities for IGFs and that readily bind to

free IGFs in the blood and extracellular fluids. IGFBPs have a two-pronged effect on IGF activity: IGF signaling activity is reduced when bound to most IGFBPs, but half-life is increased through protection from proteases (Baxter 2000). In human males, the half-life of free IGF-I or -II is only about 10-12 minutes, while bound IGFs may be stable for days. There are numerous IGF binding proteins (IGFBPs 1-7) that can attenuate or accentuate IGF activity and potency. There are 7 characterized IGFBPs, and each of these may be expressed as several variants with unique affinities for IGF-I and -II, and in many cases the affinity can be altered by various environmental factors. For example, a reduction in pH from 7.2 – 6.0 causes an increase in the affinity of IGFBP-1 and -3, but a 4 fold decrease in affinity to IGFBP-2 (Clemmons 1991).

IGFBP-2 has been implicated in the regulation of IGF activity in most mammalian tissues and organs (Park, Kim et al. 2015), and is known to be upregulated in oncogenic tissues along with IGF-I or -II (Catsburg, Gunter et al. 2015, Myers, Lin et al. 2015). IGFBP2 is also thought to play an important role in the differentiation of human corneal fibroblasts (Park, Kim et al. 2015), and cell mobility as observed in human lung cancer metastasis (Hu, Huang et al. 2014). This increase in IGF signaling that appears to be stimulated by IGFBP-2 is potentially due to a unique effect on the action of IGF1R mediated through the heparin binding domain (HBD) motif on IGFBP-2, as has been illustrated in vascular smooth muscle cells (Shen, Xi et al. 2012). If the large increase in transcripts for IGFBP-2 that is observed in escape embryos translates to increased excretion of this protein, then it would be expected to increase the activity of IGF signaling in embryos developing along the escape trajectory.

While IGFBP-2 can have positive effects on IGF signaling, it is very unique and the other IGFBPs that have been studied appear to reduce signaling activity. For example, IGFBP-4, which was nearly significantly upregulated in diapause-bound embryos, is a strong negative regulator of growth, differentiation, and mobility. Transgenic mice that over express IGFBP-4 have reduced organ sizes (Schneider, Lahm et al. 2000, Durai, Davies et al. 2006). Additionally, over expression of IGFBP-4 has a strong inhibitory effect on the growth of colon and prostate cancer cells (Shen and Singh 2004, Durai, Davies et al. 2006). Thus, increased expression of IGFBP-4 in diapause-bound embryos may further reinforce a reduction in IGF signaling activity that would be expected based on the relatively low level of observed protein expression.

Growth Hormone and Growth Hormone Receptor Transcripts

Interestingly, GHR transcripts are expressed at the highest levels just after fertilization. Again, this level of maternal provisioning may indicate a key role for GHR in early developmental events, and may be interpreted as a need for GHR activity before or shortly after the activation of the zygotic genome. The pattern of GH transcript expression is opposite of that observed for GHR. This may be due to the relatively short half-life of GH (under 45 min in humans) and a need for continued GH signaling to fuel rapid cell growth and proliferation associated with development (Faria, Veldhuis et al. 1989, Mullis, Rani Pal et al. 1992). In contrast, GHR likely has a much longer half-life, as is often the case for membrane-bound receptors (Reed and Lane 1980, K, MJ et al. 1998). Of interest to this study is the presence of both necessary components of GH

signaling, GH and GHR transcripts preceding the expression of transcripts for IGFs, which may mean that IGFs are regulated during early development as they are in canonical GH-IGF axis (Davey, Xie et al. 2001, Salvatori 2004, Reinecke, Bjornsson et al. 2005).

Expression of Transcripts for IGF Related Signaling Pathways

Taking a more systems or pathway-level approach may lend strength to some of the conclusions that have been reached in this thesis, and may also highlight possible signaling pathways through which IGF signals act to alter or affect developmental rate or trajectory. Only a small handful of transcripts associated with IGF signal transduction were found to be differentially expressed in the two developmental trajectories. Based on the upregulation of transcripts for genes in the RAS-MAPK-ERK pathway, including the immediate early genes, Jun-B-like and c-Fos-like, and two histone deacetylases, it is probable that the early effects of IGF signaling in the escape pathway are mediated through the RAS-MAPK-ERK pathway and the associated AP-1 transcriptional activation complex. It is especially interesting that the Jun-B/c-Fos-like proteins are upregulated during the window of development where temperature is known to have its effects on developmental trajectory (6-16 somite embryos) (Wagner 2002, Murphy, MacKeigan et al. 2003, Eckert, Adhikary et al. 2013). This, coupled with the early increase in IGF-I expression, strongly suggests that escape-specific activators of development should have AP-1 binding sites in their promoters. With the recent completion of an annotated genome for *A. limnaeus*, it is now possible to evaluate genes that may be responsive to

AP-1. Further, the activation of transcription for the AKT signaling pathway rather late in the escape trajectory may indicate that this pathway is more important for the regulation of tissue growth and differentiation, rather than for regulation of developmental trajectory.

Diapause-specific gene expression appears to point to an early role for the RAS-MAPK-ERK pathway in mediating IGF signaling as well. However, it is worth pointing out that an apparent increase in expression in the diapause-bound embryos can be due to both a bona fide increase in expression, as well as a decrease in expression in escape embryos as is the case for IGF-I transcripts (discussed earlier). For example, the first and most consistently “upregulated” transcript in the diapause-bound embryos is MAP kinase binding protein 1 (MAPKBP1), a protein classified as active in the RAS-MAPK-ERK pathway (Glaab, Baudot et al. 2010). However, a proteomics study in murine C2C12 myoblast cells identified MAPKBP1 as a protein that is strongly down-regulated in response to IGF-I signaling (King, Bouic et al. 2009). Thus, it is highly possible that transcripts found to be “upregulated” in the diapause-bound embryos are simply remaining stable in the face of more dynamic changes in the escape trajectory embryos that are responding to IGF signaling. Another major difference between the escape and diapause trajectories is the “upregulation” of several TBC proteins that are thought to be activated by AMP-activated protein kinase, and may play a role the regulation of metabolism (O'Neill 2013) associated with entrance into diapause. Interestingly, transcripts for STAT-3 and -5 are apparently upregulated in diapause-bound embryos. The implications of this are not yet known.

One final transcript that deserves discussion is the apparent upregulation of FOXO3 in diapause-bound embryos at the 20 somite stage. Increased DAF-16/FOXO signaling has been shown to induce diapause-specific phenotypes in a variety of invertebrates (Greer, Dowlatshahi et al. 2007, Arden 2008, Gao, Wang et al. 2010, Hesp, Smant et al. 2015, Sim, Kang et al. 2015). However, to our knowledge this is the first report of upregulated FOXO expression in a vertebrate model of metabolic dormancy. This is a significant finding, and leads us to hypothesize that there is a common mechanism for regulation of diapause-like dormancies across all animals. The evolutionary implications of a common regulatory mechanism to induce dormancy in animals have far reaching implications.

Conclusion

Large-scale gene expression profiling of diapause- and escape-bound embryos has revealed a number of interesting trends that agree with previous observations and with existing theory on the regulation of entrance into metabolic dormancy in a variety of animals. One of the most significant observations is the combined upregulation of IGF-I transcript and protein expression in concert with an upregulation of IGFBP2 early in development. These events all point towards an expected increase in IGF activity in escape embryos, and thus reinforce a role for decreased IGF-I signaling in the promotion of dormancy associated with diapause. Most if not all of the genes required for an active IGF signaling pathway are present in early embryos of *A. limnaeus*. The next steps will

require an examination of IGF regulators, such as GH, to explore the mechanisms that regulate the key differences in IGF expression that have been detailed in this thesis.

Table 4.1: Comparison of the vertebrate and invertebrate IGF/FOXO pathway

| Complex* | <i>C. elegans</i> † | <i>A. limnaeus</i> | Component | Affects |
|----------|---------------------|--------------------|---|--|
| | DAF-2 | IGF1R | IGF or Insulin receptor | Receptor for IGF ligands Transcription factor for arrested development related genes, and activator of growth regulatory elements |
| | DAF-16 | FOXO | FOXO family of Transcription Factors | Activation through AMPK and activates FOXO. Inhibits NF-Kb |
| | SIR-2.1 | SIRT | Sirtuins | Dephosphorylates PIP3 to PIP2 |
| | DAF-18 | PTEN | Phosphatase and tensin homolog | Inhibitor of IGF1R and IRS |
| | | PTPN | Protein tyrosine phosphatase, non-receptor type 1 | Phosphorylates PIP2 to PIP3 |
| | AGE-1 | PI3K | Phosphatidylinositol 3-kinase catalytic subunit | Activates AKT, IKK-β, and SGK |
| | PDK-1 | | 3-phosphoinositide dependent protein kinase 1b | Signaling adapter that couples activated IGF1R, activates PI3K |
| | IST-1 | IRS | Insulin receptor substrate 1/2 | Ligand for GHR |
| | | GH | Growth Hormone | Activates JAK2, MAPK pathway, and release of IGF |
| | | GHR | Growth Hormone Receptor | JAK2 phosphorylates STAT proteins |
| | JAK2 | | Janus Kinase 2 | Transcription factor for growth related genes including IGFs |
| | Sta-1 | STAT1 | Signal transducer and activator of transcription 1 | Transcription factor for growth related genes including IGFs |
| | Sta-1 | STAT3 | Signal transducer and activator of transcription 3 | Transcription factor for growth related genes including IGFs |
| | Sta-1 | STAT5 | Signal transducer and activator of transcription 5 | Inhibitor of IGF1R and INSR |
| | | LAR | Receptor-type tyrosine-protein phosphatase F | Activator of mTORC1, IKK-β, GSK-3β, and BCL-2. Suppressor of FOXO, p27kip1, BAD, and TSC1/2 |
| | AKT-1 | AKT | V-AKT murine thymoma viral oncogene homolog | Inhibits FOXO |
| | SGK-1 | SGK | Serine/threonine-protein kinase sgk-1 | Inhibit FOXO and Apoptosis |
| | IKK-β | | Inhibitor of nuclear factor kappa-B kinase subunit beta | |

| Complex* | <i>C. elegans</i>† | <i>A. limnaeus</i> | 5'-AMP-activated protein kinase, catalytic alpha subunit Component | Activated by energy and hypoxic stress and activates TSC1/2. Affects |
|--------------------|---------------------------|---------------------------|---|--|
| | AAK-1 | AMPK | | Activated by energy and hypoxic stress and activates TSC1/2. |
| | BCL-2 | | | Activates survival and suppression of apoptosis |
| | BAD | | Bcl-2-antagonist of cell death | Activates apoptosis |
| | Casp9 | | Caspase 9 | Activates apoptosis |
| | HAD-2 | HDAC | | Deacetylates DNA to suppress transcription |
| | C44H4.6 | GSK-3β | Glycogen synthase kinase 3 beta | Activates the glucose metabolism pathway |
| | TSC2 | | Tuberous sclerosis 2 | Complexes with TSC1 and inhibits mTORC1 |
| | TSC1 | | Tuberous sclerosis 1 | Complexes with TSC2 and inhibits mTORC1 |
| | Rheb-1 | Rheb | Ras homolog enriched in brain | mTORC1 activator |
| | CKI-2 | p27Kip1 | Cyclin-dependent kinase inhibitor | CDK-1 inhibitor |
| mTOR Complex Genes | | RICTOR | RPTOR independent companion of MTOR | mTORC2 is a complex of RICTOR, mTOR, Deptor, mSin1, Protor, MLST8, TEL2, and TTI1. mTORC2 activates AKT and promotes mobility. mTORC1 is a complex of mTOR, PRAS40, Raptor, MLST8, and Deptor to form mTORC1, which activates S6K and transcription and proliferation. |
| | | TTI1 | TELO2 interacting protein 1 | |
| | | TEL2 | Telomere maintenance 2 | |
| | | Protor | Proline rich 5 | |
| | | mSin1 | Mitogen-activated protein kinase associated protein 1 | |
| | | Deptor | DEP domain containing MTOR-interacting protein | |
| | Let-363 | mTOR | Mechanistic target of rapamycin | |
| | DAF-15 | RAPTOR | Regulatory associated protein of MTOR | |
| | | PRAS40 | | |
| | | MLST8 | MTOR associated protein, LST8 homolog | |
| | R04A9.7 | S6K | Ribosomal protein S6 | Transcription of growth and proliferation related genes |
| | CDK-2 | | Cyclin-dependent kinase 2 | Phosphorylates Cyclins |
| | Cyclin E | | Cyclin E1 | Transcription of growth and proliferation related genes |
| | Cyclin A | | Cyclin A1 | Transcription of growth and proliferation related genes |

| Complex* | <i>C. elegans</i>† | <i>A. limnaeus</i> | Component | Affects |
|-----------------|---------------------------|---------------------------|---|---|
| | SHC-1 | SHC | Src Homology 2 domain Containing transforming protein 2 | Signaling adapter that couples activated IGF1R |
| | SOS-1 | SOS | Son of sevenless | Signal adapter that couples with GRB2 and SHC to activate RAS |
| | Sem-5 | GRB2 | Growth factor receptor bound protein 2 | Signal adapter that couples with SOS and SHC to activate RAS |
| | Let-60 | RAS | Ras-like protein | Signal protein that activates RAF |
| | Lin-45 | RAF | Raf-1 proto-oncogene, serine/threonine kinase a | Signal protein that activates MEK1/2 |
| | MEK-2 | MEK 1/2 | Mitogen-activated protein kinase kinase 2b | Signal protein that activates ERK |
| | MPK-1 | ERK | Mitogen-activated protein kinase 1 | Signal protein that activates ELK1 and eIF4E |
| | Lin-1 | ELK1 | ETS domain-containing protein Elk-1 | Transcription of growth and proliferation related genes |
| | IFE-3 | eIF4E | Eukaryotic translation initiation factor 4 | Transcription of growth and proliferation related genes |
| AP-1 Complex | | FOXM1 | Forkhead box protein M | Suppressor of p27kip1 |
| | | ATF | Activating transcription factor | AP-1 is a complex of ATF, c-Jun and c-FOS as transcription factors. |
| | | C-Jun | Jun proto-oncogene | |
| | | C-FOS | Proto-oncogene protein c- fos | |

*Complexes are highlighted when applicable.

† Names that appear only in this column and not the following means they are the same in both species.

(Kanehisa, Sato et al. 2016)

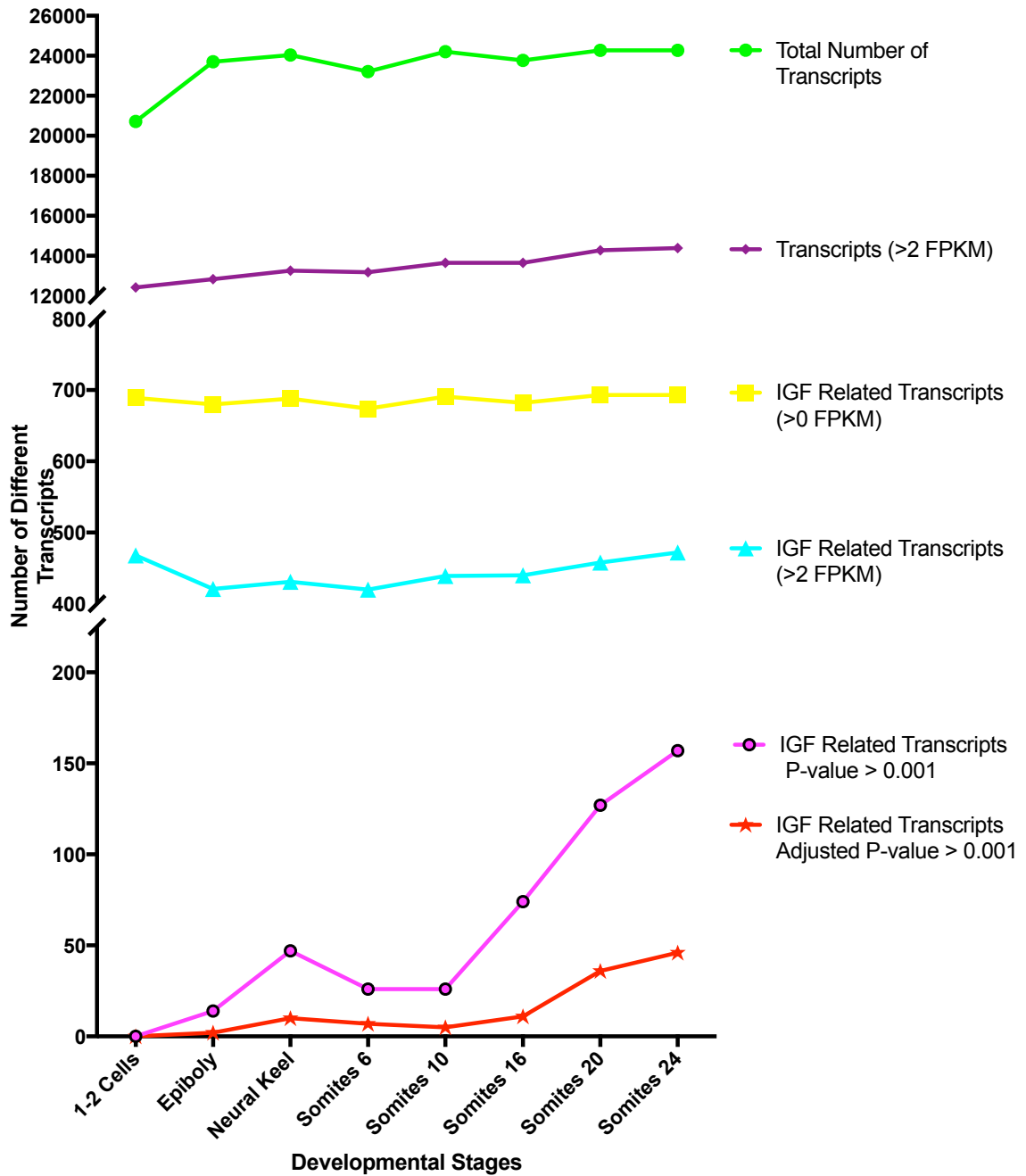


Figure 4.1 Summary of the number of mRNA transcripts identified in embryos of *Austrofundulus limnaeus* in this study.

Data reduction was performed to better focus the exploration of IGF related gene expression. Differential expression of transcripts are discussed that are present in at least 2 FPKM with an FDR adjusted P-value of 0.001. Symbols are totals of genes meeting the expressed criteria.

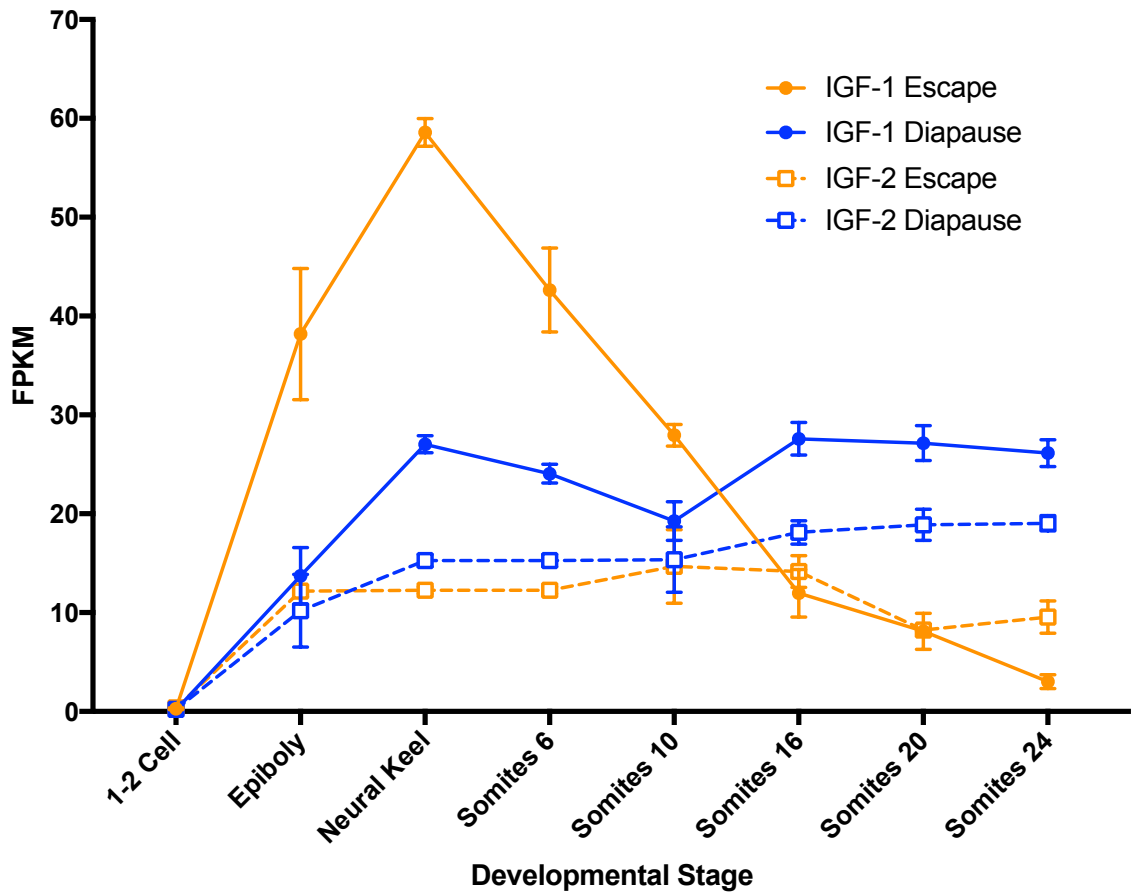


Figure 4.2. Expression of insulin-like growth factor I and II transcripts in embryos incubated at 20°C and 30°C and developing along the diapause and escape trajectories, respectively.

IGF-I transcripts are significantly upregulated in escape embryos compared to diapause-bound embryos starting at the completion of epiboly and through the 10 somite stage. Symbols are means \pm s.e.m. (n=3).

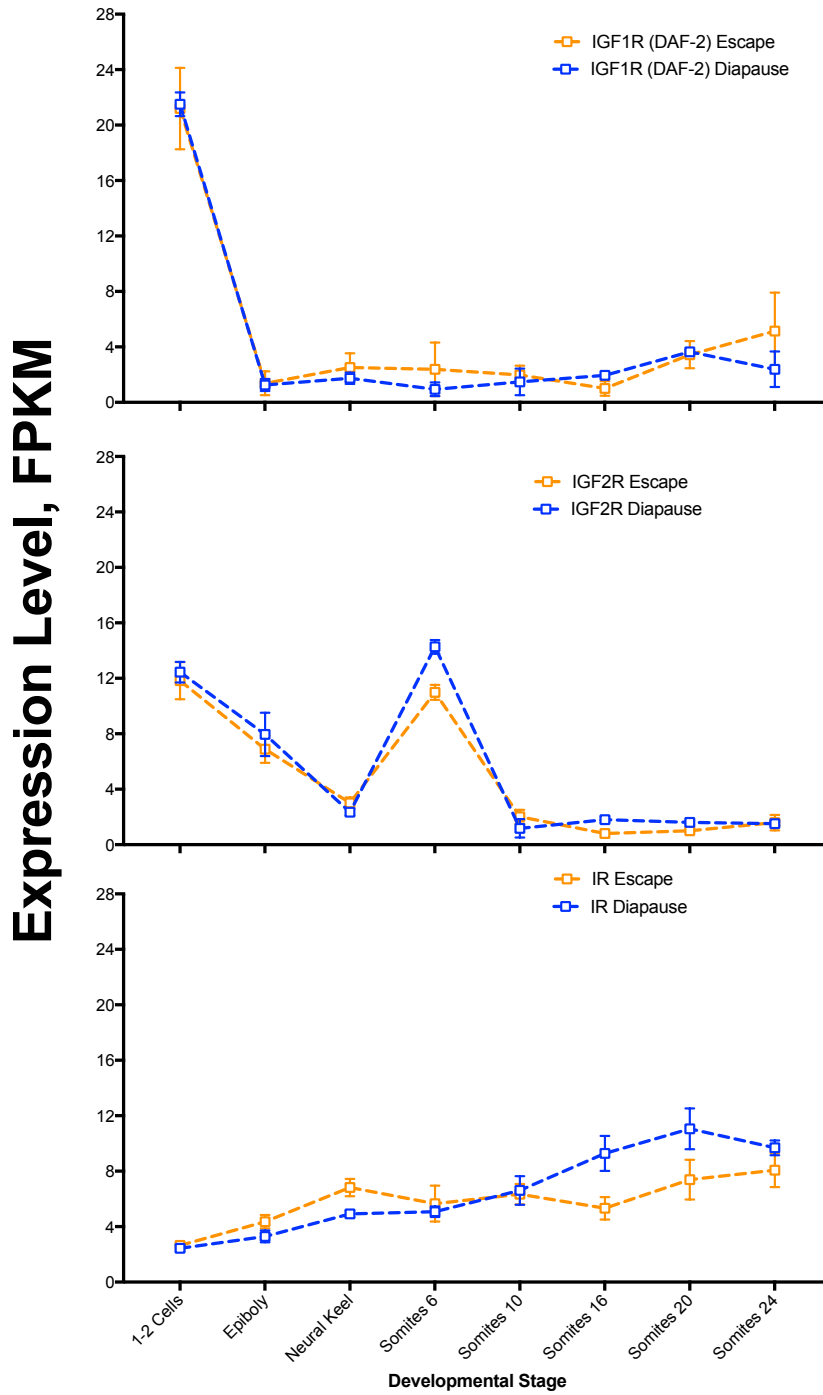


Figure 4.3. Expression levels of plasma membrane receptors known to be active in insulin-like growth factor signaling in embryos developing along the diapause (20°C) and escape (30°C) trajectories.

Expression of mRNA transcripts is stage-specific, but not altered by incubation temperature and thus likely not related to developmental trajectory. Insulin receptor has the highest expression

early 1-2 cell stage embryos, followed by IGF2R, with IGF1R exhibiting the lowest expression. Symbols are means \pm s.e.m. (n=3).

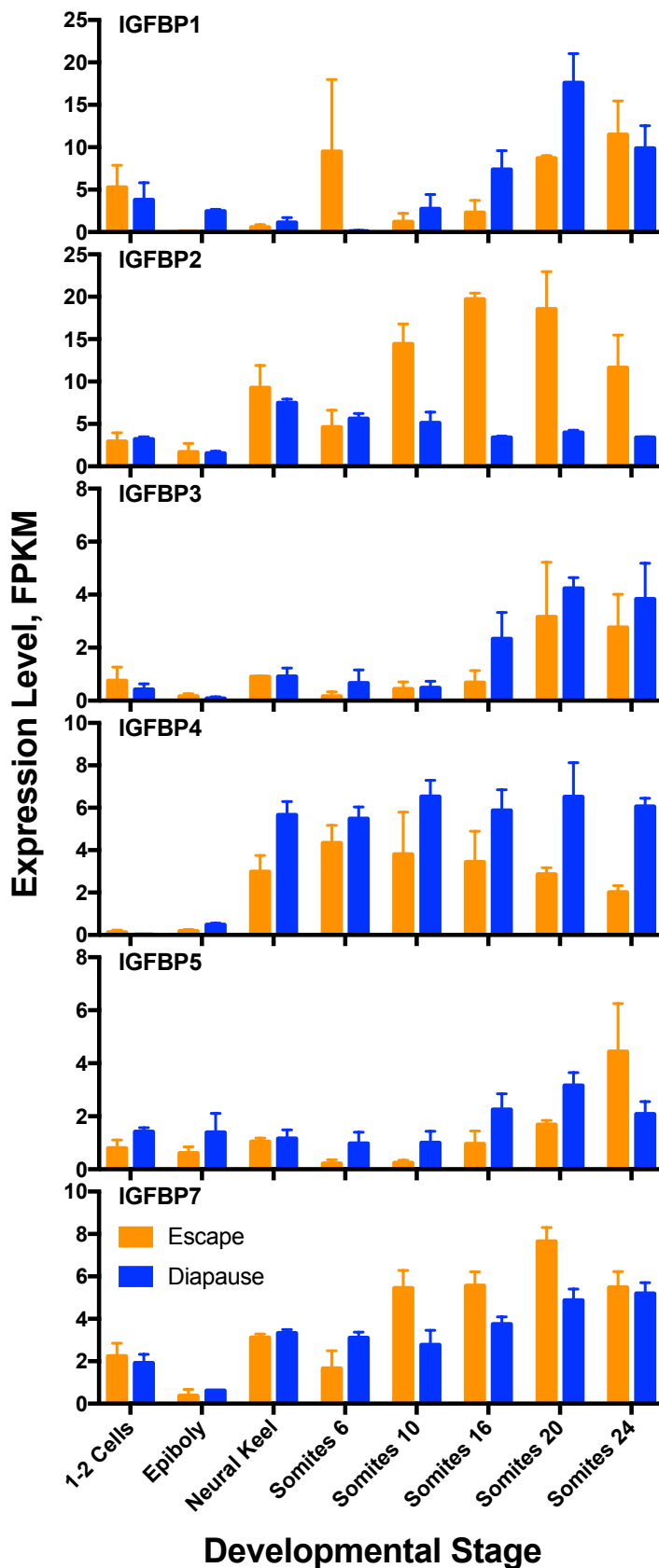


Figure 4.4. Expression level of insulin-like growth factor binding proteins during development in embryos incubated at 20°C and 30°C and developing along the diapause or escape trajectories, respectively.

There is an overall increase in the number of transcripts expressed for IGFBPs as embryos progress through development. There are also several IGFBPs that appear to be maternally packaged. IGFBP2 is significantly upregulated in escape embryos starting at the 16 somite stage (p-value < 0.001). Bars are means \pm s.e.m. (n=3).

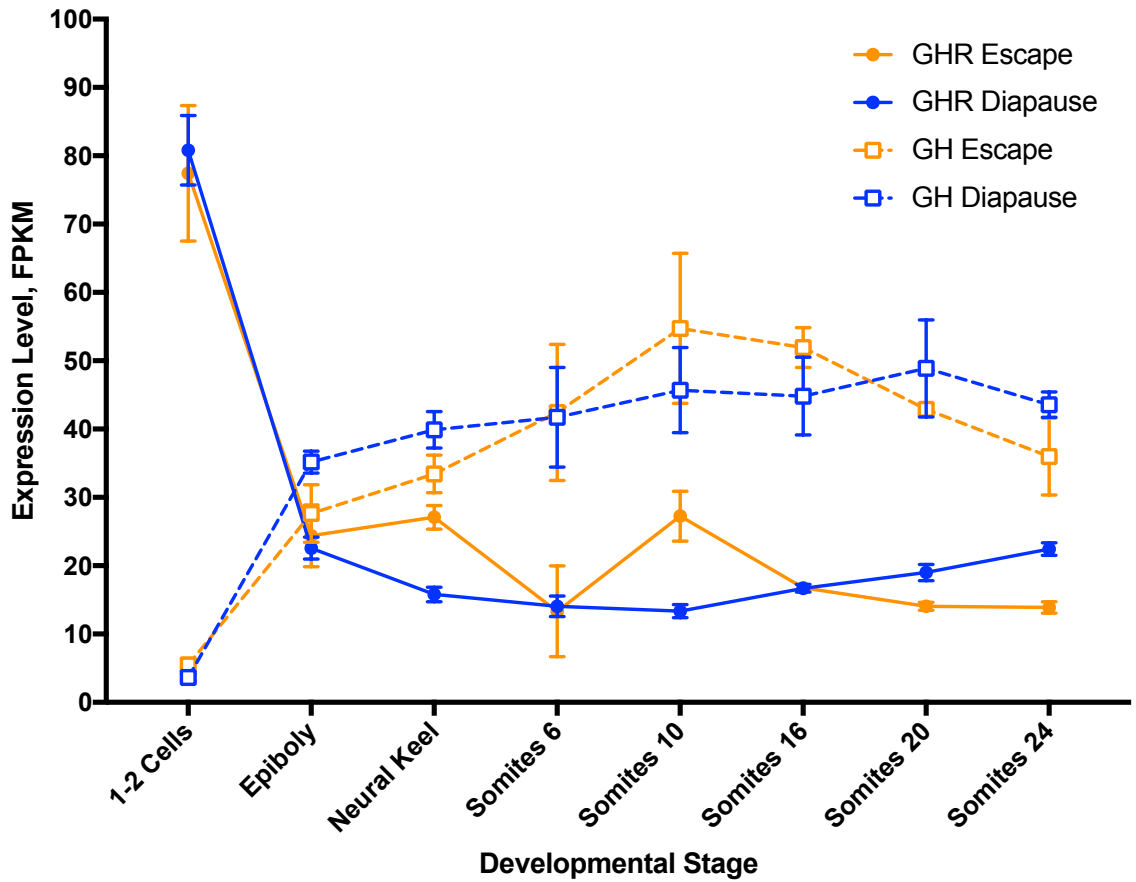


Figure 4.5. Expression of growth hormone (GH) and GH receptor (GHR) in embryos developing along the diapause (20°C) and escape (30°C) trajectories.

Expression of both the hormone and receptor are similar for both developmental trajectories. GHR transcripts are highest in 1-2 cell stage embryos indicating significant maternal provisioning of this transcript followed by a large decline by the completion of epiboly. GH transcript levels increase during early development reaching peak levels around the 6 somite stage. Symbols are means \pm s.e.m. (n=3).

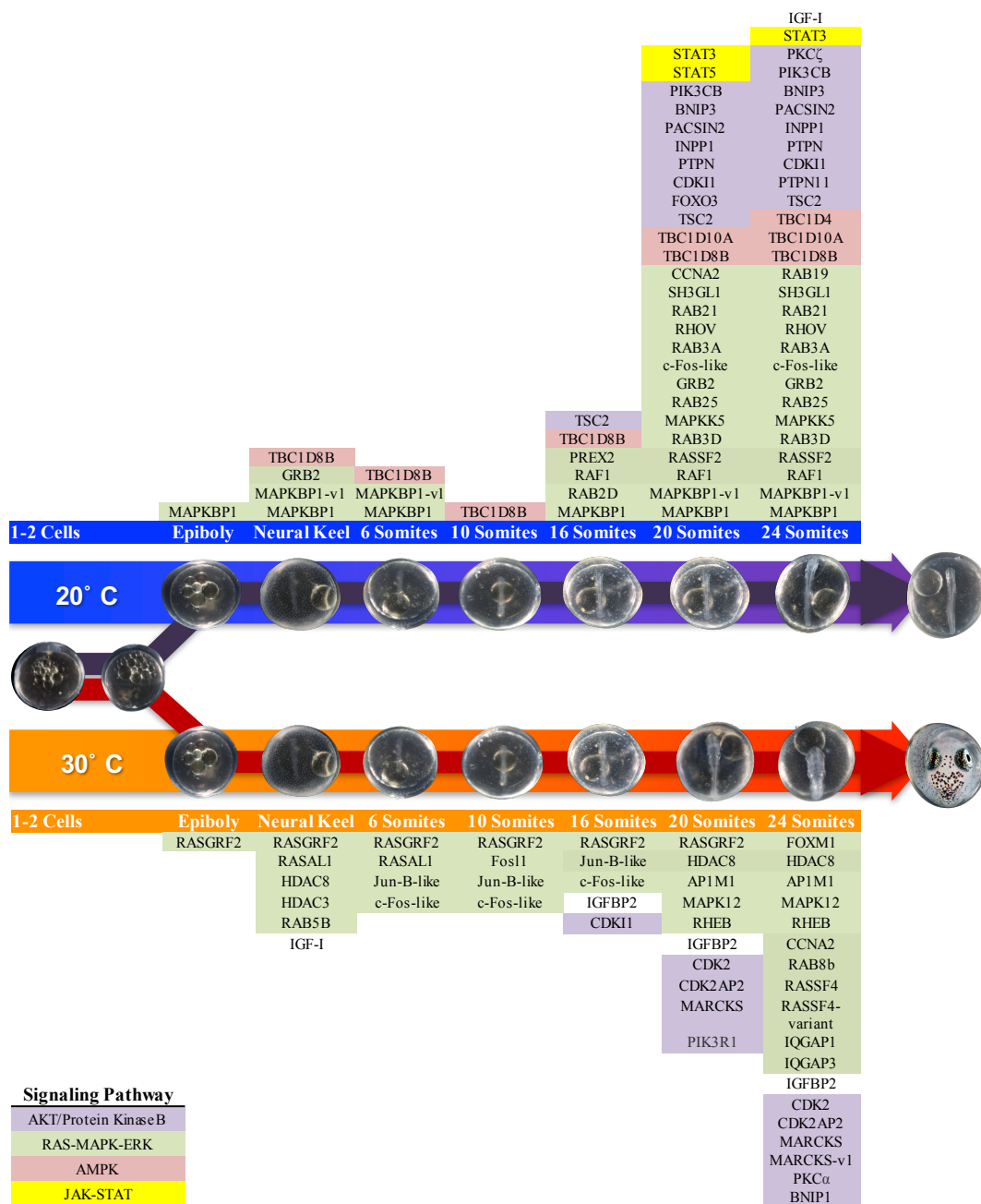


Figure 4.6. Differential expression of transcripts associated with the diapause or escape developmental trajectories.

Statistical significance was set at an FDR adjusted P-value of 0.001, for transcripts expressed at 2 FPKM or greater. Strong induction of genes that positively regulate the RAS-MAPK-ERK

pathway are observed in escape trajectory embryos, while transcripts known to regulate AMP-activated kinase and JAK-STAT signaling are upregulated in diapause-bound embryos at 20 and 24 somites.

Table 4.2 Gene name key for Figure 4.6

Gene key:

AP1M'X' - Adaptor protein complex 1 subunit MU
BNIP'X' - BCL2/adenovirus E1B 19 kDa protein-interacting protein
c-Fos-like – Fos proto-oncogene
CCNA'X' - Cyclin-A
CDK'X' – Cyclin-dependent Kinase
CDK2AP - Cyclin-dependent Kinase Adapter Protein
CDKI'X' - Cyclin-dependent Kinase Inhibitor
FOX'X' – Forkhead Box
GRB2 - Growth factor receptor-bound protein 2
HDAC'X' – Histone deacetylase
IGF-'X' – Insulin-like Growth Factor
IGFBP'X' – Insulin-like Growth Factor Binding Protein
INPP'X' - Inositol Polyphosphate-1-Phosphatase
IQGAP'X' - Ras GTPase-activating-like protein
Jun-B-like - Transcription factor jun-B
MAPK'X' – Mitogen-activated Protein Kinase
MAPKBP'X' - MAPK binding Protein
MARCKS - Myristoylated alanine-rich C-kinase substrate
PACSIN'X' - Protein Kinase C And Casein Kinase Substrate In Neurons
PIK3CB - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Beta
PKC'X' – Protein Kinase C 'Subunit'
PREX2 - Phosphatidylinositol-3,4,5-Trisphosphate-Dependent Rac Exchange Factor 2
PTPN'X' - Protein tyrosine phosphatase, non-receptor type
RAB'X' – Ras related G proteins
RAF'X' - RAF proto-oncogene serine/threonine-protein kinase
RASAL'X' – Ras protein activator-like
RASGRF2 - Ras protein-specific guanine nucleotide-releasing factor 2
RASSF'X' - Ras-association domain family
RHEB – Ras homolog enriched in brain
RHOV – Ras Homolog Family Member V
STAT'X' - Signal transducer and activator of transcription
TBC1D'X' – TBC1 domain family member
TSC'X' – Tuberculosis Sclerosis

Chapter 5: Conclusions

Development is a complex, life-long process that involves untold numbers of signaling molecules that regulate the formation and function of phenotypic characters. Both temporal and spatial gene expression patterns determine progress through development and the faithful reproduction of species-specific developmental patterns. While many patterns of gene expression during development are highly conserved across taxa, novel traits evolve through modifications to existing programs that yield novel and beneficial results. Thus, novel adaptive traits such as the ability to enter into a state of reversible metabolic arrest are likely rooted in small alterations to existing signaling pathways. Species-specific adaptive phenotypes can be achieved through a variety of mechanisms including mutation, epigenetic regulation, and alterations in sensitivities to environmental factors. In *Austrofundulus limnaeus*, the ability to enter into diapause, a state of metabolic and developmental arrest is associated with a suite of characters that include different developmental rates, differences in timing of major morphological and physiological events, and reprogramming of bioenergetics pathways. Importantly, many of these changes occur relatively early in the developmental program, during a time when theory suggests that selection pressure should favor conservation of function, rather than novelty. Further, the metabolic phenotype at the completion of embryonic development is unique for embryos that enter diapause compared to those that do not. These traits are known to be driven by different molecular and biochemical processes during

development as revealed through ongoing studies at the proteomic and transcriptomic levels. In this thesis I explored the role of insulin-like growth factor (IGF) signaling, a signaling program that is highly conserved across all multicellular animals, and its possible role in regulating developmental phenotype in embryos of *A. limnaeus*.

Insulin-like growth factors (IGF) I and II exert positive effects on rates of cellular growth, metabolism, and proliferation (Jones and Clemmons 1995, Baxter 2000). Blocking of IGF signaling has been shown to be an integral part of entrance into developmental dormancy in a variety of animals, the most well studied of which is the nematode worm, *C. elegans* (Kimura, Tissenbaum et al. 1997, Ogg and Ruvkun 1998, Honda and Honda 1999, Tissenbaum and Guarente 2001, Hesp, Smant et al. 2015). Downregulation of IGF signaling is also known to be associated with other forms of dormancy in a variety of species such as fish, insects, and mammalian hibernators (de Pablo, Perez-Villamil et al. 1993, Kenyon 2001, Schmidt and Kelley 2001, Genade, Benedetti et al. 2005, Donahue, Galley et al. 2006, Kenyon 2010, Kamei, Ding et al. 2011, Lucas-Sánchez, Almaila-Pagán et al. 2014). Downregulation of the IGF pathway leads to a suite of characters such as decreased metabolic activity, decreased mitochondrial electron transport, decreased rates of protein synthesis, cell cycle arrest, and an increase in the production of enzymes that prevent oxidative damage and repair DNA (Honda and Honda 1999, Tran, Brunet et al. 2002). All of these traits are similar to characteristics documented in embryos of *A. limnaeus* during diapause (Podrabsky and Hand 1999, Podrabsky and Hand 2000, Podrabsky and Somero 2007, Podrabsky, Tingaud-Sequeira et al. 2010, Anderson and Podrabsky 2014). Many of these traits,

including the increase in DNA repair and ROS-mitigating enzymes have been shown to be regulated through the activity of the FOXO/DAF-16 transcription factor, which is activated through release of inhibition driven through IGF1R/DAF-2 activation (Dorman, Albinder et al. 1995, Kimura, Tissenbaum et al. 1997, Zheng, Yang et al. 2007, Hesp, Smant et al. 2015, Sim, Kang et al. 2015). This pathway appears to be conserved in various organisms that have a period of arrested development in relation to longevity (Kimura, Tissenbaum et al. 1997, Kenyon 2001, Genade, Benedetti et al. 2005, Kenyon 2010).

With all of these factors in mind, I investigated the possible role IGFs, their receptors, and related proteins that are affected through the IGF pathway in the possible regulation of diapause in embryos of *A. limnaeus*. First, I demonstrated that the IGF-I and -II proteins have stage-specific expression patterns during development, and these expression patterns are unique in embryos developing along the diapause and escape developmental trajectories. These differences in protein expression are due both to changes in embryonic expression patterns of IGF-I as well as through maternal packaging of IGF-II. These data illustrated upregulation of IGF-I and -II at specific stages of development in embryos along the escape developmental trajectory. However, given the role of IGFs in promoting cellular growth and proliferation, these data did not definitively link blockage of IGF signaling to diapause in *A. limnaeus*. To further connect a reduction in IGF signaling to diapause in *A. limnaeus*, we set out to pharmacologically inhibit IGF signaling through the insulin-like growth factor 1 receptor (IGF1R) under conditions that should induce the escape phenotype. Inhibition of IGF1R results in a diapause-mimic

state, even under conditions that should lead to escape embryo development. Many of the developmental characteristics of these embryos mimicked embryos developing along the diapause trajectory. However, the inhibited phenotype was not a perfect phenocopy of diapause, suggesting that IGF-I signaling was either not completely blocked, or the presence of other signaling pathways in the regulation of the diapause phenotype. Together, the protein expression data and pharmacological inhibition data suggest that blocking of IGF signaling is likely a necessary part of a larger suite of molecular signals that dictate entry into diapause. To further investigate the IGF signaling pathway in the regulation of diapause in *A. limnaeus* I explored a large transcriptomic data set generated for embryos developing along the diapause and escape trajectories.

To focus my studies, I took a large transcriptomic dataset for diapause- and escape-bound embryos and filtered the data for all genes known to be involved in the IGF signaling pathway in vertebrates. The genes evaluated ranged from cell signaling factors, and a variety of effector proteins, to the key proteins involved in the regulation of the IGF pathway including growth hormone signaling and the association processes that lead to IGF transcription and exocytosis. These analyses lead to a relatively small number of genes that are differentially expressed in embryos developing along the two developmental trajectories. However, within this small set of differentially expressed transcripts are potent regulators of IGF signaling that seem to suggest how IGF signaling may be exerting its effects on accelerated rates of development associated with the escape trajectory. From these data it is clear that the RAS-MAPK-ERK signaling pathway is likely to be a major regulator of early IGF signaling in both developmental trajectories,

with the AKT/Protein Kinase B signaling pathway becoming more important later in development, after developmental trajectory has already been established. Importantly, in terms of IGF-I expression, the transcriptomic data is consistent with the protein data. Additionally, transcripts for a positive regulator of IGF activity, IGFBP2, has been identified as a likely candidate for increasing IGF signaling in embryos along the escape trajectory. (Myers, Lin et al. 2015, Park, Kim et al. 2015). This combined information suggests that early expression of IGF-I and increased half-life of IGF-I without blocking receptor binding through the action of IGFBP2 may support escape embryo development. In addition to these added insights, a number of other genes upregulated in both trajectories have been identified that deserve further investigation.

The data presented in this thesis set the stage for a more complete and informed investigation of the cellular signaling pathways that may regulate diapause in embryos of *A. limnaeus*. Diapause is an intriguing developmental process that occurs in various species from vertebrates to invertebrates and is characterized by diverse suite of characters involving arrest of cellular processes, and a concomitant increase in tolerance of environmental stress.

I would propose more manipulation experiments, specifically overexpression of IGFs in diapause-bound embryos. By investigating how increased IGFs affect diapausing embryos we could determine if IGF downregulation is necessary for embryos to enter diapause. In addition, investigation of additional regulators of development such as growth hormone (GH) or 5'-AMP activated protein (AMPK) appear to be promising areas for future studies (Salvatori 2004, MacRae 2010). Exploring more deeply the

function of IGF regulated genes that have been identified in other species might also lead to a better understanding of the suite of signals necessary to support entrance into metabolic depression and survival in diapause.

Growth hormone signaling is an especially promising target of interest, because of its role in regulating IGF transcription and secretion (Davey, Xie et al. 2001, Salvatori 2004). GH controls growth largely through regulating IGF concentrations, but it also has been shown to act independent of IGF signaling. For example, GH is thought to be involved in increased lipolysis and stimulation of metabolism during periods of food deprivation (Clemmons 2004). Also, it has been shown that GH and IGF-I stimulate embryos during early development in mammals and are critical in the proper formation of the blastocyst (Palma, Muller et al. 1997, Moreira, Paula-Lopes et al. 2002). Due to the nature of the relationship of IGF and GH expression, and their possible synergistic effects, exploring GH expression and inhibition in developing *A. limnaeus* embryos is very likely to provide useful information on the molecular regulation of diapause in this species. For example, the increase of IGF-I protein observed in escape embryos after epiboly may correlate with a spike in GH or the effects of IGF-I may be enhanced through GH signaling. Studies into the expression of GH during *A. limnaeus* development are already underway.

Blocking of the IGF signaling pathway is almost certainly one of the key components for regulating entrance into diapause in *A. limnaeus*, but it is most likely not the only pathway that regulates entrance into diapause and the diapause phenotype. In *C. elegans*, it is abundantly clear that regulation of IGF-I and the insulin pathway are

important for dauer formation, but it has also been shown that other signals regulate many aspects of *C. elegans* cell identity, function, and survival in the dauer state.

Transforming growth factor-beta (TGF-beta) superfamily ligands are one such group of signaling molecules that regulate dauer phenotype in *C. elegans* (De Robertis 2008). The TGF-beta superfamily is a large group made up of many signaling factors that are known to be key regulators of growth such as activin, Nodal, growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs) (Herpin, Lelong et al. 2004). There are many TGF-beta signals identified in *C. elegans* such as DBL-1 and DAF-7 with receptors such as SMA-6, DAF-1, and DAF-4 (all serine/threonine kinase receptors) (ten Dijke and Hill 2004). These signaling factors activate receptors and the SMAD pathway, which in *C. elegans* has been shown to increase body size, tail development, and dauer (Suzuki, Yandell et al. 1999). Ren et al. found that DAF-7 expression decreases when larvae enter dauer dormancy, but then increases during exit from the dauer state.

Additionally, DAF-7 loss of function mutants spontaneously enter dauer dormancy (Gumienny and Savage-Dunn. 2005). TGF-beta signaling is important in vertebrates as well, and thus it is possible that parallel pathways may be acting in *A. limnaeus* to promote active development, and thus be blocked during entrance into diapause. Another possible target is the homolog of DAF-11, a guanylyl cyclase related gene that also inhibits dauer formation in *C. elegans* (Thomas, Birnby et al. 1993). The data required to initially explore these pathways is already available, and through a more global analysis of gene expression patterns in diapause- and escape-bound embryos it may be possible to highlight the possible routes through which diapause and development are regulated in *A.*

limnaeus. While additional transcriptomic analyses may allow for some pathways to be eliminated from consideration due to a lack of expression, almost certainly more proteomic and functional work will be required to verify if hypotheses generated from transcriptomic data actually lead to functional phenotypic differences.

The data we have gathered concerning IGFs and IGF signaling in *A. limnaeus* embryonic development have shown that the IGF signaling is differentially regulated during entry into diapause in this species. This data is the first of its kind that actually shows this relationship of IGF and arrested development in vertebrates. These data suggest an evolutionary conservation of insulin-like signaling in the regulation of dormancy across all animals. However, these studies are only the first steps in elucidating the role of IGF signaling in the regulation of diapause in *A. limnaeus*. Future work will need to be focused on the many ways that IGF signaling may be regulated in these embryos. In addition, the possible role of other signaling pathways in regulating tissue-specific dormancy during entrance into diapause needs to be explored.

Citations

- Anderson, S. N. and J. E. Podrabsky (2014). "The effects of hypoxia and temperature on metabolic aspects of embryonic development in the annual killifish *Austrofundulus limnaeus*." Journal of Comparative Physiology B **184**: 355-370.
- Antebi, A., et al. (2000). "Daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*." Genes and Development **14**: 1512-1527.
- Arden, K. C. (2008). "FOXO animal models reveal a variety of diverse roles for FOXO transcription factors." Oncogene **27**(16): 2345-2350.
- Baker, D. A. and S. Russell (2009). "Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause." BMC Genomics **10**: 242.
- Baxter, R. C. (2000). "Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities." American Journal of Physiology-Endocrinology and Metabolism **278**(6): E967-E976.
- Belfiore, A., et al. (1999). "Insulin/IGF-I hybrid receptors play a major role in IGF-I signaling in thyroid cancer." Biochimie **81**(4): 403-407.
- Berois, N., et al. (2015). Annual Fishes: Life History Strategy, Diversity, and Evolution: Cellular and Molecular Aspects. Annual Fishes: Life History Strategy, Diversity, and Evolution. N. Berois, G. García and R. O. d. Sá, CRC Press.
- Berven, K. A., et al. (1979). "Countergradient Selection in the Green Frog, *Rana clamitans*." Evolution **33**(2): 609.
- Bid, H. K., et al. (2013). "Dual targeting of the type 1 insulin-like growth factor receptor and its ligands as an effective antiangiogenic strategy." Clin Cancer Res **19**(11): 2984-2994.
- Bjorge, J. D., et al. (1990). "Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation." PNAS **87**(10): 3816-3820.
- Bodenheimer, F. S. and A. Shulov (1951). "Egg-development and diapause in the Moroccan locust." Bulletin of the Research Council of Israel **1**: 59-75.

Boersma, B., et al. (2002). "Catch-Up Growth and Endocrine Changes in Childhood Celiac Disease." Hormone Research **58**(Suppl. 1): 57-65.

Bolger, A., et al. (2014). "The genome of the stress-tolerant wild tomato species *Solanum pennellii*." Nat Genet **46**(9): 1034-1038.

Bonafe, M., et al. (2003). "Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span control." J Clin Endocrinol Metab **88**(7): 3299-3304.

Bondy, C. A., et al. (1990). "Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression." Mol Endocrinol **4**(9): 1386-1398.

Brummett, A. R. and J. N. Dumont (1981). "A Comparison of Chorions from Eggs of Northern and Southern-Populations of *Fundulus-Heteroclitus*." Copeia **1981**(3): 607-614.

Carboni, J. M., et al. (2009). "BMS-754807, a small molecule inhibitor of insulin-like growth factor-1R/IR." Mol Cancer Ther **8**(12): 3341-3349.

Carter, C. A. and J. P. Wourms (1993). "Naturally occurring diblastodermic eggs in the annual fish *Cynolebias*: Implications for developmental regulation and determination." J. Morph. **215**: 301-312.

Carter, C. S., et al. (2002). "A critical analysis of the role of growth hormone and IGF-1 in aging and lifespan." Trends in Genetics **18**(6): 295-301.

Cassoni, P., et al. (2001). "Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines." J Clin Endocrinol Metab **86**(4): 1738-1745.

Catsburg, C., et al. (2015). "Serum IGFBP-2 and Risk of Atypical Hyperplasia of the Breast." J Cancer Epidemiol **2015**: 203284.

Chang, L., et al. (2004). "Insulin signaling and the regulation of glucose transport." Mol Med **10**(7-12): 65-71.

Chastant, S. (1994). "Localization and quantification of insulin-like growth factor-I (IGF-I) and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors in pig embryos during early pregnancy." Biology of Reproduction **51**(4): 588-596.

Cheng, C.-L. (2005). "Role of insulin/insulin-like growth factor 1 signaling pathway in longevity." World Journal of Gastroenterology **11**(13): 1891.

Chennault, T. and J. E. Podrabsky (2010). "Aerobic and anaerobic capacities differ in embryos of the annual killifish *Austrofundulus limnaeus* that develop on alternate developmental trajectories." J Exp Zool A Ecol Genet Physiol **313**(9): 587-596.

Clemmons, D. R. (1991). Insulin-like Growth Factor Binding Proteins. Insulin-like Growth Factors: Molecular and Cellular Aspects. D. LeRoith. Boca Raton, Florida, CRC Press, Inc.: 151-179.

Clemmons, D. R. (2004). "The relative roles of growth hormone and IGF-1 in controlling insulin sensitivity." J Clin Invest **113**(1): 25-27.

Cohen, E., et al. (2009). "Reduced IGF-1 signaling delays age-associated proteotoxicity in mice." Cell **139**(6): 1157-1169.

Colman, R. J., et al. (2009). "Caloric restriction delays disease onset and mortality in rhesus monkeys." Science **325**(5937): 201-204.

Cotterill, A. M., et al. (1993). "The regulation of insulin-like growth factor binding protein (IGFBP)-1 during prolonged fasting." Clinical Endocrinology **39**(3): 357-362.

Daniel, J. C., Jr. and R. S. Krishnan (1969). "Studies on the relationship between uterine fluid components and the diapausing state of blastocysts from mammals having delayed implantation." Journal of Experimental Zoology **172**(3): 267-282.

Davey, H. W., et al. (2001). "STAT5b is required for GH-induced liver IGF-I gene expression." Endocrinology **142**(9): 3836-3841.

de Pablo, F., et al. (1993). "IGF-I and the IGF-I receptor in development of nonmammalian vertebrates." Mol Reprod Dev **35**(4): 427-432; discussion 432-423.

De Robertis, E. M. (2008). "Evo-devo: variations on ancestral themes." Cell **132**(2): 185-195.

DeChiara, T. M., et al. (1991). "Parental imprinting of the mouse insulin-like growth factor II gene." Cell **64**(4): 849-859.

Denduluri, S. K., et al. (2015). "Insulin-like growth factor (IGF) signaling in tumorigenesis and the development of cancer drug resistance." Genes Dis **2**(1): 13-25.

Denlinger, D. L. (1981). "Basis for a Skewed Sex Ratio in Diapause-Destined Flesh Flies." Evolution **35**(6): 1247.

Denlinger, D. L. (2002). "Regulation of Diapause." Annual Review of Entomology **47**(1): 93-122.

Dillin, A., et al. (2002). "Timing requirements for insulin/IGF-1 signaling in *C. elegans*." Science **298**(5594): 830-834.

Donahue, S. W., et al. (2006). "Parathyroid hormone may maintain bone formation in hibernating black bears (*Ursus americanus*) to prevent disuse osteoporosis." J Exp Biol **209**(Pt 9): 1630-1638.

Dorman, J. B., et al. (1995). "The age-1 and daf-2 genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*." Genetics **141**(4): 1399-1406.

Duboule, D. (1995). "Vertebrate Hox genes and proliferation: an alternative pathway to homeosis?" Current Opinion in Genetics & Development **5**(4): 525-528.

Dupont, J. and M. Holzenberger (2003). "Biology of insulin-like growth factors in development." Birth Defects Res C Embryo Today **69**(4): 257-271.

Durai, R., et al. (2006). "Biology of insulin-like growth factor binding protein-4 and its role in cancer (review)." international journal of oncology.

Eckert, R. L., et al. (2013). "AP1 transcription factors in epidermal differentiation and skin cancer." J Skin Cancer **2013**: 537028.

Eivers, E., et al. (2004). "Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo." Int J Dev Biol **48**(10): 1131-1140.

Erickson, C. A. and T. L. Goins (1995). "Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes."
." Development **121**(3): 915-924.

Faria, A. C., et al. (1989). "Half-time of endogenous growth hormone (GH) disappearance in normal man after stimulation of GH secretion by GH-releasing hormone and suppression with somatostatin." J Clin Endocrinol Metab **68**(3): 535-541.

Felder, S., et al. (1990). "Kinase-Activity Controls the Sorting of the Epidermal Growth-Factor Receptor within the Multivesicular Body." Cell **61**(4): 623-634.

Fielenbach, N. and A. Antebi (2008). "C. elegans dauer formation and the molecular basis of plasticity." Genes Dev **22**(16): 2149-2165.

Finkelstein, G. P., et al. (2013). "Catch-up growth: cellular and molecular mechanisms." World Rev Nutr Diet **106**: 100-104.

Fordham, D., et al. (2006). "Compensation for inundation-induced embryonic diapause in a freshwater turtle: achieving predictability in the face of environmental stochasticity." Functional Ecology **20**(4): 670-677.

Froesch, E. R., et al. (1985). "Actions of insulin-like growth factors." Annu Rev Physiol **47**: 443-467.

Furness, A. I. (2015). "The evolution of an annual life cycle in killifish: adaptation to ephemeral aquatic environments through embryonic diapause." Biological Reviews.

Furness, A. I., et al. (2015). "Convergent evolution of alternative developmental trajectories associated with diapause in African and South American killifish." Proceedings of the Royal Society B: Biological Sciences **282**: 20142189.

Furstenberger, G. and H. J. Senn (2002). "Insulin-like growth factors and cancer." Lancet Oncol **3**(5): 298-302.

Gao, X., et al. (2010). "Identification of hookworm DAF-16/FOXO response elements and direct gene targets." PLoS ONE **5**(8): e12289.

Garcia-Echeverria, C., et al. (2004). "In vivo antitumor activity of NVP-AEW541—A novel, potent, and selective inhibitor of the IGF-IR kinase." Cancer Cell **5**(3): 231-239.

Genade, T., et al. (2005). "Annual fishes of the genus *Nothobranchius* as a model system for aging research." Aging Cell **4**(5): 223-233.

Ghosh, P. and S. Kornfeld (2004). "The GGA proteins: key players in protein sorting at the trans-Golgi network." Eur J Cell Biol **83**(6): 257-262.

Gillooly, J. F., et al. (2002). "Effects of size and temperature on developmental time." Nature **417**(6884): 70-73.

Gillooly, J. F., et al. (2002). "Effects of size and temperature on developmental time." Nature **417**(6884): 70-73.

Glaab, E., et al. (2010). "Extending pathways and processes using molecular interaction networks to analyse cancer genome data." BMC Bioinformatics **11**: 597.

Gomez-Mestre, I. and D. R. Buchholz (2006). "Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity." Proceedings of the National Academy of Sciences of the United States of America **103**(50): 19021-19026.

Greene, M. W. and T. T. Chen (1997). "Temporal expression pattern of insulin-like growth factor mRNA during embryonic development in a teleost, rainbow trout (*Onchorynchus mykiss*)." Molecular Marine Biology and Biotechnology **6**(2): 144-151.

Greer, E. L. and A. Brunet (2009). "Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*." Aging Cell **8**(2): 113-127.

Greer, E. L., et al. (2007). "An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in *C. elegans*." Curr Biol **17**(19): 1646-1656.

Griffiths, G., et al. (1988). "The mannose 6-phosphate receptor and the biogenesis of lysosomes." Cell **52**(3): 329-341.

Gumienny, T. L. and C. Savage-Dunn. (2005). TGF- β signaling in *C. elegans*. WormBook: The Online Review of *C. elegans* Biology. NCBI.

Guo, S., et al. (1999). "Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence." Journal of Biological Chemistry **274**(24): 17184-17192.

Guppy, M., et al. (1994). "Biochemical principles of metabolic depression." Comp. Biochem. Physiol. **109B**(2/3): 175-189.

Hales, C. N. and S. E. Ozanne (2003). "The dangerous road of catch-up growth." The Journal of Physiology **547**(1): 5-10.

Hand, S. C. and J. E. Podrabsky (2000). "Bioenergetics of diapause and quiescence in aquatic animals." Thermochimica Acta **349**(1-2): 31-42.

- Herpin, A., et al. (2004). "Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans." Dev Comp Immunol **28**(5): 461-485.
- Herrington, J. (2001). "Signaling pathways activated by the growth hormone receptor." Trends in Endocrinology and Metabolism **12**(6): 252-257.
- Hers, I., et al. (2011). "Akt signalling in health and disease." Cellular Signalling **23**(10): 1515-1527.
- Hesp, K., et al. (2015). "Caenorhabditis elegans DAF-16/FOXO transcription factor and its mammalian homologs associate with age-related disease." Exp Gerontol **72**: 1-7.
- Honda, Y. and S. Honda (1999). "The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans." FASEB J **13**(11): 1385-1393.
- Honda, Y. and S. Honda (1999). "The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans." FASEB J **13**(11): 1385-1393.
- Hondo, E. and C. L. Stewart (2005). "Profiling gene expression in growth-arrested mouse embryos in diapause." Genome Biol **6**(1): 202.
- Hsu, C. Y. and Y. C. Chiu (2009). "Ambient temperature influences aging in an annual fish (Nothobranchius rachovii)." Aging Cell **8**(6): 726-737.
- Hsu, C. Y., et al. (2008). "Age-Related Markers Assayed at Different Developmental Stages of the Annual Fish Nothobranchius rachovii." The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **63**(12): 1267-1276.
- Hu, Q., et al. (2014). "Is insulin-like growth factor binding protein 2 associated with metastasis in lung cancer?" Clin Exp Metastasis **31**(5): 535-541.
- Jones, J. I. and D. R. Clemmons (1995). "Insulin-like growth factors and their binding proteins: biological actions." Endocr Rev **16**(1): 3-34.
- Jones, J. I. and D. R. Clemmons (2013). "Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions." Endocr Rev **16**(1).
- K, A., et al. (1998). "Surface expression and metabolic half-life of AMPA receptors in cultured rat cerebellar granule cells." Neuropharmacology **37**(10-11): 1345-1353.

Kajimura, S., et al. (2005). "Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation." Proc Natl Acad Sci U S A **102**(4): 1240-1245.

Kajimura, S., et al. (2005). "Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation." Proceedings of the National Academy of Sciences of the United States of America **102**(4): 1240-1245.

Kamei, H., et al. (2011). "Role of IGF signaling in catch-up growth and accelerated temporal development in zebrafish embryos in response to oxygen availability." Development **138**(4): 777-786.

Kanehisa, M., et al. (2016). "KEGG as a reference resource for gene and protein annotation." Nucleic Acids Res **44**(D1): D457-462.

Kanfi, Y., et al. (2012). "The sirtuin SIRT6 regulates lifespan in male mice." Nature **483**(7388): 218-221.

Kasuya, J., et al. (1993). "Characterization of human placental insulin-like growth factor-I/insulin hybrid receptors by protein microsequencing and purification." Biochemistry **32**(49): 13531-13536.

Kenyon, C. (2001). "A Conserved Regulatory System for Aging." Cell **105**(2): 165-168.

Kenyon, C. J. (2010). "The genetics of ageing." Nature **464**(7288): 504-512.

Kim, D. and J. Chung (2002). "Akt: versatile mediator of cell survival and beyond." Journal of Biochemistry and Molecular Biology **35**(1): 106-115.

Kim, D. H., et al. (2006). "Mechanical analysis of chorion softening in prehatching stages of zebrafish embryos." IEEE Trans Nanobioscience **5**(2): 89-94.

Kim, J. J. and D. Accili (2002). "Signalling through IGF-I and insulin receptors: where is the specificity?" Growth Hormone & IGF Research **12**(2): 84-90.

Kimura, K. D., et al. (1997). "*daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*." Science **277**: 942-946.

King, C. C., et al. (2009). "A fractionation method to identify quantitative changes in protein expression mediated by IGF-1 on the proteome of murine C2C12 myoblasts." Proteome science **7**(1): 1.

King, O. D. and J. Masel (2007). "The evolution of bet-hedging adaptations to rare scenarios." Theor Popul Biol **72**(4): 560-575.

Koide, T., et al. (2005). "Xenopus as a model system to study transcriptional regulatory networks." Proc Natl Acad Sci U S A **102**(14): 4943-4948.

Kwast, K. E. and S. C. Hand (1996). "Acute depression of mitochondrial protein synthesis during anoxia contributions of oxygen sensing, matrix acidification, and redox state." J. Biol. Chem. **271**(13): 7313-7319.

Lau, M. M., et al. (1994). "Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality." Genes Dev **8**(24): 2953-2963.

LeRoith, D., et al. (1995). "Molecular and cellular aspects of the insulin-like growth factor I receptor." Endocr Rev **16**(2): 143-163.

Li, P., et al. (2011). "IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development." Development **138**(9): 1795-1805.

Liang, J. and J. M. Slingerland (2003). "Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression." Cell Cycle **2**(4): 339-345.

Lin, K., et al. (2001). "Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling." Nat Genet **28**(2): 139-145.

LINGER, D. and D. L (2013). "11 Hormonal Control of Diapause." Endocrinology II **8**: 353.

Liu, J.-P., et al. (1993). "Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r)." Cell **75**(1): 59-72.

Lowe, W. L. J. (1991). Biological Actions of the Insulin-like Growth Factors. Insulin-like Growth Factors: Molecular and Cellular Aspects. D. LeRoith. Boca Raton, Florida 33431, CRC Press: 49-85.

Lucas-Sánchez, A., et al. (2014). "*Nothobranchius* as a model for aging studies. A review." Aging and Disease **5**: 281-291.

- Ma, J., et al. (1999). "Prospective Study of Colorectal Cancer Risk in Men and Plasma Levels of Insulin-Like Growth Factor (IGF)-I and IGF-Binding Protein-3." JNCI Journal of the National Cancer Institute **91**(7): 620-625.
- Macaulay, V. M. (1992). "Insulin-like growth factors and cancer." Br J Cancer **65**(3): 311-320.
- MacRae, T. H. (2010). "Gene expression, metabolic regulation and stress tolerance during diapause." Cellular and Molecular Life Sciences **67**(14): 2405-2424.
- Mariani, T. J., et al. (2003). "A variable fold change threshold determines significance for expression microarrays." FASEB J **17**(2): 321-323.
- Massague, J. and M. P. Czech (1982). "The Subunit Structures of Two Distinct Receptors for Insulin-like Growth Factors I and II and Their Relationship to the Insulin Receptor." The journal of Biological Chemistry **257**(9): 5038-5045.
- Meller, C. L., et al. (2012). "Cell cycle arrest associated with anoxia-induced quiescence, anoxic preconditioning, and embryonic diapause in embryos of the annual killifish *Austrofundulus limnaeus*." Journal of Comparative Physiology B **182**: 909-920.
- Milton, S. L., et al. (2008). "Adenosine modulates ERK1/2, PI3K/Akt, and p38MAPK activation in the brain of the anoxia-tolerant turtle *Trachemys scripta*." J Cereb Blood Flow Metab **28**(8): 1469-1477.
- Morali, O. G., et al. (2001). "IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition." Oncogene **20**(36): 4942-4950.
- Moreira, F., et al. (2002). "Effects of growth hormone and insulin-like growth factor-I on development of in vitro derived bovine embryos." Theriogenology **57**(2): 895-907.
- Morohoshi, S. (1959). "Hormonal studies on the diapause and non-diapause eggs of the silkworm, *Bombyx mori* L." Journal of Insect Physiology **3**(1): 28-31.
- Mullis, P. E., et al. (1992). "Half-life of exogenous growth hormone following suppression of endogenous growth hormone secretion with somatostatin in type I (insulin-dependent) diabetes mellitus." Clinical Endocrinology **36**(3): 255-263.

- Murphy, L. O., et al. (2003). "A Network of Immediate Early Gene Products Propagates Subtle Differences in Mitogen-Activated Protein Kinase Signal Amplitude and Duration." Molecular and Cellular Biology **24**(1): 144-153.
- Myers, A. L., et al. (2015). "IGFBP2 modulates the chemoresistant phenotype in esophageal adenocarcinoma." Oncotarget **6**(28): 25897-25916.
- Myers, G. S. (1952). "Annual fishes." Aquarium Journal **23**: 125-141.
- Nico, L. G. and J. E. Thomerson (1989). "Ecology, food habits and spatial interactions of Orinoco Basin annual killifish." Acta Biologica Venezuelica **12**(3-4): 106-120.
- Nishimura, E. K., et al. (1999). "Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification." Dev Biol **215**(2): 155-166.
- Nissley, P., et al. (1991). The Insulin-like Growth Factor-II/Manose 6-Phosphate Receptor. Insulin-like Growth Factors: Molecular and Cellular Aspects. D. LeRoith. Boca Raton, Florida 33431, CRC Press: 113-142.
- O'Dell, S. D. and I. N. M. Day (1998). "Molecules in focus - Insulin-like growth factor II (IGF-II)." International Journal of Biochemistry & Cell Biology **30**(7): 767-771.
- O'Neill, H. M. (2013). "AMPK and Exercise: Glucose Uptake and Insulin Sensitivity." Diabetes Metab J **37**(1): 1-21.
- Ogg, S. and G. Ruvkun (1998). "The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway." Mol Cell **2**(6): 887-893.
- Oldham, S. and E. Hafen (2003). "Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control." Trends Cell Biol **13**(2): 79-85.
- Ozanne, S. E. and C. N. Hales (2004). "Lifespan: catch-up growth and obesity in male mice." Nature **427**(6973): 411-412.
- Palma, G. A., et al. (1997). "Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced in vitro." Reproduction **110**(2): 347-353.
- Park, S. H., et al. (2015). "The Role of Insulin-Like Growth Factor Binding Protein 2 (IGFBP2) in the Regulation of Corneal Fibroblast Differentiation." Invest Ophthalmol Vis Sci **56**(12): 7293-7302.

Pera, E. M., et al. (2001). "Neural and Head Induction by Insulin-like Growth Factor Signals." Developmental Cell **1**(5): 655-665.

Peters, N. (1963). "Embryonale Anpassungen oviparer Zahnkarpfen aus periodisch austrocknenden Gewässern." Int.Rev.ges.Hydrobiol. **48**(2): 257-313.

Pierce, S. B., et al. (2001). "Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse *C. elegans* insulin gene family." Genes Dev **15**(6): 672-686.

Podrabsky, J., et al. (2015). Tolerance of Environmental Stress. Annual Fishes. Life History Strategy, Diversity, and Evolution. N. Berois, G. García and R. De Sá. Boca Raton, FL USA, CRC Press, Taylor & Francis: 159-184.

Podrabsky, J., et al. (2015). Alternative Developmental Pathways. Annual Fishes. Life History Strategy, Diversity, and Evolution. N. Berois, G. García and R. De Sá. Boca Raton, FL USA, CRC Press, Taylor & Francis: 63-73.

Podrabsky, J. E. (1999). "Husbandry of the annual killifish *Austrofundulus limnaeus* with special emphasis on the collection and rearing of embryos." Environmental Biology of Fishes **54**(4): 421-431.

Podrabsky, J. E., et al. (2010). "Alternative developmental pathways associated with diapause regulated by temperature and maternal influences in embryos of the annual killifish *Austrofundulus limnaeus*." J Exp Biol **213**(Pt 19): 3280-3288.

Podrabsky, J. E. and S. C. Hand (1999). "The bioenergetics of embryonic diapause in an annual killifish, *Austrofundulus limnaeus*." Journal of Experimental Biology **202**(19): 2567-2580.

Podrabsky, J. E. and S. C. Hand (2000). "Depression of protein synthesis during diapause in embryos of the annual killifish *Austrofundulus limnaeus*." Physiological and Biochemical Zoology **73**(6): 799-808.

Podrabsky, J. E., et al. (1998). "Physical and chemical characteristics of ephemeral pond habitats in the Maracaibo basin and Llanos region of Venezuela." Hydrobiologia **362**(1-3): 67-77.

Podrabsky, J. E. and G. N. Somero (2004). "Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*." J Exp Biol **207**(Pt 13): 2237-2254.

Podrabsky, J. E. and G. N. Somero (2007). "An inducible 70 kDa-class heat shock protein is constitutively expressed during early development and diapause in the annual killifish *Austrofundulus limnaeus*." Cell Stress & Chaperones **12**(3): 199-204.

Podrabsky, J. E., et al. (2010). Metabolic dormancy and responses to environmental desiccation in fish embryos. Dormancy and resistance in harsh environments. E. Lubzens, J. Cerda and M. Clark, Springer: 203-226.

Polačik, M., et al. (2014). "Alternative intrapopulation life - history strategies and their trade - offs in an African annual fish." Journal of Evolutionary Biology **27**(5): 854-865.

Puig, O., et al. (2003). "Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway." Genes Dev **17**(16): 2006-2020.

Rahman, M. S. and P. Thomas (2014). "Hypoxia-induced global DNA methylation and IGF regulation in a marine teleost, red snapper." Integrative and Comparative Biology **54**: E335-E335.

Ratovondrahona, D., et al. (1998). "Prolactin stimulation of phosphoinositide metabolism in CHO cells stably expressing the PRL receptor." Biochem Biophys Res Commun **243**(1): 127-130.

Reed, B. C. and M. D. Lane (1980). "Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes." Proc Natl Acad Sci U S A **77**(1): 285-289.

Reinecke, M., et al. (2005). "Growth hormone and insulin-like growth factors in fish: where we are and where to go." Gen Comp Endocrinol **142**(1-2): 20-24.

Ren, P., et al. (1996). "Control of *C. elegans* Larval Development by Neuronal Expression of a TGF-beta Homolog." Science **274**(5291): 1389-1391.

Renehan, A. G., et al. (2004). "Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis." The Lancet **363**(9418): 1346-1353.

Richard-Parpaillon, L., et al. (2002). "The IGF pathway regulates head formation by inhibiting Wnt signaling in *Xenopus*." Dev Biol **244**(2): 407-417.

Rogina, B. and S. L. Helfand (2004). "Sir2 mediates longevity in the fly through a pathway related to calorie restriction." PNAS **101**(45): 15998-16003.

Rosen, C. J. and M. Pollak (1999). "Circulating IGF-I: New Perspectives for a New Century." Trends in Endocrinology & Metabolism **10**(4): 136-141.

Ruvkun, G. (1998). "The Taxonomy of Developmental Control in *Caenorhabditis elegans*." Science **282**(5396): 2033-2041.

Saenger, P., et al. (2007). "Small for gestational age: short stature and beyond." Endocr Rev **28**(2): 219-251.

Saltiel, A. R. and C. R. Kahn (2001). "Insulin signalling and the regulation of glucose and lipid metabolism." Nature **414**(6865): 799-806.

\

Salvatori, R. (2004). "Growth hormone and IGF-1." Rev Endocr Metab Disord **5**(1): 15-23.

Samani, A. A., et al. (2007). "The role of the IGF system in cancer growth and metastasis: overview and recent insights." Endocr Rev **28**(1): 20-47.

Schmidt, K. and K. Kelley (2001). "Down-regulation in the insulin-like growth factor (IGF) axis during hibernation in the golden-mantled ground squirrel, *Spermophilus lateralis*: IGF-I and the IGF-binding proteins (IGFBPs)." J Exp Zool **289**(1): 66-73.

Schneider, M. R., et al. (2000). "Transgenic mouse models for studying the functions of insulin-like growth factor-binding proteins." FASEB J **14**(5): 629-640.

Scott, J., et al. (1985). "Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues." Nature **317**(6034): 260-262.

Shen, Q. and P. Singh (2004). "Identification of a novel SP3 binding site in the promoter of human IGFBP4 gene: role of SP3 and AP-1 in regulating promoter activity in CaCo2 cells." Oncogene **23**(14): 2454-2464.

Shen, X., et al. (2012). "Insulin-like growth factor (IGF) binding protein 2 functions coordinately with receptor protein tyrosine phosphatase beta and the IGF-I receptor to regulate IGF-I-stimulated signaling." Mol Cell Biol **32**(20): 4116-4130.

Sim, C. and D. L. Denlinger (2008). "Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*." Proc Natl Acad Sci U S A **105**(18): 6777-6781.

Sim, C., et al. (2015). "Identification of FOXO targets that generate diverse features of the diapause phenotype in the mosquito *Culex pipiens*." Proceedings of the National Academy of Sciences **112**(12): 3811-3816.

Simao, F. M., et al. (2007). "Short communication. Zebrafish embryo development can be reversibly arrested at the MBT stage by exposure to a water temperature of 16 C." Spanish Journal of Agricultural Research **5**(2): 181-185.

Smith-Gill, S. J. (1983). "Developmental Plasticity: Developmental Conversion versus Phenotypic Modulation." American Zoologist **23**(1): 47-55.

Soos, M. A., et al. (1993). "Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity." Biochemical Journal **290**(2): 419-426.

Soto, N., et al. (2003). "Insulin sensitivity and secretion are related to catch-up growth in small-for-gestational-age infants at age 1 year: results from a prospective cohort." J Clin Endocrinol Metab **88**(8): 3645-3650.

Spicer, J. I. and W. W. Burggren (2003). "Development of physiological regulatory systems: altering the timing of crucial events." Zoology **106**(2): 91-99.

Spicer, J. I. and M. M. El-Gamal (1999). "Hypoxia accelerates the development of respiratory regulation in brine shrimp - but at a cost." Journal of Experimental Biology **202**(24): 3637-3646.

Spicer, J. I. and S. D. Rundle (2007). "Plasticity in the timing of physiological development: Physiological heterokairy -- What is it, how frequent is it, and does it matter?" Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology **148**(4): 712-719.

Stenvinkel, P., et al. (2016). "Nutrients and ageing: what can we learn about ageing interactions from animal biology?" Curr Opin Clin Nutr Metab Care **19**(1): 19-25.

Stockard, C. R. (1921). "Developmental rate and structural expression: An experimental study of twins, "double monsters" and single deformities, and the interaction among embryonic organs during their origin and development." American Journal of Anatomy **28**(2): 115-277.

Storey, K. B. and J. M. Storey (1990). "Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation." Q.Rev.Biol. **65**(2): 145-174.

Storz, B. and J. Travis (2007). "Temporally Dissociated, Trait-Specific Modifications Underlie Phenotypic Polyphenism in *Spea multiplicata* Tadpoles, Which Suggests Modularity." The Scientific World **7**: 715-726.

Suzuki, Y., et al. (1999). "A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*." Development **126**(2): 241-250.

Tamemoto, H., et al. (1994). "Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1." Nature **372**(6502): 182-186.

Tanner, J. M. (1963). "Regulation of Growth in Size in Mammals." Nature **199**(4896): 845-850.

ten Dijke, P. and C. S. Hill (2004). "New insights into TGF-beta-Smad signalling." Trends Biochem Sci **29**(5): 265-273.

Thomas, J. H., et al. (1993). "Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*." Genetics **134**(4): 1105-1117.

Thomerson, J. (1987). "Information for aquarists on *Autrofundulus limnaeus*." J.Am.Killifish Assoc. **20**(6): 220-222.

Tissenbaum, H. A. and L. Guarente (2001). "Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*." Nature **410**(6825): 227-230.

Tollefsen, S. E., et al. (1989). "Insulin-like growth factors (IGF) in muscle development. Expression of IGF-I, the IGF-I receptor, and an IGF binding protein during myoblast differentiation." J Biol Chem **264**(23): 13810-13817.

Tollefsen, S. E., et al. (1989). "Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation." Proc Natl Acad Sci U S A **86**(5): 1543-1547.

Tran, H., et al. (2002). "DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein." Science **296**(5567): 530-534.

UniProt, C. (2015). "UniProt: a hub for protein information." Nucleic Acids Res **43**(Database issue): D204-212.

van Heemst, D. (2010). "Insulin, IGF-1 and longevity." Aging Dis **1**(2): 147-157.

Vecchio, L., et al. (2006). "DADLE induces a reversible hibernation-like state in HeLa cells." Histochemistry and Cell Biology **125**(1): 193-201.

Vincent, A. M. and E. L. Feldman (2002). "Control of cell survival by IGF signaling pathways." Growth Horm IGF Res **12**(4): 193-197.

Von Baer, K. E. (1828). Über Entwicklungsgeschichte der Thiere: Beobachtung und Reflexion. Königsberg, Bei den Gebrüdern Bornträger.

Vu, T. H. and A. R. Hoffman (1994). "Promoter-specific imprinting of the human insulin-like growth factor-II gene." Nature **371**(6499): 714-717.

Waddington, C. H. (1942). "Canalization of Development and the Inheritance of Acquired Characters." Nature **150**(3811): 563-565.

Wagner, E. F. (2002). "Functions of AP1 (Fos/Jun) in bone development." Annals of the Rheumatic Diseases **61**(Supplement 2): 40ii-42.

Walter, H. J., et al. (1997). "Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain." Endocrinology **138**(7): 3024-3034.

Werner, H., et al. (2008). "Similarities and differences between insulin and IGF-I: structures, receptors, and signalling pathways." Arch Physiol Biochem **114**(1): 17-22.

West-Eberhard, M. J. (2005). "Developmental plasticity and the origin of species differences." Proceedings of the National Academy of Sciences of the United States of America **102**(Suppl 1): 6543-6549.

White, M. F. and L. Yenush (1998). "The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action." Curr Top Microbiol Immunol **228**: 179-208.

Wilkinson, R. J., et al. (2004). "Development and characterization of a competitive polyclonal antibody enzyme-immunoassay for salmon insulin-like growth factor-II." Comp Biochem Physiol B Biochem Mol Biol **139**(2): 193-201.

Willmore, K. E., et al. (2007). "Phenotypic Variability: Its Components, Measurement and Underlying Developmental Processes." Evolutionary Biology **34**(3-4): 99-120.

Wilson, P. N. and D. F. Osbourn (1960). "Compensatory growth after undernutrition in mammals and birds." Biol Rev Camb Philos Soc **35**: 324-363.

Wood, A. W., et al. (2005). "Targeted knockdown of insulin-like growth factor binding protein-2 disrupts cardiovascular development in zebrafish embryos." Mol Endocrinol **19**(4): 1024-1034.

Wourms, J. P. (1972). "Developmental biology of annual fishes I. Stages in the normal development of *Austrofundulus myersi* Dahl." Journal of Experimental Zoology **182**(2): 143-168.

Wourms, J. P. (1972). "The developmental biology of annual fishes III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes." Journal of Experimental Zoology **182**(3): 389-414.

Wourms, J. P. (1972). "The developmental biology of annual fishes. II. Naturally occurring dispersion and reaggregation of blastomeres during the development of annual fish eggs." Journal of Experimental Zoology **182**(2): 169-200.

Zar, J. H. (1996). Biostatistical Analysis. Upper Saddle River, N.J., Prentice Hall.

Zheng, X., et al. (2007). "FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress." Proceedings of the National Academy of Sciences of the United States of America **104**(40): 15899-15904.