8-13-2013

The Effects of Topical Dose Delivery of Corticosterone on the Development and Hatching Success of the Zebra Finch

Ethan Dyer
Georgia State University

THE EFFECTS OF TOPICAL DOSE DELIVERY OF CORTICOSTERONE ON THE DEVELOPMENT AND HATCHING SUCCESS OF THE ZEBRA FINCH

An Honors Thesis

Submitted in Partial Fulfillment of the

Requirements for Graduation with

Undergraduate Research Honors

Georgia State University

2013

by

Ethan Dyer

Committee:

Dr. Laura L. Carruth, Honors Thesis Director

Dr. Sarah Cook, Honors College Associate Dean

Date

THE EFFECTS OF TOPICAL DOSE DELIVERY OF CORTICOSTERONE ON THE DEVELOPMENT AND HATCHING SUCCESS OF THE ZEBRA FINCH

by

ETHAN DYER

Under the Direction of Laura L. Carruth, Ph.D

ABSTRACT

The Australian Zebra Finch (*Taeniopygia guttata*) is an important animal model for vertebrate development and behavior. New research initiatives in the fields of epigenetics rely heavily on injecting hormones and environmental toxins directly into the eggs of different bird species such as zebra finches and other passerine songbirds to replicate the effects maternal condition on offspring. However, the widely used method of egg-injections does not accurately replicate physiological conditions, as the injected substances remain concentrated at the injection site for extended periods and do not diffuse into the developing tissues. Therefore, we propose an alternative method to injection protocols that takes advantage of the porous nature of eggs. Corticosterone (CORT), a major vertebrate stress hormone, dissolved in ethyl alcohol was applied to the surface of zebra finch eggs daily. The effect of this treatment on decreasing hatching success shows that topical hormonal treatments are a viable alternative to egg injection.

INDEX WORDS: Zebra Finch, Epigenetics, Topical Egg Treatment, Corticsoterone, CORT, Hatching success

THE EFFECTS OF TOPICAL DOSE DELIVERY OF CORTICOSTERONE ON THE DEVELOPMENT AND HATCHING SUCCESS OF THE ZEBRA FINCH

by

ETHAN DYER

An Honors Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Bachelors of Biological Sciences

in the College of Arts and Sciences

Georgia State University

2013

THE EFFECTS OF TOPICAL DOSE DELIVERY OF CORTICOSTERONE ON THE DEVELOPMENT AND HATCHING SUCCESS OF THE ZEBRA FINCH

by

ETHAN DYER

Honors Thesis Director: Laura L. Carruth, Ph.D
Honors College Associate Dean: Dr. Sarah Cook

Electronic Version Approved:

GSU Honors College
Georgia State University
August 2013

DEDICATION

I dedicate this paper to my senior cousin, Vonette Anglin, whose backyard dissection of a *Periplaneta Americana* kindled an interest in biological sciences within me at the tender age of 10.

ACKNOWLEDGEMENTS

I, first and foremost, would like to thank Dr. Carruth for her continued guidance and support, without which none of this would be possible. It truly a pleasure to work under the direction of such a professor, one who continually goes beyond her responsibility to ensure the success of dedicated students.

I also would like to acknowledge fellow researcher Bao Chau Ly who assisted me, without compensation, for many hours with the collection and organization of data. I wish to thank all members of the Carruth Lab that has made me feel like a welcome member of the group.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The Australian Zebra Finch (*Taeniopygia guttata*) is a popular model species in research and has been used to study a variety of topics that range from, the development of the brain and behavioral sexual dimorphisms (Duncan et al., 2011) to spatial memory and learning (Watanabe, 2004). The popularity of this species for study is due, largely in part, to their ability to thrive in captivity as opportunistic breeders. Additionally, zebra finches have behavioral parallels and neuroendocrine systems homologous to mammals, which can be experimentally manipulated (Banerjee, 2011; Shahbazi, 2011). Therefore, they are a powerful model for understanding neurological processes, such as the effect of stress on hypothalamic-pituitary-adrenal-axis (HPA). The field of epigenetics (defined as changes in cellular phenotype or gene that does not result from changes in DNA sequence) has expanded in recent years and the importance of the zebra finch as a model has increased considerably.

The recent surge of interest in the field of epigenetics has placed greater emphasis on manipulating conditions during embryological development of egg laying vertebrates. Birds of the order Passeriformes ("perching birds") have become central model organisms in pioneering epigenetic research studies. Treating female European starlings (*Sturnus vulgaris*) with the adrenal stress hormone corticosterone (CORT) implants causes a propensity towards female-biased reproductive investment (Love et al., 2005). This demonstration of the theory of sex-allocation showed that hormonal changes, such as stress-induced surges in maternal plasma CORT levels, may be inextricably linked to alterations of the phenotype of progeny. Eggs are advantageous because the chick develops in a stable external environment in which offspring's phenotype is determined both by genetics and maternal condition at egg formation. Furthermore, eggs studies allows researchers to manipulate developmental conditions independent of the

mother in order to elucidate mechanisms by which maternal or environmental conditions may affect progeny. Because much of zebra finch development, physiology and behavior are well understood the species has become a model organism for this type of epigenetic study. In order to manipulate the hormonal environment in which embryos develop eggs are either injected with or have hormone applied topically.

Egg injection protocols, which are quickly becoming a standard for egg manipulation studies, may be limited in their ability to mimic physiological conditions. CORT injections have been used to demonstrate the effects of prenatal stress on offspring. In European Starlings *in ovo* CORT injections led to a decrease in mass of male birds at hatching and caused reduced HPA-axis responses in both sexes (Love and Williams, 2008). Further studies have shown that testosterone exposure *in ovo*, of Japanese Quail (*Coturnix japonica*) eggs via a similar injection protocol affected male affiliation preferences and caused acoustic changes in crowing in males, while boosting growth in females (Schweitzer et al., 2013). The egg-injection method has also been adapted for use in toxicology studies. Toxins, such as environmental contaminants, dissolved in a DMSO carrier may be injected into zebra finch eggs to assay their epigenetic and carcinogenic effects of such chemicals (Winter et al., 2013). Research aimed at assessing the effects of maternal condition generally conducted hormone injections within-24 hours of an egg being lain and prior to parental incubation (e.g., Love and Williams, 2008; Hayward et al., 2006). However, the hypothesis that hormone injections correctly emulate physiological changes caused by maternal condition may be innately flawed.

Uneven distribution of treatment target agents and vehicle-embryo interactions after the egg injection procedure raises concerns as to the validity of this approach. In studies involving hormone treatment an oil vehicle such as sesame seed oil (Love et al., 2005) or refined corn oil

(Schweitzer et al., 2013) are generally used as a solvent (since steroid hormones are cholesterol derivatives and lipid-soluble), while in toxicology studies other organic solvents such as DMSO (Winter et al., 2013) are employed. However, solvent interactions may be toxic to a developing embryo. It was found that the embryo is most susceptible at the earliest stages of development (Heinz et al., 2006). Therefore, further investigation should be conducted to determine if results of many of these pre-incubation injection studies may be skewed by the solvent-embryo interactions. Another concern is that when CORT and the adrenal and gonadal androgen testosterone (both steroid hormones) are dissolved or suspended in oil and injected near the yolk of chicken (*Gallus domesticus*) eggs the hormone remains concentrated at the injection site beyond six days of incubation and therefore exposes the developing chick to pharmacological levels of the hormone (von Engelhardt et al., 2009) during critical stages of development. These concerns warrant investigating alternative methods for hormone treatment for *in ovo* developmental studies.

Topical treatment protocols are a viable alternative to egg-injection protocol as it facilitates gradual infusion of chemical compounds by exploiting the porous nature of egg shells. This technique has been applied to reptilian models to evaluate the effects of sex steroid hormones such as estradiol on development (e.g., Crews et al., 1991) and many toxicology studies employ this strategy to assess the impact of toxins (e.g. Muller, et al., 2007; Gale, et al., 2002). A potential limitation of topical treatment is that studies show low and wide ranged rates of absorption of experimental agents into the egg. Additionally, these rates have been shown to be both species and dose specific. This may be due to differences in pore density and permeability across species. Therefore, before for this technique can be used to assess the effects of physiological levels, it must first be shown that topical treatments of the target chemical agent,

such as a hormone or teratogen, can indeed cause physiological or developmental changes due to topical application.

In this experiment a CORT-ethanol suspension was applied to zebra finch eggs to determine if that development was affected by treatment. A vehicle control was used to validate that the solvent interactions with the developing embryo did not contribute towards observed changes.

METHODS

Animal husbandry and breeding:

Zebra finches were housed in two separate flight cages dimensions (60 3/8" W/30 3/8" D/78 ¼"H) labeled Flight Cage One (FC1) and Flight Cage Two (FC2). There were 11 nest boxes in FC1 and 12 nest boxes in FC2. Birds were kept under controlled environmental conditions (temperature 21.1–23.8 °C, humidity 50–65%, constant light schedule of 12L: 12D, lights on at 08:00) at the Georgia State University animal care facility in the Petit Science Center. All finches received a mixed seed (Fort-Diet Pro Health Finch, Kaytee Products, Wisconsin) diet, water, grit and cuttlefish bone (calcium) *ad libitum*. All animal procedures were approved by the Georgia State University Institutional Use and Animal Care Committee. Nest boxes within one FC1 were randomly assigned either as untreated or sham treated and all FC2 nest boxes were designated for hormone treatment. All nest boxes were checked daily between 8.00 am and 1.00 pm and new eggs marked systematically. All eggs were weighed daily to obtain data on weight changes, clutch size and laying intervals.

Hormone application:

Six nest boxes in FC1 were designated for sham treatment; eggs laid in these nest boxes (n=37) received daily applications of approximately 0.1 ml of 70% ethyl alcohol vehicle using cotton tipped swab within 24 hours of laying (day 0) and up to hatching. Two of the remaining nest boxes in FC1 (n=22) remained untreated. Eight nest boxes in FC2 (n=19) were treated with daily applications of approximately 0.1 ml of a 40 µg/ml CORT dissolved in 70% ethyl alcohol. Care was taken to apply treatment to less than 20% of the egg surface in order to mitigate the obstruction of gas exchange through shell pores.

Assessment of development:

All eggs were weighed, treated and candled daily. Candling involved holding eggs held 10-20 cm away from a cool bright lamp (Steris Amsco Examiner 10, 34,500 lux) and oriented such that the developing embryo and surrounding vascular structure would be most visible. Anatomical terminology and morphological changes used to track and describe developmental progress correlated with a zebra finch embryological staging atlas (Murray, et al., 2013). Digital images were captured during candling of eggs (Canon EOS Rebel XT 350D) and annotated with Photoshop to represent various stages of development.

Statistical analysis:

Eggs weighing less than 0.60 g or those that showed no development after 7 days after being laid were assumed to be infertile and not used in statistical analysis. Also, eggs damaged by natural means, either accidentally or by the parents during incubation or by handling process, and those which went missing were excluded.

The final stages of development reached by each embryo were categorized based on visual observations of the intact egg. Termination of development was categorized into early stage embryo death (ES), mid stage embryo death (MS), late stage embryo (LS) and hatching success (HS). ES began at the first visible sign of vascularization (generally a faint red streak or ring within the yolk) and lasted until the yolk become stationary and heartbeat was clearly visible. Then MS began and lasted until the interior of the egg was a deep red and the embryo became difficult to observe. LS lasted from then until hatching. ES, MS and LS generally corresponded with expected progress after 2-3, 4-8 and 9- 14 days respectively according to

literature (Winter et al., 2013). Because incubation and hatching synchrony often varies (Gilby, 2013), the transitions between each stage were estimated based on observed developmental changes rather than fixed time intervals.

All statistical analyses were conducted using the statistical software R (Version 3.01, 2013). Due to the small sample size Fischer's exact test (FET) was used to compare both eggs treated with ethyl alcohol and those treated with CORT dissolved in ethyl alcohol to untreated birds.

RESULTS

Ethyl alcohol control vs. untreated:

There was no significant difference in the any of the parameters analyzed in this experiment between eggs treated with ethyl alcohol alone (VC) and those eggs which were untreated (NT). There was no difference in the hatching success (HS) or eggs treated with ethyl alcohol to untreated birds ($p > 0.74$; FET). The rates of late stage embryo death (LS) for the untreated eggs and controls were comparable ($p > 0.06$; FET). When comparing rates of early stage embryo death (ES) and mid stage embryo death (MS) control and untreated egg were the same ($p > 0.99$; FET).

Cort vs. untreated:

CORT treated eggs had a lower rates of hatching success and higher rates of mid stage abortion when compared to NT eggs. HS of CORT treated birds was significantly lower than untreated birds ($p<0.02$; FET). CORT treated eggs had a significantly higher probability of MS than NT eggs ($p>0.00$; FET).

Table 1. Hatching success (HS), late stage embryo death (LS), mid stage embryo death (MS), early stage embryo death (ES) for no treatment controls (NT), vehicle only control (VO) and CORT treated (CT) eggs.

Treatment	HS	LS	MS	ES
NT	0.562	0.375	0	0.062
VO	0.437	0.156	0.031	0.062
CT	0.105	0.315	0.421	0.158

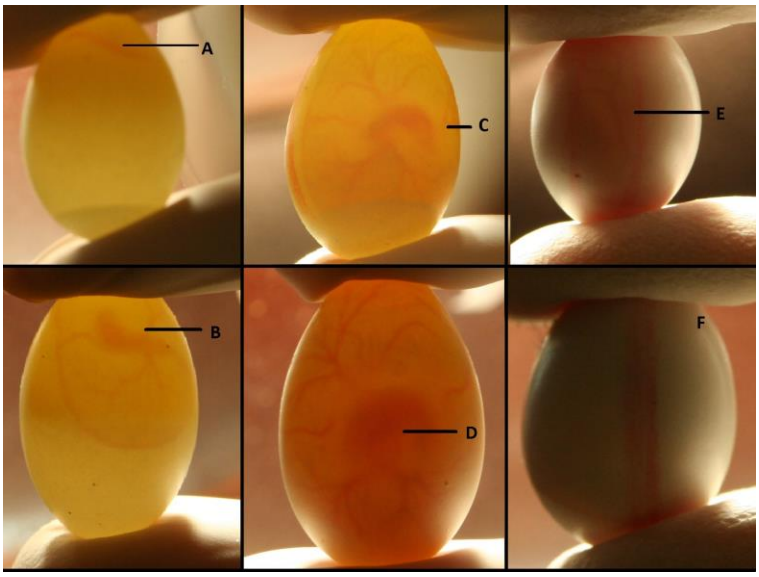

Figure 1. Development stages of sample eggs to illustrate various stages of development. Eggs labeled A and B are in the early stages of development. C and D are at mid stages of development and E and F are at late stage embryos.

DISCUSSION

The results strongly support the hypothesis that topical treatment of CORT on zebra finch eggs can impact development and hatching success. The findings suggest that daily treatment of CORT caused a significant decrease in hatching success. For this validation study we were not concerned with maintaining CORT levels at physiological levels. However, there is some evidence linking levels of maternal CORT to decreased hatching success in other avian species such as the Common Eider (*Somateria mollissima*), a large seabird, in which the effect of baseline levels of CORT was correlated with lower hatching success in birds that were nesting in exposed sites (D'Alba et al., 2011).

The porosity of eggs allow for the gradual diffusion of chemicals across a larger surface area than egg injection protocols. Eggs from the same clutch have similar eggshell thickness and pore density in birds such as the Magellanic Penguin (*Spheniscus magellanicus*) (Boersma, 2009). If this holds true for zebra finches then eggs within the clutch would have the same diffusion rate of topically applied chemicals into the egg, which would allow for direct comparisons to be made between eggs treated with varying concentrations.

Although increasing maternal stress levels would achieve physiological distributions of CORT in yolk, limitations in application necessitate a reliable alternative. Passerines, such as the Great Tit (*Parus major*) have been shown to increase CORT in response to acute stress which was passed on to the eggs laid the following day (Pitk, et al., 2012). This technique makes it possible to vary eggs with elevated CORT levels and controls within a clutch. However, the study also indicated CORT deposition varied and with differences in behavioral responses by mothers. Therefore without sacrificing the egg or hatchling it is impossible to determine how much hormone was deposited into each egg. Also the technique of inducing a physiological

changes to alter maternal hormone levels may be more difficult with other hormones such as the androgen Testosterone. Another method used is to alter maternal circulating hormone levels by implantation. However, this method is also not precise; one study found that manipulating circulating CORT levels of barn owls (*Tyto alba*) resulted in significant variations in CORT levels of eggs which peaked at day 3 of treatment (Almasi, et al., 2012). The unpredictability of these hormone treatments limits the experimenter from being able to precisely manipulate conditions for the developing embryo and makes it difficult to quantitatively compare the results of varying concentrations of hormones elevations.

Although our experimental results show that CORT treatment of eggs correlated with a significant decrease hatching success and an increase in mid stage death further analysis may be necessary to gain a better understanding of the cause of these results. First, assaying hormone distribution within the egg at various stages of development would help verify that pharmacological exposure resulted to CORT caused embryo failure and also could be used to determine rates of absorption of applied hormone by the egg. Second, gaining an accurate value of egg weight loss of CORT treated birds and controls could give more insight. Experiments shows that, in the case of Japanese quails, embryos that die prematurely show excessive weight loss compared to hatch eggs independent of shell thickness and porosity and suggests that some other functional component of the egg may be the cause (Soliman et al., 1994). Based on that conclusion it would be interesting to determine if CORT treated eggs experience, on average, greater weight loss than control. When analyzed egg weight loss showed no pattern and had a wide range. Upon close inspection of the shells exterior we found that because the eggs would often gain debris such as fragments of feathers and excrement which may have skewed weight results.

CONCLUSIONS

The Topical application model of hormone treatments has strong potential to be more physiologically relevant than inject protocols while being more precise and having broader applications than manipulating maternal conditions directly. Injected chemicals remain concentrated at the point of injection adding scrutiny to the physiological relevance of results. Further research is necessary to determine what dosages and timing necessary to expose the developing embryo to physiological treatment. However, this experiment shows definitively that CORT was able to affect embryological development.

FUTURE DIRECTIONS

It is important that the experiments describing the hormonal processes that occur during embryonic development be validated in order to determine if they accurately simulate physiological conditions. The results and conclusions of this preliminary research will support our understanding of the role and mechanisms maternal condition plays in health and development of offspring. There are several important "next steps" in research that will expand upon these findings. It will be important to determine the actual absorption rates of topical treatments of the CORT-ethyl alcohol preparations in the eggs. This can be determined by conducting porosity eggshell experiments. In addition, comparing egg weight loss as a result of different CORT concentrations that alter embryo development may provide a better understanding of the cause and timing for drastically high proportions of mid stage embryo death found in this experiment.

Other future studies include determining and administering a topical hormone dose at which known concentrations of hormone across the albumen, yolk layers and enter the developing embryo. We know how much we applied to the eggs but not how much hormone accumulated in the yolk and embryonic tissues. We have collected yolk and will be using radioimmunoassay for CORT to measure the amount of hormone in the yolk. This will allow for future comparisons hormone treated eggs to eggs in which maternal incubations conditions were directly manipulated. Finally, topical treatment doses should be compared to traditional injection methods and evaluated by additional research and review of past literature to resolve the issue of which technique would be most efficacious to epigenetic studies moving forward.

REFERENCES

Almasi, B., Rettenbacher, S., Muller, M., Sandra, B., Wanger, H., Lukas, J., 2012. Maternal cortecosterone is transferred into the egg yolk. Gen. Comp. Endocrinol. 178 139-144.

Banerjee, S.B., Adkins-Regan, E., 2011. Effect of isolation and conspecific presence in a novel environment on corticosterone concentrations in a social avian species, the zebra finch (*Taeniopygia guttata*). Horm Behav. 60, 233-238.

Boersma, P.D., Rebstock, G.A., 2009. Magellanic Penguin eggshell pores: does number matter? Environment Comlete. 151, 535-540.

Crews, D., Bull, J.J., Wibbels, T., 1991. Estrogen and sex reversal in turtles: A dose-dependent phenomenon. Gen. Comp. Endocrinol. 81:357–364.

D'Alba, L., Spencer, K.A., Nager, R.G., Monaghan, P., 2011. State dependent effects of elevated hormone: Nest site quality, corticosterone levels and reproductive performance in the common eider. Gen. Comp. Endocrinol. 172:218-224.

Duncan, K.A., Jimenez, P., Carruth, L.L., 2011. Distribution and sexually dimorphic expression of steroid receptor coactivator-1 (SRC-1) in the zebra finch brain.
Gen. and Comp. Endocrinol. 170, 408-414.

Gale, R.W., Bergeron, J.M., Willingham, E.J., Crews, D., 2002. Turtle sex determination assay: Mass balance and responses to 2,3,7,8- tetrachlorodibenzo-p-dioxin and 3,3',4,4',5-pentachlorobiphenyl. Environ. Toxicol. Chem. 21:2477–2482.

Gilby, A. J., Mainwaring, M. C., Griffith, S. C., 2013. Incubation behaviour and hatching synchrony differ in wild and captive populations of the zebra finch. Anim. Behav. 85, 1329-1334.

Hayward, L.S., Richardson, J.B., Grogan,M.N.,Wingfield, J.C., 2006. Sex differences in the organizational effects of corticosterone in the egg yolk of quail. Gen. Comp. Endocrinol. 146, 144–148.

Muller J.K., Gross, T.S., Borgert, C.J., 2007. Dose verification following topical treatment of alligator eggs. Environ. Toxicol. Chem. 26:908–913.

Murray, J.R., Varian-Ramos, W.C., Welch, Z.S., Saha, M.S., 2013. Embryological staging of the Zebra Finch, *Taeniopygia guttata*. J. Morphol doi: 10.1002/jmor.20165. [Epub ahead of print]

Love, O.P., Chin, E.H.,Wynne-Edwards, K.E.,Williams, T.D., 2005. Stress hormones: A link between maternal condition and sex-biased reproductive investment. Am. Nat. 166, 751–766.

Love, O.P., Williams, T.D., 2008. Plasticity in the adrenocortical response of a free living vertebrate: The role of pre- and post-natal developmental stress. Horm. Behav. 54, 496–505.

Pitk, M., Tilgar, V., Kilgas, P., Mand, R., 2012. Acute stress affects the corticosterone level in bird eggs: A case study with great tits (*Parus major*). Horm. Behav. 62, 475-479.

Schweitzer, C., Goldstein, M.H., Place, N.J., Adkins-Regan, E., 2013. Long-lasting and sex-specific consequences of elevated egg yolk testosterone for social behavior in Japanese qual. Horm. Behav. 63, 80-87.

Shahbazi, M., Schmidt, M., Carruth, L.L., 2011. Distribution and subcellular localization of glucocorticoid receptor-immunoreactive neurons in the developing and adult male zebra finch. Gen. Comp. Endocrinol. 174, 354-361.

Soliman, F.N., Rizke, R.E., Brake, J., 1994. Relationship between shell porosity, shell thickness egg weight loss, and embryonic development in Japanese quail eggs. Poult. Sci. 73, 1607-1611.

von Engelhardt, N., Henriksen, R., Groothuis, T.G.G., 2009. Steroids in chicken egg yolk: Metabolism and uptake during early embryonic development. Gen. Comp. Endocrinol. 163, 175–183.

Watanabe, S., Bischof, H., 2004. Effects of hippocampal lesions on acquisition and retention of spatial learning in zebra finches. Behave. Brain Res. 155, 147-152.

Winter, V., Elliott, J.E., Letcher, R.J, Williams, T.D., 2013. Validation of an egg-injection method for embryotoxicity studies in a small, model songbird, the zebra finch (*Taeniopygia guttata*). Chemosphere. 90, 125-131.

APPENDICES

SAMPLES OF EMBRYO DEVELOPMENT

Over 1000 pictures were systematically taken of the developing eggs for this experiment. This sample of a series of early stage death, mid stage death and late stage death and day 1 hatchlings. Colored pencil markings were used systematically label each egg.

Figure 2. Series of early stage death of a zebra finch embryo from day 1 to day 5 of incubation.

Figure 3. Series of mid stage death of a zebra finch embryo day 2-6 of incubation

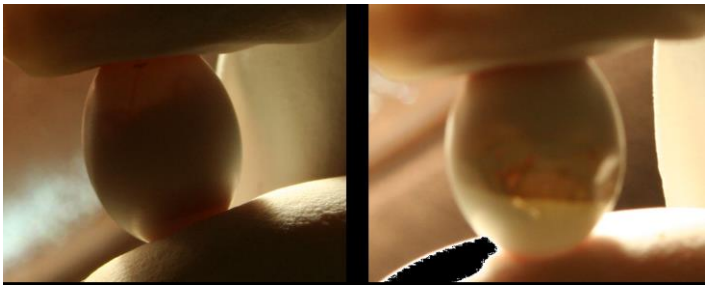

Figure 4. Before and after late stage cell death

Figure 5. Day 1 hatchling

SAMPLE DATA COLLECTION SHEET

FCS- Cort Date: 4/6

Time: 8 AM

Special note:

	Nest Box H/8	Date:4/6/2013	Time: 0810
	Egg	**Weight**	**Comments:**
	O 12	1.01g	Dark, intact vascular structure; embryo hidden
	U 11	1.13g	Small suspended mass; no vascular development;
	XR 9	0.36g	Suspended brown mass; crack in egg
*	XG 7		
	XP 7	1.17g	Intact vascular structure; embryo visualized; moving

	Nest Box I/9	Date:4/6/2013	Time:0820
	Egg	**Weight**	**Comments:**
	G14	0.39g	Hole in egg
	P 13	0.83g	Intact vascular structure; embryo hidden; red inside
	O 12	0.89g	Some vascular structure intact; some vascular structure aggregated; unable to distinguish mass
	U 11	0.93g	Vascular structure intact; red egg, moving mass

	Nest Box J/10	Date:4/6/2013	Time:0835
	Egg	**Weight**	**Comments:**
	R 15	0.76g	Yellow-brown inside; brown embryo; vascular structure aggregated
	O 12	0.74g	No development
	U 11	0.39g	Egg cracked on handling; no vascular structure; yellow-brown embryo; no movement
	W 10	1.00g	Questionable mass; no vascular structure
*	XR 8		Broken and discarded
*	XG 7	0.40g	Fractured egg; broken and discarded

	Nest Box K/11	Date:4/6/2013	Time:0845
	Egg	**Weight**	**Comments:**
	R 15	1.14g	Deep red; intact vascular structure; embryo hidden
	G 14	0.87g	Intact vascular structure; red inside egg; embryo hidden
	O 12	0.93g	No distinct development
*	U 11		
	W 10	0.95g	Small brown embryo; no vascular structure
	XR 9	1.00g	Intact vascular structure; moving embryo;
	XG 8	0.90g	Some intact vascular structure; embryo hidden