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Non-invasive method for leptin supplementation in zebrafish (*Danio rerio*)

Regan McNamara
rbm36@zips.uakron.edu

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Non-invasive Method for Leptin Supplementation in Zebrafish (*Danio rerio*)

Regan B. McNamara

Department of Biology

Honors Research Project

Submitted to

*The Williams Honors College
The University of Akron*

Approved:

Date:

Honors Project Sponsor (signed)

Honors Project Sponsor (printed)

Date:

Reader (signed)

Reader (printed)

Date:

Reader (signed)

Reader (printed)

Accepted:

Date:

Honors Department Advisor (signed)

Honors Department Advisor (printed)

Date:

Department Chair (signed)

Department Chair (printed)

Non-invasive method for leptin supplementation in zebrafish (*Danio rerio*)

Regan B. McNamara

ABSTRACT

I tested the hypothesis that recombinant leptin protein can be introduced to zebrafish *in vivo* through non-invasive soaking in a solution containing the protein. One way to study various molecules' effects *in vivo* is through intraperitoneal or intracerebroventricular injections during the embryonic or larval stage, which is invasive, difficult to administer, and can have a high mortality rate. 48 hours post fertilization (hpf) zebrafish were soaked in a His-tagged recombinant leptin protein solution at 10 nM and 100 nM concentrations (produced by Genscript). After soaking, zebrafish larvae were washed extensively to remove all recombinant protein on their exterior before homogenization. The homogenate was evaluated for presence of His-tagged leptin protein using a western blot, which was quantified using ImageJ densitometry. Western blot results were indeterminate, and One-way ANOVA statistical analysis suggested there was no significant difference in mean protein uptake among larvae soaked in 0, 10 nM, or 100 nM leptin ($p=0.5378$). Relative target protein normalization with inconsistent β -actin loading control is likely to have affected results. Although results were inconclusive, this soaking method is still in its preliminary stages and should be explored in greater detail. Downstream applications of this technique include testing the effects of leptin on metabolic rate and whether leptin increases signal transduction in JAK-STAT pathways.

INTRODUCTION

Leptin is known as the “satiety hormone” because it can reduce appetite (Zhou and Rui, 2013). As an adipostat, leptin controls weight and energy balance by signaling to the brain that the body has sufficient fat stores (Deck et al., 2017; Kastin and Pan, 2000). While leptin is mainly associated with long-term energy expenditure and metabolism, it can exert its hormonal effects on multiple biological systems (Austin and Marks, 2009). Other functions include immune system stimulation, bone deposition, and blood pressure control through sympathetic nervous system activation (Faggioni et al., 2001; Tune and Considine, 2007). Leptin is also needed for normal reproductive function because it determines the start of puberty, stimulates hypothalamic gonadotropic-releasing hormone (GnRH) release, and controls ovulatory cycles through its functions in energy balance (Hausman et al., 2012). Although leptin mutations are rare in humans, leptin dysregulation is implicated in obesity, hearing and vision loss, heart failure, and multiple psychological disorders such as clinical depression and dementia (Paz-Filho et al., 2010).

Hunger and satiety sensations are mediated by the arcuate nucleus and ventromedial hypothalamus (Zhou and Rui, 2013). Their neurons contain anorexigenic and orexigenic peptides, which are either stimulated or inhibited by hormones of peripheral tissues (Klok et al., 2006). Anorexigenic peptides are pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART), which increase sensations of fullness. Orexigenic peptides include neuropeptide Y (NPY) and agouti-related peptide (AgRP), which promote hunger (Austin and Marks, 2009; Lanfray and Richard, 2017). In humans, leptin is secreted into the bloodstream by white adipose tissue (WAT), where it then crosses the blood brain barrier, and binds its receptor in the hypothalamus (Londrville et al., 2017; Kastin and Pan, 2000). By binding leptin receptors

of hypothalamic neurons, leptin upregulates the actions of POMC and CART and downregulates NPY and AgRP (Austin and Marks, 2009). Through this feedback mechanism, the brain plays a crucial role in weight maintenance (Londraville et al., 2017).

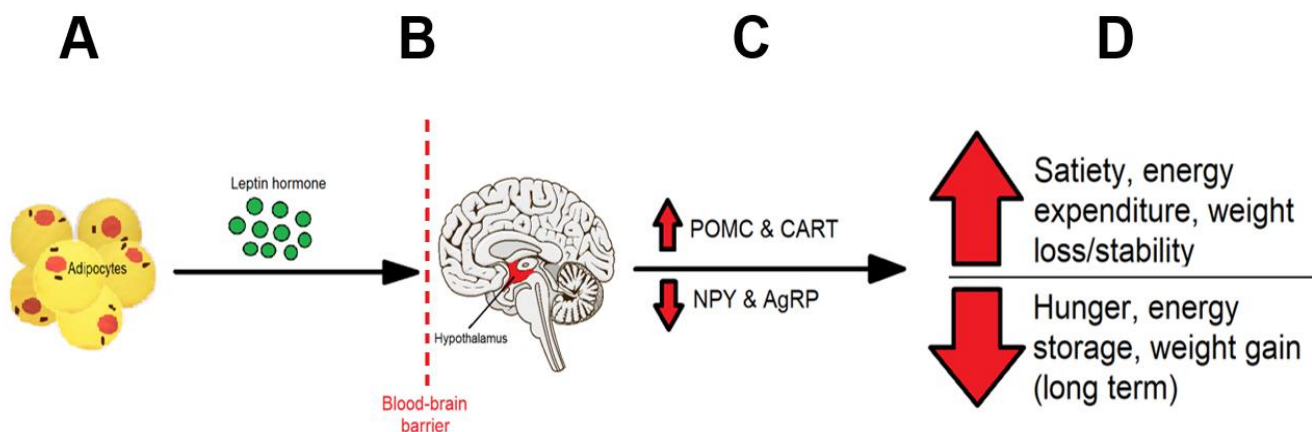


Figure 1. Leptin exerts its anorexigenic effects by targeting the hypothalamus. (A) White adipose tissue (WAT) secretes leptin hormone into the bloodstream. (B) Leptin binds its target receptor in the hypothalamus and crosses the blood-brain barrier via receptor-mediated transcytosis. It upregulates anorexigenic POMC and CART and downregulates orexigenic NPY and AgRP. These actions increase satiety and energy expenditure for long-term weight maintenance (Austin and Marks, 2009).

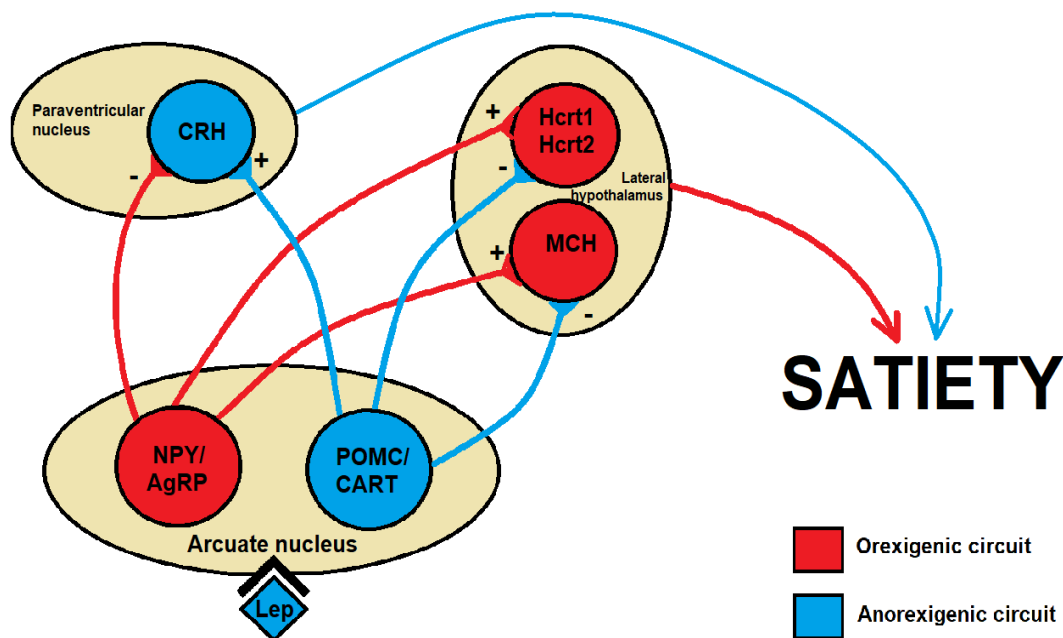


Figure 2. Hypothalamic circuits involved in appetite control. Hormones from peripheral tissues enter the arcuate nucleus and activate anorexigenic (blue) or orexigenic (red) neuron circuits. Downstream neurons are activated by receptor binding in the paraventricular nucleus (CRH) and lateral hypothalamus (Hcrt1/Hcrt2 and MCH). Leptin activates the anorexigenic circuit (adapted from Lanfray and Richard, 2017).

Under normal physiological conditions, weight maintenance is stable and governed by the proper functioning of appetite control hormones leptin and ghrelin. Ghrelin is secreted as a hunger hormone in the body (Klok et al., 2006; Cummings, 2006). Ghrelin stimulates orexigenic peptides NPY and AgRP of the arcuate nucleus, and thus works in opposition to leptin (Austin and Marks, 2009). This stimulation increases gastric acid secretion and motility of the gastrointestinal tract, thus preparing the body to transport and process incoming food (Asakawa et al., 2000). Leptin and ghrelin dictate the long and short-term functioning and outcomes of energy expenditure, respectively. Compared to leptin, ghrelin's actions on the body are rapid and short-lived because of its role in mealtime hunger (Cummings, 2006; Asakawa et al., 2000). While high leptin and low ghrelin levels are paradoxically observed in obese individuals, abnormal functioning of either hormone can lead to obesity. However, leptin is prioritized in

obesity research because of its long-term effects on food-intake, weight, and energy expenditure (Klok et al., 2006).

Either leptin hormone deficiency or leptin resistance can contribute to obesity. While humans with congenitally low leptin exist, it is a rare mutation that cannot account for the current obesity epidemic (Paz-Filho et al., 2010). In general, circulating leptin hormone concentrations are directly proportional to Body Mass Index (BMI) and body fat percentage (Al-Maskari and Alnaqdy, 2006). Most obese individuals have high blood leptin concentrations but compromised intracellular signaling, resulting in leptin insensitivity (Szczesna and Zieba, 2015).

Based on structures of leptin and its receptor, leptin (Ob) belongs to the cytokine family and uses Janus kinase/signal transducer and activator of transcription (JAK/STAT) as its main pathway (Faggioni et al., 2001; Paz-Filho et al., 2010). The LEP gene encodes leptin protein which is 167 amino acid residues in length including a 21-amino acid signal sequence (Zhang et al., 1997). Signal sequence cleavage converts leptin to its mature, secreted form that is 146 amino acids in length and has molecular mass 16 kDa (Ghadge and Khaire, 2019). Leptin forms a bundle of four α -helices and shares homology with the long-chain helical cytokine family in its secondary structure. This family includes interleukins IL-6, IL-11, and IL-12 (Faggioni et al., 2001). Leptin receptor (Ob-R) is a member of the class I cytokine receptor family and is alternatively spliced to produce four different isoforms in the human body: Ob-Ra, Ob-Rb, Ob-Rc, and Ob-Re (Ghadge and Khaire, 2019; Paz-Filho, 2010). Of these isoforms, Ob-Ra and Ob-Rb are best studied. The Ob-Ra isoform facilitates leptin hormone passage through the blood-brain barrier, and the Ob-Rb isoform is the most involved in appetite regulation due to its high abundance in hypothalamic feeding centers (Paz-Filho et al., 2010).

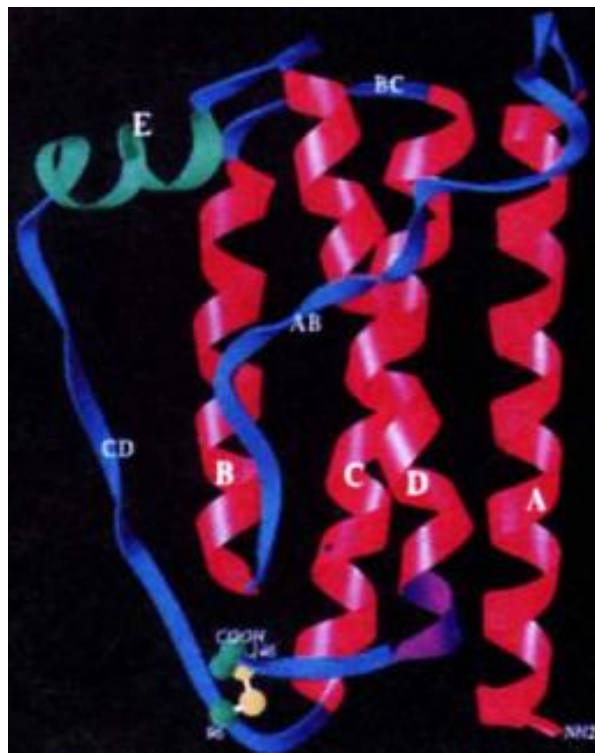


Figure 3. Crystal structure of obesity protein leptin E-100 reported by Zhang et al. (1997). Its four α -helices are shown in red.

The onset of leptin resistance can be explained by dysfunctional signal transduction pathways and failure of leptin to cross the blood brain barrier and involves receptor isoforms Ob-Rb and Ob-Ra, respectively (Clément et al., 1998; Paz-Filho et al., 2010; Kastin and Pan, 2000). Leptin binding to Ob-Rb activates JAK/STAT signaling pathways that can have widespread biological effects. In particular, obesity and leptin resistance arise from mutations in JAK2/STAT3, JAK2/STAT5, SHP2/ERK, and IRS/PI3K signal transduction cascades (Zhou and Rui, 2013). The Ob-Ra isoform uses receptor-mediated transcytosis to move leptin through the blood-brain barrier to anorexigenic circuits in the hypothalamus. Mutations in Ob-Ra structure would prevent leptin from entering the brain and promoting satiety (Kastin and Pan, 2000; Paz-Filho et al. 2010).

Zebrafish are an effective model organism for understanding biological systems because of their fully sequenced genome, external fertilization, and rapid growth (Zang et al., 2018; Avdesh et al., 2012). Physiological systems are highly conserved between zebrafish and humans, making them an attractive model for investigating mechanisms of action and treatments in disease (McRae and Peterson, 2003; Aluru, 2017). Zebrafish continue to be useful for studying obesity pathogenesis and developing drug therapies to address leptin resistance (Faillaci et al., 2018). Furthermore, zebrafish are also ideal models for studying small molecules *in vivo*. Transparency during the embryonic and larval stages allow the small molecule's effects to be observed in real-time (McRae and Peterson, 2003). For a substance to be considered a small molecule, its molecular mass must fall below the 900 Dalton limit (Nwibo et al., 2014), and as a 16 kDa polypeptide, leptin is not considered a small molecule (Zhang et al., 1997). However, small molecule leptin derivatives show pharmacological promise in treating leptin resistance and obesity (Roujeau et al., 2014; Nwibo et al., 2014). Lowering molecular mass allows for easier passage through the blood-brain barrier and in one study increased leptin receptor activation through allosteric binding (Roujeau et al., 2014). Embryonic and larval zebrafish are ideal for testing novel leptin-derived therapeutics *in vivo* (McRae and Peterson, 2003). In addition, high fecundity, low cost, ease of maintenance, and widespread availability also make zebrafish a practical choice for laboratory use (Avdesh et al., 2012).

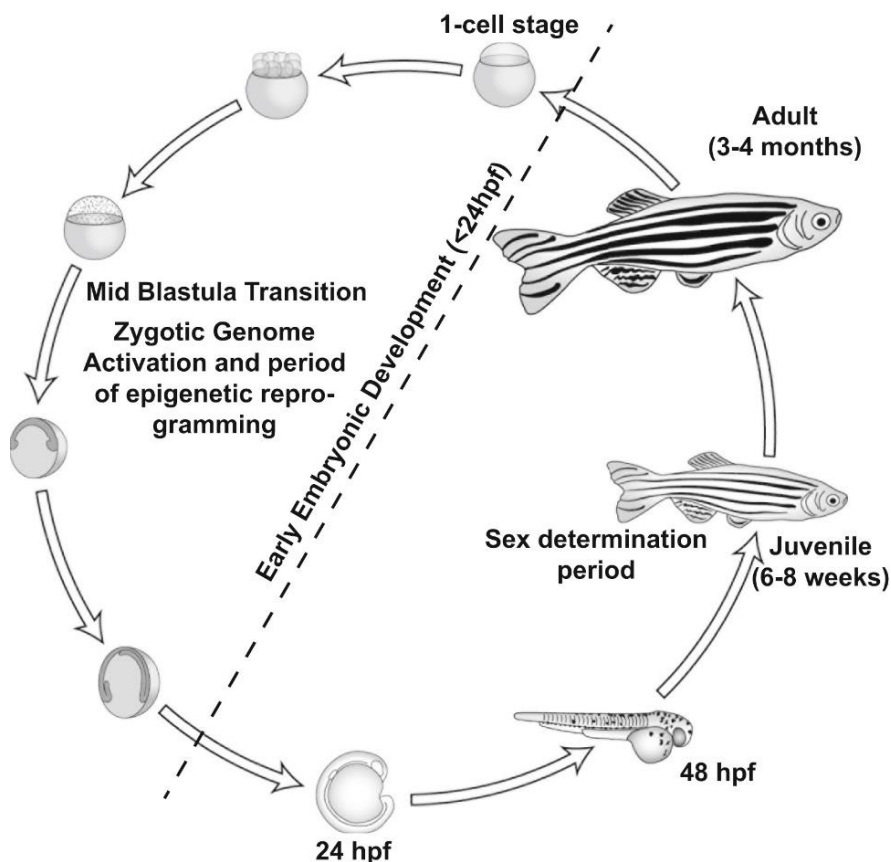


Figure 4. Life cycle of *Danio rerio* illustrated by Aluru (2017). Zebrafish are ideal model organisms for toxicology studies *in vivo*.

Recently, zebrafish embryos and larvae have become instrumental in drug delivery and toxicology research. Bioactive substances are usually delivered to zebrafish embryos and larvae via microinjection or aqueous exposure depending on the situation (Maes et al., 2012; Schubert et al., 2014). Microinjections are direct and precise and can be performed using manual or automated methods (Wang et al., 2007; Zhao et al., 2018). While manual microinjection is more effective in minimizing cell damage and mortality, it is slow, laborious, and only logical in small-scale compound screening (Wang et al., 2007; Maes et al., 2012). Automated microinjections are fast and autonomous in operation, and are therefore better suited for high-throughput compound screening (Wang et al., 2007; Zhao et al., 2018). However, the

convenience of high-throughput microinjection comes at the cost of higher mortality, since zebrafish embryos are fragile and prone to damage (Schubert et al., 2014; Wang et al., 2007). Microinjection methods require a great deal of technical skill and complicated, expensive equipment. In general, microinjection can be overly invasive, cause undue stress on the organism, and adversely affect experimental outcomes (Wang et al., 2007; Maes et al., 2012). Likewise, it is often unnecessary in that cell membranes are permeable to most small molecules (Maes et al., 2012). Compounds can be delivered to zebrafish embryos and larvae by aqueous exposure (Kirta et al., 2018). Despite being less direct and precise than microinjection, soaking methods are easier to implement, have lower mortality rates, do not require expensive equipment (Maes et al., 2012).

Toxicity assays in Maes et al. (2012) evaluated the tolerance of AB wild-type zebrafish embryos and larvae to twelve organic solvents and two carriers, which are used to facilitate the transport of poor water-soluble molecules into biological systems (Maes et al., 2012). Thirty total zebrafish embryos and larvae were tested per solvent per developmental stage. Zebrafish and larvae at 2-4 cells, 4 hpf, and 1, 2, 3, 4, and 7 dpf were incubated in water containing solvents and carriers of multiple concentrations and examined for signs of toxicity after 24 hours (Maes et al., 2012). All embryos and larvae were examined again at 9 dpf to ensure that all signs of toxicity were detected. Across all developmental stages, propylene glycol and methanol were the most tolerated solvents and 1% cyclodextrin (HPBCD) was the better carrier, which may be significant for compound screenings in future research (Maes et al., 2012).

The protocol from Maes et al. (2012) was analogous to the soaking technique used in this project. However, zebrafish larvae were soaked in His-tag recombinant leptin solutions in this project, rather than organic solvents, and were examined for protein uptake instead of toxicity.

While still in its preliminary stages, this protocol establishes a framework for soaking assays in leptin research. Likewise, it has potential to lower zebrafish mortality rate by providing an alternative to microinjection methods.

MATERIALS AND METHODS

Zebrafish (*Danio rerio*) larvae were soaked in histidine-tagged recombinant leptin buffered in tank water (protocol designed by Buo, 2019), and His-tagged leptin uptake through the gills was quantified using Western blotting to differentiate between naturally occurring leptin protein in the zebrafish larvae and leptin taken up by soaking. Since leptin protein is too large to permeate the inner membrane of the chorion (Coward et al., 2002), zebrafish embryos were dechorionated at 48 hpf prior to soaking. Ten dechorionated embryos (n=10) were placed in 1 mL 10 and 100 nM His-tagged recombinant leptin solutions for 48 hours. Larvae were removed from solution and washed with 10 mM Tris Buffer (Bio-Rad) three times in one-minute intervals to remove leptin protein from the zebrafish exterior prior to homogenizing with 10 mM Tris buffer.

Total protein concentrations for each sample were measured using a Bicinchoninic acid (BCA) protein assay (Smith et al., 1985; Olsen and Markwell, 2007). SDS-PAGE separated samples by size (Manns, 2011). Prior to electrophoresis, samples were denatured using a dry heat bath at 60°C for ten minutes. Recombinant zebrafish Leptin A protein (GenScript), tris buffer from final 100 nM wash, and zebrafish larvae that were not soaked in leptin-containing solution served as controls for the Western blot. BLUEstain™ 11-245 kDa protein ladder (Goldbio) was used to estimate molecular weight of the samples, and anti β -Actin (Cell Signaling Inc.) was used as a loading control for recombinant zebrafish leptin A protein in the western blot.

The resulting SDS-PAGE gel was transferred to a Polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus (Ghosh et al., 2014). 50 mL Transfer buffer was prepared containing 10 mM CAPS, 10% methanol, pH 11. A PVDF membrane was soaked in methanol and rinsed in transfer buffer along with two filter papers. The apparatus interior was covered in transfer buffer, and the transfer unit was assembled. Gel proteins were transferred to the PVDF membrane for 2 hours at 90 mA/gel.

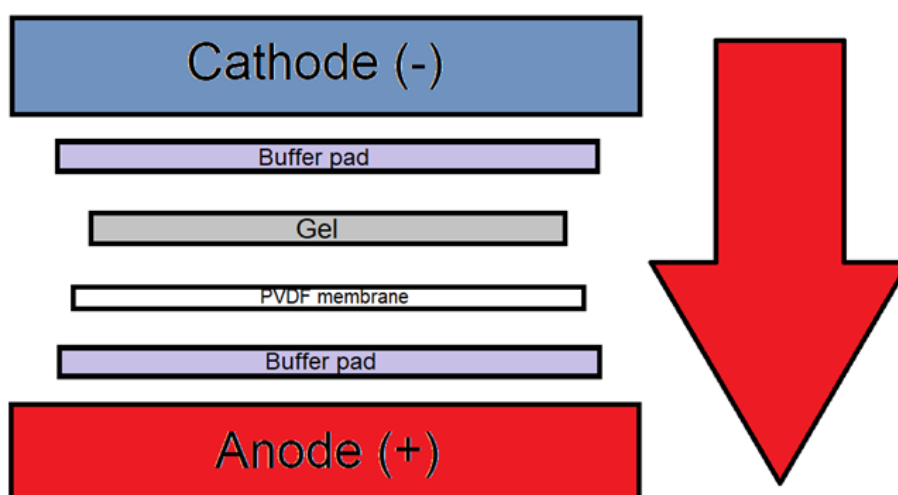


Figure 5. Transfer apparatus for semi-dry transfer. Arrow represents gel bands migrating to PVDF membrane by “running to red,” cathode to anode.

After transfer, the PVDF membrane was soaked in 5% Carnation non-fat dry milk in PBS for one hour at room temperature. In western blotting, milk functions as a blocking agent to prevent nonspecific antibody binding (Mahmood and Yang, 2012). Anti-His-tag Mouse monoclonal primary antibody (1:1000 Applied Biological Materials Inc.) or anti β -actin polyclonal antibody (Cell Signaling Inc.) was applied with agitation overnight at 4°C, then washed the following day with 1X PBS. Then anti-mouse IgG secondary antibody (1:1000

Sigma Aldrich) was added to the PVDF membrane and was washed with 1X PBS after incubating for an hour at room temperature.

The Western blot was developed with Millipore Immobilon Chemiluminescent HRP Substrate and imaged using GeneSnap software (MilliporeSigma, 2005, pp.1-4). Prior to imaging, a working HRP substrate solution was made by combining 5 mL each of HRP Substrate Peroxide Solution and HRP Substrate Luminol Reagent and added to the PVDF membrane. The PVDF membrane was incubated for 5 minutes at room temperature and then drained of excess liquid prior to GeneSnap imaging. β -actin was tested using the same method. Exposure time was 30 seconds and western blot images were recorded for further analysis.

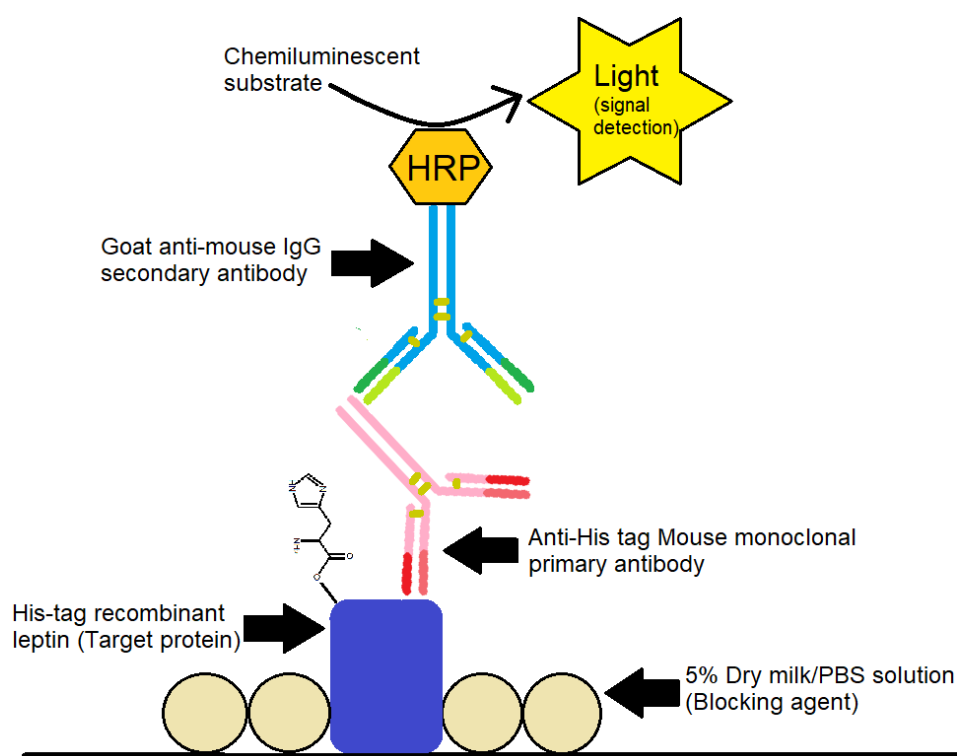


Figure 6. Indirect western blot detection method. Primary antibody binds target protein, and secondary antibody binds primary. Horseradish peroxidase (HRP) enzyme on secondary antibody reacts with chemiluminescent substrate for signal detection in GeneSnap. Blocking agent prevents nonspecific antibody binding. Black line represents PVDF membrane.

Since western blotting only confirms the presence or absence of the protein of interest, bands were quantified with densitometry using ImageJ software (Ferreira and Rasband, 2012). The western blot image was opened as a tiff file in ImageJ and converted to grayscale (8-bit). Sample and β -actin control bands were selected using the rectangle tool, and their intensity plots were generated. Intensities were measured by first using the straight-line tool to seal off peaks and then by using the wand tool to select the area enclosed by the peak and straight-line. Band intensities were automatically recorded as areas in the results notepad, along with their area sizes as percentages of the total area of all selected peaks. Notepad results were opened in an Excel spreadsheet to record relative and normalized relative densities. Relative densities of samples were determined with respect to His-tag recombinant leptin soaking solution in Lane 2, which functioned as the standard. β -actin relative densities were also determined with respect to Lane 2. Percentage of total area for each band was divided by percentage for the standard. Relative density was normalized by dividing sample protein by loading control relative densities (Heidebrecht et al., 2009). A One-way Analysis of Variance (ANOVA) statistical test compared leptin protein uptake of control, 10 nM-soaked, and 100 nM-soaked zebrafish larvae based on their normalized relative densities.

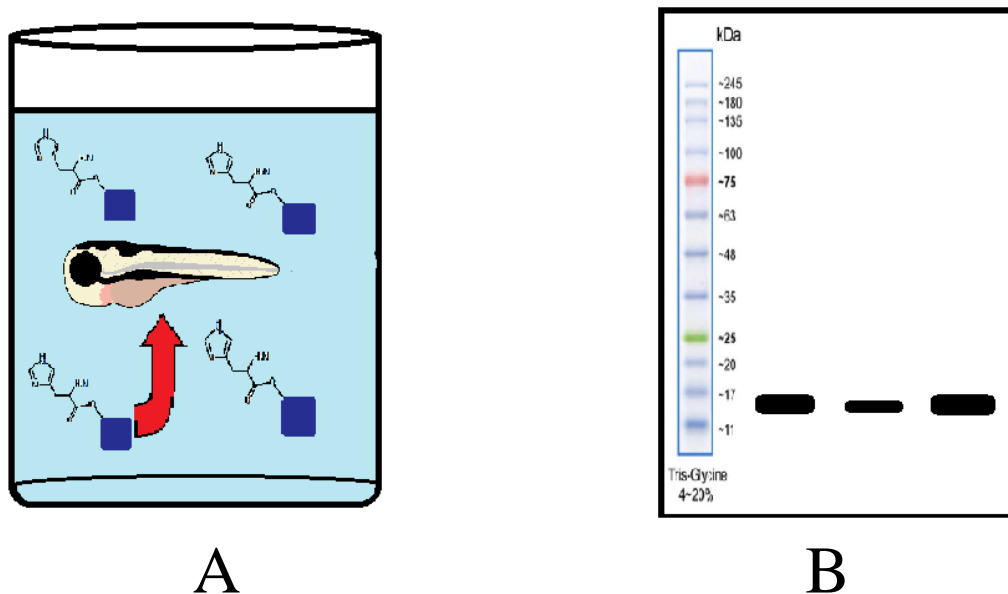


Figure 7. Soaking procedure of *Danio rerio* using His-Tag recombinant protein. (A) Larvae were soaked for 24 hours in 10 and 100 nM His-tag leptin solution. Arrow represents leptin protein uptake by larvae. (B) Protein uptake was quantified by western blotting. His-tag leptin is expected to appear at 16 kDa. Protein ladder (11-245 kDa) pictured on left side of gel.

RESULTS AND DISCUSSION

This experiment tested the hypothesis that recombinant leptin protein could be introduced to zebrafish larvae *in vivo* using a non-invasive soaking method. Zebrafish larvae were placed in one of three conditions; they were either soaked in 10 nM or 100 nM recombinant leptin protein solutions or were not soaked (control). This experiment called for BCA protein assays, SDS-PAGE, and western blotting. Ultimately, protein uptake was detected using a western blot and quantified using densitometry on ImageJ image analysis software. Band densities for the three conditions were compared using One-way ANOVA statistical analysis.

After zebrafish larvae were soaked overnight in their respective conditions, total protein concentration was determined for each group using a BCA protein assay (Table 1). Total protein concentrations were 3.459 $\mu\text{g/mL}$, 2.766 $\mu\text{g/mL}$, and 3.674 $\mu\text{g/mL}$ for control, 10 nM-soaked,

and 100 nM-soaked larvae, respectively. While total protein concentration was low for all groups, it was lowest for 10 nM-soaked larvae. Differences in total protein concentration can be attributed to different protein content of embryos, or incomplete homogenization after soaking (Vuong et al., 2012).

Differences in total protein concentration were accounted for when preparing samples for SDS-PAGE. Samples were loaded into the gel in 20 μL volumes. This value was divided by total protein concentration ($\mu\text{g}/\text{mL}$) to obtain protein sample volume needed for SDS-PAGE. 5X loading dye made up one-fifth, or 4.0 μL , of the 20 μL sample for all groups. 10 mM Tris buffer was used for the remaining volume. Three times the calculated sample amounts were made to account for possible sample loss from the dry heat bath. This ensured that samples could be loaded in duplicate for SDS-PAGE.

	Protein sample (μL)		5x Loading dye (μL)		10 mM Tris (μL)	
	Calc. Amt.	Total	Calc. Amt.	Total	Calc. Amt.	Total
Control	5.8	17.4	4.0	12.0	10.2	30.6
10 nM	7.2	21.6	4.0	12.0	8.8	26.4
100 nM	5.4	16.2	4.0	12.0	10.6	31.8

Table 1. Preparation of SDS-PAGE samples. Protein sample, 5x loading dye, and 10 mM Tris volumes added based on standard curve concentration calculations.

Problems encountered in SDS-PAGE may explain abnormal western blot results (Figures 8 and 9). Sample loading error accounted for most of these problems. Although recommended loading volumes for a 12-well gel plate are 20 μL (ThermoFisher Scientific, 2015, p. 26-29), overflow of loaded samples suggested that a smaller volume should have been used. Sample spillage into neighboring lanes caused loaded volumes and protein concentration to vary across the gel. Evidence of this is seen in Lane 12 (Figure 8). Sample overflow from Lane 11 resulted in a faint band in Lane 12, which was not supposed to contain any sample. Another source of error

during sample loading was the use of improper pipette tips. Standard micropipette tips were used due to a shortage of gel loading tips. This was another complicating factor that likely contributed to sample volume differences across the gel. Aside from sample loading errors, a small leak in the electrophoresis chamber occurred while the gel was running. Electrophoresis was paused twice for the chamber to be refilled. Periodic pausing and restarting of electrophoresis may explain crooked gel lanes.

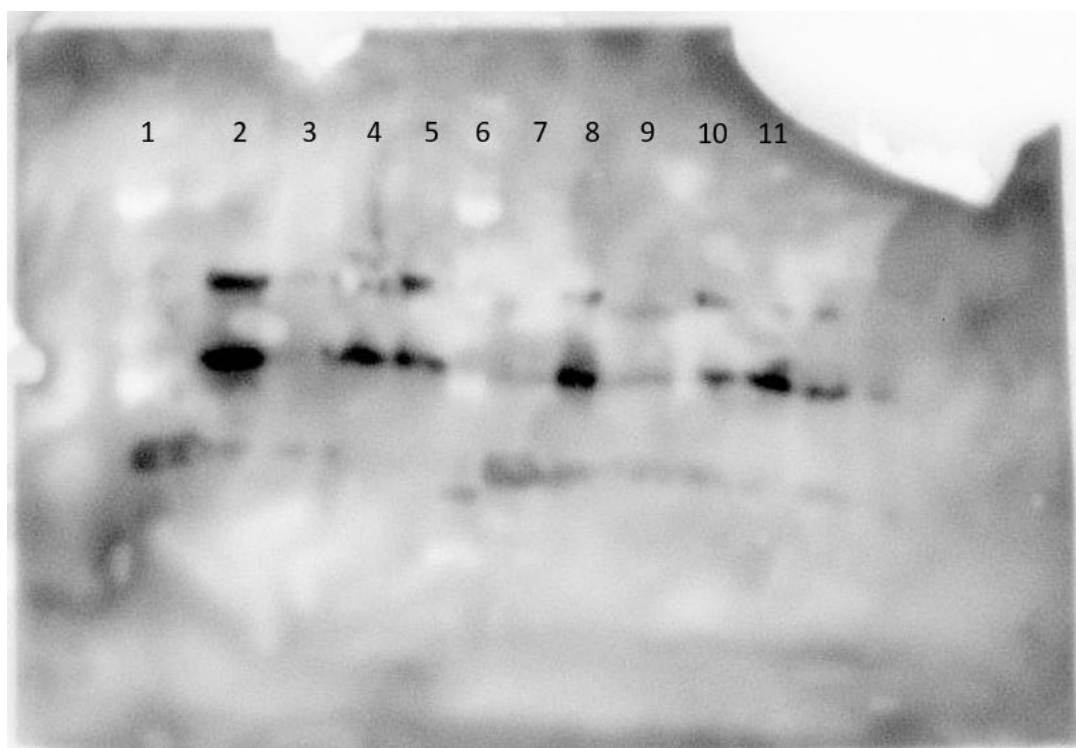


Figure 8. Western blot imaged using GeneSnap software. Lane 1: Protein ladder, Lane 2: His-tag leptin soaking solution positive control, Lane 3: Control larvae, Lane 4: 10 nM soaked larvae, Lane 5: 100 nM soaked larvae, Lane 6: Protein ladder, Lane 7: Tris Buffer negative control from final 100 nM wash, Lane 8: His-tag leptin soaking solution positive control duplicate, Lane 9: Control larvae duplicate, Lane 10: 10 nM soaked larvae duplicate, Lane 11: 100 nM soaked larvae duplicate.

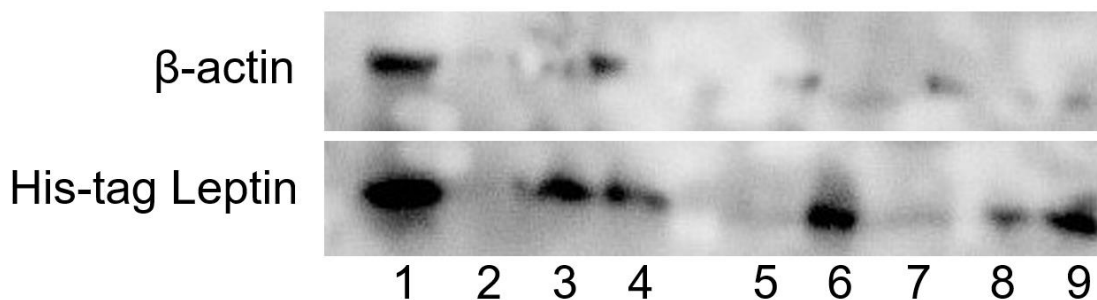


Figure 9. Western blot analysis of His-tag recombinant leptin uptake via soaking by *Danio Rerio*. β -actin (top) was loading control for His-tag recombinant leptin (bottom). Band 1: His-tag leptin soaking solution positive control, Band 2: Control larvae, Band 3: 10 nM soaked larvae, Band 4: 100 nM soaked larvae, Band 5: Tris Buffer negative control from final 100 nM wash, Band 6: His-tag leptin soaking solution positive control duplicate, Band 7: Control larvae duplicate, Band 8: 10 nM soaked larvae duplicate, Band 9: 100 nM soaked larvae duplicate.

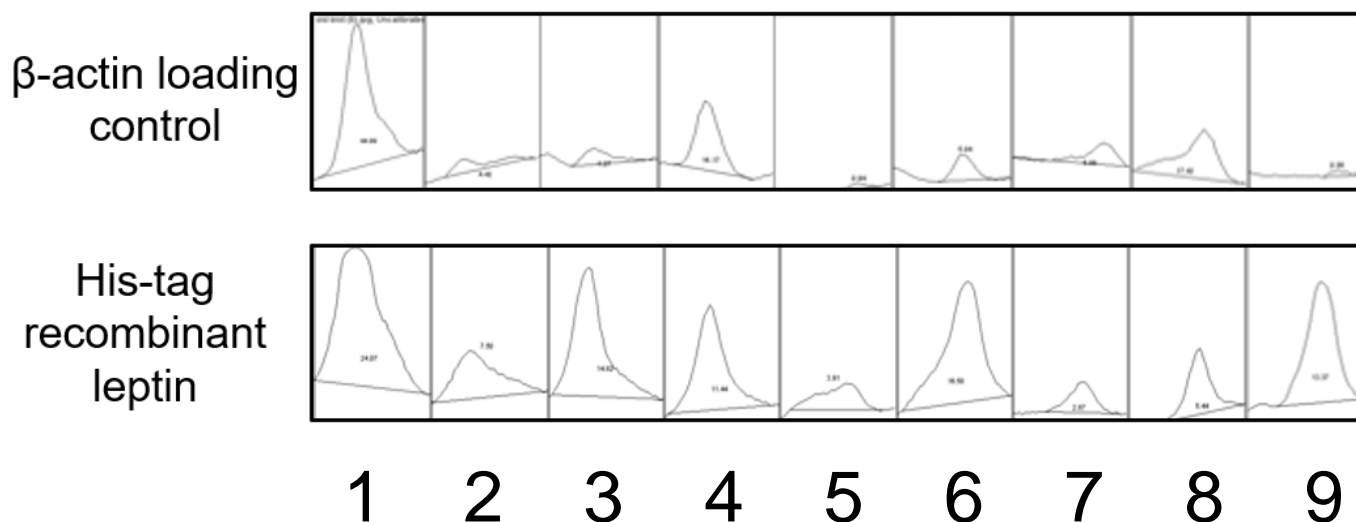


Figure 10. Densitometry plots for β -actin loading control protein and His-tag recombinant leptin protein. Larger area under the curve corresponds to higher band density. Band 1 was used as standard to calculate relative density for loading control and leptin proteins.

Band	Pixel Area (Actin)	Pixel Area (Leptin)	Adj. Rel. Density
1(S)	9808.016	14356.016	1
2	963.619	4470.024	3.1691
3	930.891	8836.752	6.4844
4	3525.468	6823.752	1.3223
5	204.849	2334.083	7.7836

6	1098.184	9885.38	6.1499
7	1281.912	1708.79	0.9105
8	3797.246	3245.832	0.5839
9	187.849	7974.146	28.9927

Table 2. His-tag recombinant leptin protein quantification. Area and Percent measured by ImageJ Densitometry. Relative density was calculated with respect to His-tag leptin soaking solution positive control. Adjusted Relative Density of leptin (yellow) was normalized using β -actin.

Adjusted Relative Density by Condition		
Control	10 nM	100 nM
3.1691	6.4844	1.3223
0.9105	0.5839	28.9927

Table 3. Adjusted Relative Densities of Control (Bands 2 & 7), 10 nM-soaked (Bands 3 & 8), and 100 nM-soaked larvae (Bands 4 & 9).

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	2	4.0796	2.0398	2.550637
10 nM	2	7.0683	3.53415	17.40795
100 nM	2	30.315	15.1575	382.8255

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	206.2729	2	103.1365	0.768177	0.537801	9.552094
Within Groups	402.7841	3	134.2614			
Total	609.057	5				

Figure 11. One-way ANOVA test for adjusted relative densities of control, 10 nM, and 100 nM-soaked samples (Table 2).

His-tag recombinant leptin protein uptake was detected with enhanced chemiluminescence (ECL), and western blot images were recorded using GeneSnap software (Figures 8 and 9). ImageJ densitometry software calculated signal densities over selected areas, which were relativized using the His-tag leptin positive control as a standard (Figure 10; Table 2). A β -actin loading control was used to normalize relative sample densities. A one-way ANOVA test was performed to determine whether there were significant differences in the means of the three treatment conditions (Figure 11).

Based on the western blot image, His-tag recombinant leptin was present in soaked larvae at both concentrations and absent in control larvae (Figures 8 and 9). At first glance, these results reasonably support the hypothesis that aqueous exposure was effective in delivering substances to zebrafish larvae. Further quantification is required to determine relative signal densities of samples, since western blot images alone can only determine presence or absence of the protein of interest. Other factors such as normalization to a loading control, antibody specificity to the target protein, and relative abundance of the protein of interest must also be considered for reliable results (Ghosh et al., 2014).

A single factor ANOVA test showed no significant difference in mean adjusted relative signal densities among 10 nM-soaked, 100 nM-soaked, and control larvae. The null hypothesis that the mean was statistically the same for all groups could not be rejected, since p -value=0.5378 and $F < F$ -crit (Figure 11). These findings do not support the hypothesis that recombinant leptin protein is introduced in a dose-dependent manner, but in all cases, His-tagged leptin was detected in soaked fish. The presence of bands for leptin-soaked samples was undermined by many mistakes and unusual features in the western blot.

Unreliability of the β -actin loading control presented the greatest cause for concern and appeared to have the most direct effect on the outcome of the one-way ANOVA test. In western blotting, loading controls are used to normalize signals for the protein of interest by confirming protein loading was the same across the gel (Ghosh et al., 2014; Mahmood and Yang, 2012). β -actin was present at its approximate 42 kDa molecular weight but its bands were inconsistent, faint, and did not appear to have any relationship to related to leptin signals in the same lane (Figures 8 and 9). Variable expression by β -actin loading control suggested that total protein concentration was not identical across the gel. This can be explained by sample loading errors during SDS-PAGE. Total protein concentration must be the same in all wells to obtain accurate relative expression of the protein of interest. As a result, signal densities for samples could not be relativized and normalization was basically useless.

Other errors were also seen in the western blot (Figure 8). His-tag leptin appeared in the blot at approximately double its expected molecular weight, \sim 32 kDa, suggesting that it had dimerized. Dimer formation was likely due to failure to fully reduce the protein before SDS-PAGE. Samples should be heated longer using the dry heat bath for full denaturation of tertiary structure. The blot also had a high, speckled background that indicated insufficient washing of antibodies with 1 X PBS. This should be corrected by increasing the volume of the washing buffer and the number and duration of washes. Western blot results could have been improved by lowering the amount of technical errors.

Over the past three semesters, about 20-25 western blot experiments have been performed to test the effectiveness of the soaking method. While western blots are useful tools in detecting target protein, they are also easily contaminated and difficult to perform with high technical accuracy. Most of these blots could not be interpreted with densitometry because of

large regions of no-transfer, high background, low resolution, or complete lack of signal. Leptin oligomerization, oversaturation, and nonspecific antibody binding were also common issues. Other western blot results were invalid because of strong leptin signals in control larvae groups. In earlier blots, leptin uptake from ambient water could not be differentiated from naturally occurring leptin. A poly-histidine tag (His-tag) was implemented in more recent blots to account for this problem and strong negative control signals still sometimes occurred. Despite this western blot's multiple errors, it was still an anomaly in that the target protein was detected in leptin-soaked zebrafish at both 10 and 100 nM concentrations and was absent in control larvae. Many of these western blots did not work because of known technical errors, so experiments should be performed to determine the sources of inconclusive results.

CONCLUSION

The hypothesis that recombinant leptin protein could be introduced to *Danio rerio* larvae through an *in vivo* aqueous exposure method was tested at 10 nM and 100 nM concentrations. Protein uptake was detected with western blotting and semiquantitative densitometry software. A single factor ANOVA test (Figure 14) showed no significant difference in mean signal density between 10 nM-soaked, 100 nM-soaked, and control larvae ($p=0.5378$). Bands were present in soaked larvae, indicating uptake of His-tag recombinant leptin protein from the surrounding medium. However, the protein of interest was normalized to an unreliable β -actin loading control, which distorted results. Results can be improved by ensuring that total protein concentration is equal for all samples. Although western blot results were inconclusive, the soaking method is still being developed and should continue to be explored as a non-invasive, high-throughput alternative to microinjections in future research.

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