Published in final edited form as:

Biol Bull. 2015 June ; 228(3): 171–180.

Gene expression changes associated with the developmental plasticity of sea urchin larvae in response to food availability

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Abstract

Planktotrophic sea urchin larvae are developmentally plastic: in response to food scarcity, development of the juvenile rudiment is suspended and larvae instead develop elongated arms, increasing feeding capacity and extending larval life. Here, data are presented on the effect of different feeding regimes on gene expression in larvae of the green sea urchin *Strongylocentrotus droebachiensis*. These data indicate that during periods of starvation, larvae down-regulate genes involved in growth and metabolic activity while up-regulating genes involved in lipid transport, environmental sensing and defense. Additionally, we show that starvation increases FoxO activity, and that in well-fed larvae rapamycin treatment impedes rudiment growth, indicating that the latter requires TOR activity. These results suggest that the developmental plasticity of echinoplutei is regulated by genes known to control aging and longevity in other animals.

Keywords

Strongylocentrotus; echinopluteus; RNA-Seq; plasticity; development

Introduction

Developmental plasticity is the environmental elicitation of alternate phenotypes from a single genotype, which facilitates the optimization of a phenotype-environment match (Dewitt *et al.*, 1998; Agrawal, 2001; Gilbert and Epel, 2009). Marine larvae can experience significant fluctuations in temperature, pressure, salinity, nutrition, and predation. Of these factors, food availability is particularly influential on the development of marine invertebrate larvae (Strathmann *et al.*, 1992; Byrne *et al.*, 2008; Soars *et al.*, 2009).

Planktotrophic echinoid larvae (echinoplutei) are provided with limited maternal investment and rely heavily on exogenous resources (*e.g.*, phytoplankton) for growth to competency for metamorphosis. During pelagic transport larvae often experience growth-limiting conditions owing to scarcity of food (Conover, 1968, Olson and Olson, 1989). When echinoplutei encounter such conditions, development of the juvenile rudiment is suppressed, while the post-oral arms lengthen and the stomach shrinks (Fenaux *et al.*, 1988; Strathmann *et al.*,

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1992; Miner, 2005). This response was recently shown to be mediated by a decrease in dopamine signaling (Adams *et al.*, 2011). Furthermore, it has been shown that thyroid hormone-like compounds obtained from the microalgae upon which echinoplutei feed promote rudiment development and metamorphosis (Heyland and Hodin, 2004; Heyland *et al.*, 2004). In the face of food scarcity sea urchin larvae may make use of their ability to take up free amino acids for subsistence (Manahan *et al.*, 1983).

Although the food-responsive developmental plasticity of sea urchin larvae has been examined morphometrically (Boidron-Metairon, 1988; Strathmann *et al.*, 1992; Heyland and Hodin, 2004; Miner, 2005; McAlister, 2008) and molecularly (Adams *et al.*, 2011), genome-wide changes in gene expression associated with this phenomenon have not been explored. To begin filling this void we used next generation, high-throughput RNA sequencing (RNA-Seq) as well as quantitative reverse transcription and polymerase chain reaction (qRT-PCR) to investigate the gene expression changes that occur in larvae of the sea urchin *Strongylocentrotus droebachiensis* in response to different feeding regimes. Additionally, we tested the hypothesis that TOR and FoxO, key regulatory molecules known to control nutrient signaling, responsiveness to energy stress, plasticity and aging in a variety of animal models, similarly contribute to the food-responsive developmental plasticity of echinoplutei.

Materials and Methods

Procurement of gametes, larval culture, and preparation of RNA

Gametes from a parental mixture were obtained and fertilized at the University of Maine's Center for Cooperative Aquaculture Research (CCAR; Franklin, ME), using standard methods. Embryos were cultured at CCAR in 18 L Plexiglas hatching conicals at 50 eggs ml^{-1} until feeding commenced, at which time the embryos were collected by filtration using a 105 μm screen, and transferred into fluorescently backlit, flow-through 230 L fiberglass cylinders at 4 larvae ml^{-1} . Some of the larvae were transferred to the MDI Biological Laboratory (MDIBL) and cultured at 4 larvae ml^{-1} in 1 L filtered seawater in magnetic stir jars at 8° C.

Larvae were fed a 50:50 mixture of *Dunaliella tertiolecta* and *Rhodomonas salina*. For the RNA-Seq experiment *ad libitum* feeding ($>20,000$ cell larvae $^{-1}$ ml^{-1}) was carried out at CCAR, and diet-restricted feeding (200 cell larvae $^{-1}$ ml^{-1}) was carried out at MDIBL. For RNA-Seq, samples of larvae fed *ad lib* were flash frozen at 2 dpf (gastrula), 6 dpf (pre-feeding pluteus), 13 dpf (6-arm pluteus), 22 dpf (8-armed pluteus with rudiment), and 27 dpf (8-arm pluteus competent for metamorphosis); samples of diet-restricted larvae were flash frozen at 2 dpf (gastrula), 7 dpf (pre-feeding pluteus), and 38 dpf (6-armed pluteus). RNA was then extracted from each sample using RNeasy[®]-Midi total RNA isolation kit (Ambion[®]) with lithium chloride precipitation, and quality-tested using a 2100 Bioanalyzer system (Agilent Technologies). Isolated RNA was sent to the Sick Kids[®] Centre for Applied Genomics at the University of Toronto for sequencing where Illumina mRNA-Seq libraries were prepared for each sample and sequenced on an Illumina GAIIx using manufacturer's protocols.

In a second experiment, using the procedure described above, 6-armed (21 days post-fertilization) larvae at 2 larvae ml⁻¹ were fed a 50:50 *D. tertiolecta* and *R. salina* mixture every three days at 50, 500, and 5,000 cells larvae⁻¹ ml⁻¹. An additional culture was fed 5,000 cells larvae⁻¹ ml⁻¹ in the presence of 100 nM rapamycin, a concentration determined by a survey of the literature to be used routinely to inhibit TOR activity in mammalian cells (e.g., Almilaji *et al.*, 2012), and which we showed in preliminary experiments to inhibit rudiment development while having no adverse effect on larval somatic growth or survival. Rapamycin was added to the culture with each water change, which was done prior to each feeding. Once a week, one hundred larvae were removed from each culture and flash frozen. RNA was purified from the frozen aliquots using the RNeasy[®] plus mini kit (Qiagen), and quantified using a Nanodrop[®] ND-1000 spectrophotometer.

Respirometry

Metabolic rates for the larvae used in the RNA-Seq experiment were estimated by measuring the rate of decline in dissolved oxygen in a sealed, stirred, and temperature controlled chamber containing 1,000 larvae. The larvae were washed free of external microalgae by centrifugation prior to being introduced into the chamber, although the stomachs of the well-fed larvae remained full of microalgae. Dissolved oxygen levels were measured using an Ocean Optics fluorescence-based oxygen sensor (FOXY) and multifrequency phase fluorometer.

mRNA-Seq Data Analysis

Sequence reads were analyzed using CLCBio Genomics Workbench (version 4.0) using the following workflow. First, the reads were trimmed by quality after examining sequence quality and composition of the reads. Next, trimmed reads were mapped to the *Strongylocentrotus purpuratus* NCBI version 2.1 genome assembly and expression levels for each gene reported in reads per kilobase of exon model (RPKM) units. Mapped reads were at least 80% identical to the *S. purpuratus* assembly over at least 50% of their trimmed length. Mapping reads to the *S. purpuratus* genome assembly was done in lieu of *de novo* transcriptome assembly because *S. droebachiensis* is a close relative of *S. purpuratus* (Lee, 2003), so a majority of reads from the former would be expected to map to annotated genes of the latter (borne out by the results, see below and Table S1, <http://www.biolbull.org/content/supplemental>), permitting differential expression analysis. Enriched Gene Ontology annotations among differentially expressed genes were calculated using the R/topGO (Alexa and Rahnenfuhrer, 2010) (version 2.16.0) package in the R (version 3.1.1) statistical computing environment taken from Ensembl Genomes (Kersey *et al.*, 2014) (version 23) annotation of *S. purpuratus* genes obtained using BioMart (Kinsella *et al.*, 2011). For the enrichment analysis, all expressed genes were used as the background.

Quantitative reverse transcription and polymerase chain reaction (qRT-PCR)

RNA was reverse transcribed to cDNA using SuperScript[®] III (Life Technologies) and random hexamer primers, and expression levels for genes of interest were measured in triplicate by qPCR with Perfecta SYBR Green (Quanta Biosciences). Relative expression levels were calculated using the delta-delta Ct method, using 3-UTR sequences from *hprt*

(Hypoxanthine-guanine phosphoribosyltransferase, expression of which was not affected by the experimental treatments) as a reference for normalization. Primer sequences were obtained from sequence contigs retrieved by BLAST queries of the *S. droebachiensis* transcriptome for each gene of interest (Table S4, <http://www.biolbull.org/content/supplemental>), using the program Primer3Plus. The sequences of the primers are provided in Table 1.

Morphometrics and assessment of competency for metamorphosis

After four weeks of differential feeding ten larvae randomly selected from each diet were imaged on a Zeiss Axiovert 25 microscope equipped with a Zeiss AxioCam MRm. Rudiment area was measured using NIH ImageJ software (<http://imagej.nih.gov/ij/>). To determine competency for metamorphosis, 50 larvae per diet were removed from the culture and allowed to settle in glass dishes over 72 hours and monitored for metamorphosis. Post-metamorphic test size was measured following imaging of the metamorphosed juveniles as described above.

Results

RNA-Seq analysis of larval gene expression under different feeding regimes

To assess the effect of different feeding regimes on the *S. droebachiensis* larval transcriptome, we performed mRNA-Seq on total RNA extracted from larvae fed either *ad libitum* or a restricted diet (see Materials and Methods). Samples of the *ad libitum* (AL) culture were taken at 2, 6, 13, 22, and 27 days post-fertilization (dpf; larvae were at the 6-arm stage at 13 dpf, and achieved metamorphosis by ~28 dpf), while samples of the diet-restricted (DR) culture were taken at 2, 7, and 38 dpf (larvae were at the 6-arm stage at 38 dpf) (Fig. 1). Measurements of oxygen consumption rates of the sampled larvae suggested that the metabolic rate of DR larvae at 38 dpf was approximately half that of equivalently staged (6 arm) AL larvae at 13 dpf (data not shown), although whether this was due to intrinsic metabolic differences or simply to the fact that the stomachs of the AL larvae were full of microalgae that may still have been respiring cannot be ascertained.

An average of 44.4 million 76 bp reads were generated per sample and an average of 57.8% of trimmed reads per sample were mapped to the *Strongylocentrotus purpuratus* genome assembly (Sodergren *et al.*, 2006) (Table S1, <http://www.biolbull.org/content/supplemental>), with an average of 84.7% of mapped reads per sample mapping to annotated exons. Comparing expression levels between 38 dpf diet-restricted and 13 dpf *ab lib* samples relative to their respective 2 dpf samples identified 2,349 genes that were up-regulated and 1,966 genes that were down-regulated in response to starvation (Table S2, <http://www.biolbull.org/content/supplemental>). These genes were differentially expressed by at least two-fold and had more than five reads per kilobase of exon gene model (RPKM) in at least one sample. The functional context of the up- and down-regulated genes was analyzed by examining enriched Gene Ontology (Ashburner *et al.*, 2000) Biological Process (BP) terms in REVIGO (Supek *et al.*, 2011) (Table S3, <http://www.biolbull.org/content/supplemental>). Lipid transport and its descendent BP terms constituted the largest category of enriched terms for up-regulated genes (Figure S1A, <http://www.biolbull.org/content/>

supplemental). The three largest categories of enriched BP terms for down-regulated genes were DNA conformation change (including gene expression and DNA and RNA metabolism), cellular component biogenesis, and respiratory electron transport chain (Figure S1B, <http://www.biolbull.org/content/supplemental>). In general, these changes suggest that starved larvae down-regulate growth-related processes such as protein synthesis and organelle biogenesis that entail a high metabolic demand, as well as aerobic metabolism, while up-regulating processes involved in lipid mobilization, membrane biology and responsiveness to environmental stimuli (*e.g.*, neurotransmitter transport, ion transport, and signal transduction-related processes).

The three transcription factors found to be most highly over-expressed in underfed, 38-day old 6-arm plutei compared to their well-fed 13-day old counterparts were CREB, Elk, and FoxJ1 (Fig. 2). Genetic variability between the two cultures is unlikely to account for this differential expression, as none of these factors was differentially expressed between the respective 2-day (gastrula stage) samples. Furthermore, after the onset of feeding, in the well-fed larvae Elk and FoxJ1 showed constant per-larva expression throughout development to metamorphosis, suggesting that the high level of overexpression observed in the 38-day underfed larvae was due to the different feeding regime rather than to developmental or chronological time. Expression of CREB increased with development past the 6-arm stage in the well-fed larvae, which correlated temporally with development of the rudiment. However, the 38-day underfed larvae lacked rudiments, suggesting that overexpression of CREB in the latter occurred specifically in response to food scarcity. This fits with what is known from studies of mammals and flies, wherein CREB functions in the regulation of energy balance and mobilization of energy stores in response to fasting or starvation (Iijima *et al.*, 2009; Oh *et al.*, 2013).

Validation of RNA-Seq results by quantitative RT-PCR

To validate and extend the RNA-Seq findings we performed an experiment in which a culture of 3-week old, 6-arm larvae developed from a single batch of embryos was divided evenly into four cultures, three of which were respectively fed with 50, 500, and 5,000 cells of microalgae larva⁻¹ ml⁻¹ biweekly, the fourth being fed 5,000 cells larva⁻¹ ml⁻¹ biweekly in the presence of rapamycin, an inhibitor of the nutrient-sensing kinase TOR. After three weeks of these treatments, larvae in each culture had developed to the 8-armed stage. However, by this time most larvae on the low-food diet ('starved' larvae) as well as those treated with rapamycin either lacked or had very small rudiments, whereas those fed intermediate and high-food diets had well-developed rudiments (Fig. 3 and Fig. 4A), and by 4 weeks achieved competency for metamorphosis (Fig. 4B). Post-metamorphic test diameter of larvae on medium and high food diets did not significantly differ, but rapamycin-treated larvae that underwent metamorphosis displayed nearly 2-fold reduction in test diameter (Fig. 4C).

Quantitative RT-PCR was used to measure the expression of several of the genes found to be differentially expressed by RNA-Seq: the transcription factors Elk, FoxJ1, CREB (Fig. 2), as well as NFκB (RNA-Seq indicating ~1.9-fold higher expression in starved larvae); the neural excitatory amino acid transporter EAAT (~4-fold higher expression in starved

larvae); the translation inhibitor 4E-BP (~2-fold higher expression in starved larvae); the ribosomal biosynthesis factor nucleolin (Nuc, ~2-fold lower expression in starved larvae); and the mitochondria-associated proteins ATP synthase-coupling factor 6 (ATPSyn, ~2.6-fold lower expression in starved larvae), Cytochrome C (CytC, ~3.8-fold lower expression in starved larvae), and HSPE1 (~3.4-fold lower expression in starved larvae). In comparison to both intermediate and well-fed larvae, Elk ($p < 0.008$), CREB ($p < 0.0001$), FoxJ1 ($p < 0.0005$), NF κ B ($p < 0.005$), EAAT ($p < 0.0005$), 4E-BP ($p < 0.0005$), and ATPSyn ($p < 0.0003$) expression levels were significantly elevated in starved larvae, each except for ATPSyn recapitulating what was observed in the RNA-Seq experiment (Fig. 5). Expression levels of those genes also significantly increased ($p = 0.015$) in the well-fed larvae treated with rapamycin, phenocopying the situation in starved larvae (Fig. 5). Starved and rapamycin-treated larvae displayed no differential expression of ATPSyn ($p > 0.35$), EAAT ($p > 0.07$), or Elk ($p > 0.30$). In starved larvae, CytC ($p < 0.001$), Nuc ($p < 0.005$), and HSPE1 ($p < 0.00001$) expression were significantly decreased, but only HSPE1 ($p < 0.0003$) expression significantly decreased in rapamycin-treated larvae, while CytC ($p > 0.15$) remained constant, and Nuc ($p < 0.00001$) significantly increased.

It should be noted that the experimental context for the comparisons made by qRT-PCR was somewhat different than that made by RNA-Seq, in that the latter compared larvae of the same morphological stage (6-arm) but different ages (well-fed larvae at 13 dpf vs. underfed larvae at 38 dpf), whereas the former compared larvae of the same age (6 weeks) but different morphological stages (well-fed 8-arm larvae with rudiments vs. underfed 8-arm larvae without rudiments). This raises the possibility that the differences in gene expression observed by qRT-PCR might simply be due to spatially differential gene expression between the larval soma and the juvenile rudiment. However, this possibility is ruled out by both the RNA-Seq data (Fig. 2) and the time-course qRT-PCR data presented below (Fig. 6) showing that regulatory genes that are highly overexpressed in response to starvation do not display any decrease in expression in well-fed larvae between the 6-arm stage and the 8-arm stage with a fully developed rudiment.

Time-course of *FoxO* and *4E-BP* expression

In *Drosophila* the translational inhibitor 4E-BP has been shown to be upregulated in response to nutritional stress, providing a metabolic ‘brake’ to control fat metabolism (Teleman *et al.*, 2005). 4E-BP is post-translationally inhibited by TOR kinase and transcriptionally activated by FoxO, which mediates plasticity in response to decreased growth factor (e.g., insulin) signaling (Puig *et al.*, 2003; Tang *et al.*, 2011). In addition to being post-translationally regulated, FoxO is known in some contexts to positively regulate its own transcription (Essaghir *et al.*, 2009). To examine the effect of different feeding regimes on FoxO expression and activity we employed qRT-PCR to measure expression of both FoxO and 4E-BP over the entire time course of the experiment. Larvae exposed to different feeding regimes displayed dose-dependent differential expression of both FoxO and 4E-BP, with higher expression levels in starved larvae (Fig. 6). Well-fed larvae treated with rapamycin did not follow this trend, with expression profiles more closely resembling those in diet restricted larvae, albeit with a temporal lag (Fig. 6). After four weeks larvae cultured with rapamycin showed the largest increase in FoxO expression (~10 fold;

$p < 0.0001$), and those cultured on a low food diet also showed a greater (~7 fold; $p < 0.0001$) increase in FoxO than those cultured on intermediate- and high-food diets (~5 fold; $p < 0.0001$) (Fig. 6). 4E-BP displayed similar trends in expression, except expression in starved larvae was greater than in rapamycin-treated larvae at the end of four weeks (Fig. 6). After 4 weeks 4E-BP expression increased most in starved larvae (~18 fold; $p < 0.0001$), followed by rapamycin-treated (~10 fold; $p < 0.0001$), intermediate- (~7 fold; $p < 0.0001$), and high-food (~5 fold; $p < 0.0001$) diets.

Discussion

Plasticity in response to food availability serves to produce a better fit between phenotype and environment, through changes in individual chemistry, physiology, development, morphology or behavior (Dewitt *et al.*, 1998; Agrawal, 2001; Gilbert and Epel, 2009). To increase fitness and food clearance rate during periods of low food availability, sea urchin larvae elongate post-oral arms and reabsorb stomach tissues; in contrast, when food is abundant post-oral arms remain short while the stomach lengthens (Strathmann *et al.*, 1992; Miner, 2005; Adams *et al.*, 2011). This adaptation appears to be widely conserved among echinoids (Strathmann *et al.*, 1992; Heyland and Hodin, 2004; Miner, 2005; Reitzel and Heyland, 2007; Byrne *et al.*, 2008; Adams *et al.*, 2011), although subject to evolutionary variation (McAlister, 2008).

We found that starved 38-day echinoplutei of the sea urchin *S. droebachiensis* down-regulate genes associated with growth and mitochondrial activity, consistent with preliminary respirometry measurements suggesting that they have a lower metabolic rate than that of well-fed 13-day larvae of an equivalent morphological stage. Concomitantly, the starved larvae up-regulate genes that are known in other animals to regulate energy homeostasis (*e.g.*, CREB, 4E-BP, FoxO), environmental sensing and neuroplasticity (*e.g.*, CREB, Elk, EAAT), ciliogenesis (*e.g.*, FoxJ1), and stress-resistance (*e.g.*, FoxO, NF κ B) (Amara and Fontana, 2002; Li and Stark, 2002; Wang, 2002; Carter and Brunet, 2007; Iijima *et al.*, 2009; Besnard *et al.*, 2011; Hay, 2011; Oh *et al.*, 2013; Choksi *et al.*, 2014; Webb and Brunet, 2014). This suggests that during periods of nutritional hardship echinoplutei enhance their sensitivity to environmental conditions and capacity to defend against stress.

Studies using *C. elegans* and *Drosophila* have shown that diet restriction increases activity of FoxO and its target 4E-BP while decreasing TOR activity, which increases lifespan, effects that are reversed when food is abundant (Teleman *et al.*, 2005; Hay, 2011; Webb and Brunet, 2014). Our data suggest that these regulatory relationships are conserved in sea urchin larvae and control developmental transit toward metamorphosis. In addition to correlating with nutrient supply and decreased FoxO activity, rudiment growth and/or development is inhibited by rapamycin, suggesting that it requires TOR activity. It is interesting to consider this finding in light of the emerging consensus that the gene encoding TOR, a pro-growth kinase that is essential for early development, is a key pro-aging gene in adult animals, epitomizing the criteria for the antagonistic pleiotropy theory of aging proposed by George Williams in 1957 (Williams, 1957; Blagosklonny, 2010). Our findings are consistent with Williams' prediction that arresting development prior to reproductive

maturation would prevent senescence: inhibiting TOR, a kinase that promotes cell proliferation, growth, and protein synthesis (Wullschleger *et al.*, 2006), suppresses development of the adult rudiment (by as-yet unknown mechanisms) while allowing continued larval existence, possibly indefinitely.

In the wild, suspension of rudiment development in response to food scarcity could allow echinoplutei to serve as ‘life rafts’ for dispersive colonization, resuming development when food becomes available. In support of this we have found that after seven months on a restricted diet precluding rudiment development, *S. purpuratus* larvae remain competent to develop through metamorphosis when provided sufficient food (Davis and Coffman, 2011). We have been able to maintain diet-restricted larvae for up to a year in the laboratory (JAC, unpublished results). The maximum lifespan of echinoplutei remains an open question, as does, given their ability to undergo cloning (Eaves and Palmer, 2003; Vaughn and Strathmann, 2008), the possibility that they afford echinoids the potential for metagenesis (Mortensen, 1921).

Nevertheless it is clear that echinoid larvae, as well as planktonic larvae of other marine invertebrates, can remain planktonic long enough to cross oceans and maintain connectivity between populations (Scheltema, 1977; Strathmann, 1978; Todd *et al.*, 1998; Behnam *et al.*, 2012), suggesting that starvation-induced suspension of development is an effective mechanism for increasing gene flow across meta-populations and gyres. Teleplanic larvae of echinoderms, molluscs, polychaetes, and crustaceans (Scheltema, 1971; Strathmann, 1978; Shanks, 2009), may remain in the open-sea for more than 300 days and veligers of the snail *Fusitriton oregonensis* have been maintained in the lab for four-and-a-half years (Strathmann and Strathmann, 2007). Pelagic dispersal duration of around a year is believed to be common among deep-sea invertebrate larvae that require extensive transport to the nearest site suitable for settlement (Adams *et al.*, 2012). In 1973, Turner proposed that deep-sea larvae delay metamorphosis for long periods and remain in the benthic boundary layer until a suitable habitat is detected (Turner, 1973). This phenomenon may also apply to larvae that transport to surface waters from methane seeps (Arellano *et al.*, 2014) as well as hydrothermal vents. Such plasticity may be essential for long-term species survival given the unpredictability of the oceanic environment.

The fact that during periods of nutritional hardship echinoid larvae up-regulate both CREB and NFκB (Figs. 2 and 5) is interesting in light of recent studies showing that adult humans who experienced adverse socioeconomic circumstances as children display a highly-sensitized, pro-inflammatory ‘defensive’ phenotype marked by up-regulation of those same factors (Miller *et al.*, 2009). This raises the possibility that genomic encodings that mediate developmental programming in response to early-life adversity may have arisen early in metazoan evolution, and be widely conserved among animal species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Steve Eddy of the University of Maine CCAR for culturing and providing larvae and algae, Timothy Stearns of MDI Biological Laboratory for Gene Ontology enrichment analysis, and Andy Cameron of Caltech for providing helpful comments on the manuscript prior to submission. Research reported in this publication was supported by the NSF research experience for undergraduate (REU) program at the MDI Biological Laboratory (DBI 0453391), and by Institutional Development Awards (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant numbers P20-GM104318 and P20-GM103423.

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Fig 1.
Schematic of the two developmental time-courses with different feeding regimes sampled for RNA-Seq, with representative images of larvae from each sample.

Fig. 2.
RNA-Seq measurements (RPKM) for CREB, Elk, and FoxJ1 under the two feeding regimes (AL = ad libitum; DR = diet restricted), in relation to time and the larval stages from which the data were obtained.

Fig. 3. Larval morphology after three weeks of differential feeding and rapamycin treatment, beginning three weeks post-fertilization at the 6-arm pluteus stage. Scale bar = 200 μ m.

Fig. 4.

Results of four weeks of differential feeding and rapamycin treatment on (A) rudiment size, (B) competency for metamorphosis, and (C) post-metamorphic test diameter. Error bars represent the standard error of the mean (SEM).

Fig. 5.

Results of three weeks of differential feeding and rapamycin treatment on relative expression of selected genes after three weeks of treatment. Each bar represents the average of triplicate qRT-PCR measurements from a single experiment; error bars represent the SEM.

Fig. 6. Expression over time, relative to expression levels at the onset of the experiment (week 0 larvae prior to treatment, arbitrarily assigned a value of 1), of (A) FoxO and (B) 4E-BP in larvae subjected to different feeding regimes and rapamycin treatment. Each point represents average values obtained triplicate qRT-PCR measurements from a single experiment; error bars represent the SEM.

Table 1

Sequences of primers used in qRT-PCR

Gene	Forward Primer	Reverse Primer
<i>Sd-FoxO</i>	TCATTCTGGACGCGACAAA	ATCAGCGCTTGACCTGTCA
<i>Sd-4EBP</i>	CAGGCCTCTGTGAGCATTCA	ACGAGAAAGAGCTGCCGAAA
<i>Sd-ATP_{syn}</i>	CTTGCATTGGGTTTCATGCCA	ATTGGGGCGGAGTTTCTCTG
<i>Sd-CytC</i>	ATGCTGCCCCAATGTGTTTTT	GAATGCTTGTGTGTCGGAGA
<i>Sd-Nuc</i>	TGTCGACATTTTGTGAAGA	TTTTTGTGTATGTCAGTGCATAAT
<i>Sd-HSPE1</i>	ATATGCGACCACAGCCAGAG	CAGCCGTTTGCAAGACAGTG
<i>Sd-NFkB</i>	TGCCCAGGTTACAGCTAACG	AGAGAAGCGCATGTGTCACA
<i>Sd-CREB</i>	AAGACAGCCAAGGGAATCCC	AACTTCTGCTGCTCGACTCC
<i>Sd-EAAT</i>	AAACAGGAAAGCCTGGCATA	GACTTGAGATGGGCAGCAAT
<i>Sd-FoxJ1</i>	TGGCAGAATTCCATCCGTCA	AGGCGTGTGCTCTCTTCTTG
<i>Sd-Elk</i>	TTCATTGGCCCGCCATTTTG	CCGACCCGCCATTTTCGTATA
<i>Sd-HPRT</i>	CTCAACTGGAGGTCAACCCC	AAGTTGGCTTTCTGGACCCC