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Cx43 Expression Increases in Response to Increased Temperature Incubation in the Developing Chicken Embryonic Brain

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CX43 EXPRESSION INCREASES IN RESPONSE TO INCREASED TEMPERATURE INCUBATION IN THE DEVELOPING CHICK EMBRYONIC BRAIN

A thesis submitted in partial fulfillment
Of the requirement for the degree of
Master of Science

By

Erick A. Barrios
B.S., Boston College, 2015

2017
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

DATE OF DEFENSE

7/11/2017

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Erick A Barrios ENTITLED Cx43 Expression Increases in Response to Increased Temperature Incubation in the Developing Chicken Embryonic Brain BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Barrios, Erick A. M.S. Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2017. Cx43 Expression Increases in Response to Increased Temperature Incubation in the Developing Chicken Embryonic Brain.

We used the chicken embryo model to examine whether altered incubation temperature during chicken embryo development would alter the protein expression of heat shock protein 70 (hsp70), hsp90, and connexin 43 (cx43) in the brain. We incubated eggs in either optimal (37.6°C, Con), increased (39.6°C, +2), or decreased (35.6°C, -2) temperature conditions until they reached embryonic day 16 (E16). The -2 condition significantly increased incidence of embryonic mortality. As shown in the literature, the development of the eggs was accelerated or decelerated due to increased or decreased temperature respectively. To control for this, we incubated eggs at optimal temperature (37.6°C) to the developmental stage of the +2 (E17.5, HH43-44) and -2 (E12, HH38) conditions. Embryonic chicken brain protein was quantified for hsp70, hsp90, and cx43 using Western Blot analysis. We found that the +2 condition significantly increased cx43 and hsp70 protein expression, but had no effect on hsp90 protein expression.

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LIST OF ABBREVIATIONS

cx43 = connexin 43

hsp70 = heat shock protein 70

hsp90 = heat shock protein 90

HH Stages = Hamburger Hamilton Stages

HH# = Hamburger Hamilton Stage #

E# = Embryonic day #

BCA = Bicinchoninic acid

ECL = Enhanced Chemiluminescent (substrate)

SDS = sodium dodecyl sulfate

PBS = Phosphate Buffered Solution

PBST = Phosphate Buffered Solution with Tween 20

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DEDICATION

I would like to thank my parents for providing me with the opportunity to attend this program and pursue this degree and my aspirations as a scientist. I would also like to thank my brother and my friends for mitigating the stress that is ever present on the journey of life.

I. INTRODUCTION

The chicken embryonic model is a well-defined model of development, so much so that the use of chicken eggs to understand general embryonic development was implemented by the Greek philosopher Aristotle himself around 300 BC. (Cardwell, 1905, Hamburger & Hamilton, 1992). There are many benefits to using this model. For example, in the chicken embryo model the embryo is separate from the mother. Although the embryos are covered with a hard shell, techniques have been developed to allow for embryo manipulation using injection, windowing (i.e. opening an egg and adding a see-through covering) (Korn & Cramer, 2007), and environmental manipulation via temperature or humidity. Chicken embryos also develop rapidly with little required maintenance - hatching in approximately 21 days (Hamburger & Hamilton 1992, Bellairs & Osmond, 2014). However, no animal model is perfect. Although there are some promising studies for genetically engineering chicken (Lee et. al, 2015), a transgenic chicken model has not yet been developed. Additionally, there are fewer antibodies specifically made for chicken compared to the rat and mouse models. Overall, the experiments in this thesis examine the effect(s) of altered incubation temperature on development and, more importantly, protein expression of our three proteins of interest. Therefore, the chicken embryonic model is optimal for our experiment because it allows for easy and constant manipulation of incubation temperature.

In this thesis, we focused on embryonic days 12 (HH38), 16 (HH42), and 17.5 (HH43-44). It should be noted that the reasoning for including embryonic days 12 and

17.5 in the thesis is incidental and will be explained in the results section of the thesis. We intentionally selected embryonic day 16 (E16) as the day of interest for our chicken embryos for several reasons. The most important one being that around E9~E10 (HH35-36) the brain has finished developing all of its functional anatomical regions. After this timepoint, the chicken embryonic brain continues to develop in size throughout incubation and makes new connections but the morphological regions are already intact (Bellaris & Osmond, 2014, p. 91-101). Complex morphological brain development can alter our proteins of interest, and this had the potential to convolute the data analysis; therefore, we chose a developmental time-point that would exclude this variable. Because alterations in egg incubation temperatures can modify developmental staging and we wanted to specifically examine embryonic staging, we chose an embryonic day well before E19~E21 (HH45-46) (timeframes when the chick could hatch) to exclude this variable. For these reasons, we chose to grow the chicken embryos to day E16.

In the chicken development field, "Hamburger Hamilton (HH) Stages" are often used to characterize chicken development. These defined HH Stages refer to specific observable morphological characteristics. In early stages (HH1-39, E1-E13) the changes are more rapid and therefore span hours not days. In this thesis we often refer to the embryonic day of the embryo as that is the timepoint that we grew them to and is therefore necessarily descriptive of this thesis' method. Past HH38 (E12) the formation of the embryonic chicken eyelid and the development of feather germs is normally indistinct

leaving only the beak and third toe length as indicators of HH stage past that point (Bellaris & Osmond, 2014 p. 603-608, Hamburger & Hamilton, 1951).

Chicken development can be altered using temperature (Noiva et al, 2014, Merkow & Leighton, 1966). In order to study temperature-related developmental changes in the chicken, we chose to focus our studies on the heat shock protein (hsp) family. This family consists of proteins that were initially discovered due to changes in temperature, hence "heat shock" (Ritossa, 1962) but have since been shown to protect against a variety of cellular stresses (Kumar et. al, 2016, Tamura et al, 2012, Franklin, 2005). Hsp70 family proteins have also been shown to be generally cytoprotective, which is why overexpression of hsp70 is present in many different cancers (Kumar et al, 2016). Hsp70 functions primarily to fold proteins into functional forms in an ATP dependent manner (Young 2010). In addition, hsp70 acts as a chaperone, shuttling proteins to different areas of the cell (Voos, 2003). From a signaling standpoint, hsp70 acts as a key inhibitor of apoptotic machinery, further explaining its cytoprotective properties (Garrido et al, 2006), directly interacts with connexin-43 (cx43) (Hatakeyama et al, 2013), and has regulatory effects on the G1/S transition indicating a role in cellular proliferation (Hino et. al, 2015). Therefore, hsp70 has a range of important functions in the cell which may be altered by temperature.

Similarly, hsp90 is also known to be modulated by heat-shock and act as a protein-folding chaperone (Schopf et al, 2017). Hsp90 has been well studied as a mediator of morphological development in response to changes in temperature (Rohner et

al, 2013, Rutherford & Lindquist 1998). However, its signaling within a cell is thought to require binding to various proteins, including known proto-oncogenes such as Src and Raf (Pratt, 1997). Hsp90 also directly binds and acts as a chaperone protein for cx43 – regulating its cellular localization. Specifically, hsp90 is part of the TOM/TIM complex which determines whether proteins (specifically cx43) can be inserted into the inner mitochondrial membrane (Rodriguez-Sinovas et al, 2006) where cx43 plays a cytoprotective role (Boengler et al, 2013, Rodriguez-Sinovas et al, 2006). This process involves both hsp70 and hsp90 forming a complex with cx43 and several TOM proteins (Rodriguez-Sinovas et al, 2006). The concept that hsp70, hsp90, and cx43 could affect development via energy production through mitochondrial respiration is novel, beyond the scope of this thesis, and as yet untested. While we are interested in that, the current thesis focuses on whether cx43 is affected by temperature. Depending on the findings, this may serve as a gateway into further experimentation on whether cx43 affects mitochondrial respiration and energy production.

Connexins are a family of ubiquitous proteins that can form hemichannels and gap junctions, allowing adjacent cells to communicate quickly and directly (Kumar & Gilula 1996, Bennett & Zukin 2004). One connexin family member, cx43, has been proven to play a multifaceted role in cell growth, cellular mitochondrial metabolism, and cytoprotection via involvement in apoptotic pathways (Contreras et. al, 2002, Ruiz-Meana et. al, 2008, Trudeau, Muto, Roy 2012, Yin et al, 2012). More specifically, inhibition of mitochondrial cx43 has been shown to cause increased calcium currents and

cytochrome c out of mitochondria, both of which can lead to apoptosis (Goubaeva et. al, 2007). Furthermore, from a tissue perspective, loss of cx43 in astrocytes has been shown to increase the infarct size in rat brain post stroke (Nakase et al, 2004). Therefore, cx43 plays a role in cellular proliferation and cell death decisions.

In this thesis, we examined the role of temperature in the expression of hsp70, hsp90, and cx43 protein in chick embryonic development. As previously stated, heat shock proteins are known to react to changes in temperature (Ritossa, 1962). Therefore, we expected to see these changes in this study and hypothesized that temperature would also alter proteins associated with them, including cx43. We also hypothesize that cx43 protein expression will change after temperature alteration because increases in temperature outside homeostatic range are known to stress the mitochondria (Abele et al, 2002) and cx43 plays a role in protecting the mitochondria against such stressors (Goubaeva et. al, 2007, Boengler, 2013). Therefore, changing temperature should stress the cells such that cx43, hsp70, and hsp90 expression should change in order to mitigate that stress. In this experiment, we examined at cx43, hsp70, hsp90 protein expression in response to temperature in the chicken embryo model system by increasing and decreasing the temperature from its normal temperature of 37.6°C. Since changes in embryonic development have been observed by variation of 1°C (Novia et al, 2014), we chose to vary incubation by 2°C such that we observe any minor changes in protein expression via western blot.

Rationale

Temperature can be a stressor that alters cellular hsp70 and hsp90 expression. Because hsp70 and hsp90 are known to interact with cx43, we would like to test whether cx43 may also be affected in response to a change in temperature (directly or indirectly – via hsp).

Hypothesis

Cx43 protein expression will be elevated in response to increased incubation temperature and lowered in response to decreased incubation temperature.

II. MATERIALS AND METHODS

Chicken egg incubation and conditions

SPF fertilized Leghorn chicken eggs were obtained from Charles River Labs at E1 at approximately HH5-6**.** Fertilized chicken eggs were incubated for 16 days at one of three temperatures: optimal temperature (37.6°C, "Con") (Osmond & Bellairs, 2014, p.7); at a decrease from optimal temperature by 2 degrees Celsius (35.6°C, "-2"); or at an increase from optimal temperature 2 degrees Celsius (39.6°C, "+2"). Humidity was kept constant at 53%. Constant humidity ensures that the eggs don't become dehydrated; however, there doesn't appear to be a standard humidity noted in the literature (Osmond & Bellairs, 2014)**.** Self-turning Octagon 40 Cradle incubators from Brinsea were used to ensure proper turning as well as monitoring temperature and humidity. Fertilized eggs were incubated at 20°C for either one day ("-2" condition) or two days ("Con" condition) before being brought to optimal temperature in order to delay egg growth for each experiment. At 20°C, fertilized embryos can remain viable without undergoing development. Eggs were considered E1 once they had been incubated at temperatures above 30°C. The three temperature conditions had 24 eggs each. In addition, in the development experiment, all fertilized eggs were incubated at optimal temperature (37.6°C) for either 12 (E12), or 17.5 (E17.5) days and had 20 and 12 eggs respectively. The reason they did not have 24 eggs each as well was due to an incubator breaking

down and compromising the eggs. The actual n-values of the experiments vary due to death in vivo, thus changing the amount of testable embryos in a given experiment.

Chicken embryo dissection and tissue storage

The chicken eggs were weighed; however, we found no significant differences in total egg weight across conditions (data not shown). Each chicken embryo was removed from its shell, placed on a clear glass dissection plate, and photographed beside a ruler before being euthanized via swift cervical dislocation and decapitation. Whole brain was dissected, flash frozen liquid nitrogen, and the tissue stored in cryo-vials at -80°C.

Chick Brain homogenization and lysis

Whole chicken brains were homogenized in lysis buffer containing phosphatase inhibitors in order to inhibit any activation of proteases that may degrade proteins of interest post-homogenization. During lysis, all samples were kept on ice to prevent any protein degradation due to heat. Once homogenized, lysates were spun down at 12,000 rcf in a micro-centrifuge at 4°C. The supernatant containing the protein was then removed from the Eppendorf tube containing the lysate and placed into another tube and stored at -80°C. The remaining pellet was also stored at -80°C for future analysis. All heat-sensitive materials (lysates, protein ladders, antibodies, samples, etc.) were kept on ice when not noted otherwise.

BCA assay

Lysates underwent a bicinchoninic acid (BCA) assay to determine protein concentration. We used a BCA assay kit from Thermo Fisher Scientific (Thermo Fisher Scientific, Catalogue#: 23225). Standards were made using known concentrations of bovine serum albumin. Each lysate was diluted in a 1:30 dilution (in lysis buffer) in order to stay within the curve. Diluted samples (10μl) and standards were plated on a 96 well plate in duplicate. 190μL of BCA solution was added and the plate was placed on a shaking platform for 30 seconds. It was then incubated at 37.6°C for half an hour without shaking. After incubation, the protein concentrations were read on a microplate reader using a light wavelength of 562nm. Once absorption was read, the standard curve was determined, and protein concentrations calculated. The standard curve was always linear and had an r-squared correlational coefficient value greater than 0.99 for each assay (data not shown).

Antibodies

Our primary antibodies consisted of a, rabbit polyclonal anti-cx43 antibody (Abcam, ab11370) at a 1/8000 dilution, mouse monoclonal anti-hsp70 antibody (Abcam, ab2787) at a 1/1000 dilution, and a mouse monoclonal anti-hsp90 antibody (Abcam, ab13492) at a 1/500 dilution. Our secondary antibodies consisted of a HRP-conjugated horse anti-mouse IgG antibody (Cell Signaling Technology, #7076S) at a 1/10,000 dilution, and a HRP-conjugated goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000) at a

1/10,000 dilution. For our loading control, we used a HRP-linked mouse monoclonal anti-β-actin IgG_1 (Santa Cruz Biotechnology, sc-47778). All dilutions were in 5% milk prepared using PBST (0.1% Tween20) and commercially available dried milk

SDS-PAGE and Western Blot

In order to evaluate protein expression, we performed Western Blotting. Gels were made in-house and were comprised of 10% bis-acrylimde and were 1.5mm thick. The samples were diluted such that each well contained 20µg of protein. Each sample preparation contained sample (65% of the total solution, enough for 20µg of protein per well used), 99% pure (14.3M) beta-mercaptoethanol (BME, 10% of total solution) and 4X SDS (25% of total solution). After the gels were run, they underwent a wet transfer onto a nitrocellulose membrane. Ponceau stain was applied if there were any possible issues or irregularities. Membranes were then labeled (usually by cutting an edge in a certain, unique way) and then placed in 5% milk for 40 minutes. Blots were then incubated in the primary antibody of the protein of interest overnight at 4°C with rocking. The membranes were washed in PBST (.1% Tween20) for 3 washes each lasting 5 minutes. The complimentary HRP-conjugated secondary antibody was added and incubated for 1 hour at room temperature. The membranes were then washed 3 times for 10 minutes each in PBST before being incubated in ECL substrate for 5 minutes. At this point the ECL reacted with the HRP of the secondary antibody causing a chemiluminescent reaction.

These membranes were then exposed to X-ray films in a dark room. The X-ray films were then developed and recorded.

Analysis of Western Blot bands

The x-ray films containing the bands were scanned in a film scanner and then analyzed using ImageJ software provided by the National Institute of Health (NIH) (https://imagej.nih.gov/ij/). The image was inverted in order to measure intensity of the bands. A square was fit around the largest band, then moved over all the other bands to ensure that the area was kept consistent across the bands; thus ensuring accuracy of the intensity differences between bands. The intensities of the protein of interest bands were then divided by the intensity of the loading control (beta-actin) bands. Each band was then normalized to the control band; which is why the data presented is in fold-changes from the control.

Data analysis

Because there are multiple groups that were all compared to one another, fold changes and beak lengths were analyzed using an ANOVA with Tukey post-hoc analysis. Embryonic death was analyzed via a Fischer's exact test.

III. RESULTS

Morphological analysis

As noted in the methods, we exposed fertilized eggs to three temperature conditions and allowed them to gestate for 16 days. Temperature groups included an increased temperature condition at 39.6°C (+2), an optimal control temperature condition at 37.6°C (Con), and a decreased temperature condition at 35.6°C (-2). Embryos from the -2 and +2 conditions showed morphology inconsistent with the control (Fig.1A, B) indicating a temperature-induced change in developmental stage. As noted in Figure 1, decreasing the egg incubation temperature by 2 degrees caused that group of embryos to not develop feathering, have a small eyelid slit, and have an overall decrease in body length when compared to embryos at the control temperatures – indicating a delay in development. Increased incubation temperature by 2 degrees had the opposite effect and appeared to accelerate embryonic development with more heavily developed feathers (Figure 1). It was also observed that, while storing bones of the +2 condition for future immunohistochemistry experiments, they readily sank in 4% paraformaldehyde in contrast to the control and -2 conditions, which did not (data not shown). This indicates that the +2 condition had relatively denser bone structure.

In order to determine whether the morphological changes noted were consistent with developmental stage changes, we measured both beak length and feathering. Beak length is a standard for differentiating developmental stage past E12 (HH38) (Hamburger

& Hamilton, 1951). Beak length measurements (Fig. 1C), the appearance of feathering, and the presence of defined phalanges allowed for approximate determinations of equivalent chick embryo development. We determined that chicken embryos incubated in the -2 condition (35.6°C) morphologically resembled an embryo at E12 (HH38), indicating that decreasing the incubation temperature delayed development by approximately 4 days. Alternatively, incubating chicken embryos at 2 degrees higher (39.6°C, i.e. the +2 condition) resembled chicken embryos that had developed quicker than normal between E17 and E18 (HH43-44) (hence E17.5).

In order to control for these changes in development, we performed an additional developmental experiment in which all embryos were incubated at the same optimal temperature (37.6°C), but their gestation period was altered to match the morphological phenotypes noted above. The morphology, size, and beak length between these embryos were compared to those of the temperature conditions (Figure 2). The beak length for the -2 condition embryos was not significantly different than the beak length of embryos incubated at 37.6°C for 12 days (E12 condition) using ANOVA with post-hoc Tukey HSD analysis (Figure 2A,B). Similarly, the beak length for the +2 condition embryos was not significantly different than the beak length of embryos incubated at 37.6°C for 17.5 days (E17.5 condition) using ANOVA with post-hoc Tukey HSD analysis (Figure 2A,B). These conditions differed significantly with the conditions outside their developmental control (Table 1, $p<0.001$) and were within the predicted ranges for their developmental control (Table 1). In other words, the temperature conditions matched their

developmental controls (Ex. +2 was not significantly different than E17.5) but were different from all other conditions (Ex Both +2 and E17.5 were significantly different from -2, Con, and E12). Therefore we can conclude that development has been properly controlled for.

FIGURE 1

Chicken embryo general morphology and beak length.

(A)Representative images of chicken embryos at embryonic day 16 (E16) at either decreased temperature (35.6°C, -2), optimal temperature (37.6°C, Con), or increased temperature (39.6°C, +2).. (B) Representative images of chicken embryos kept at optimal temperature (37.6°C) for either 12 days (E12), 16 days (E16) or 17.5 days (E17.5). (C) Average embryonic beak length of each condition in mm.

FIGURE 1

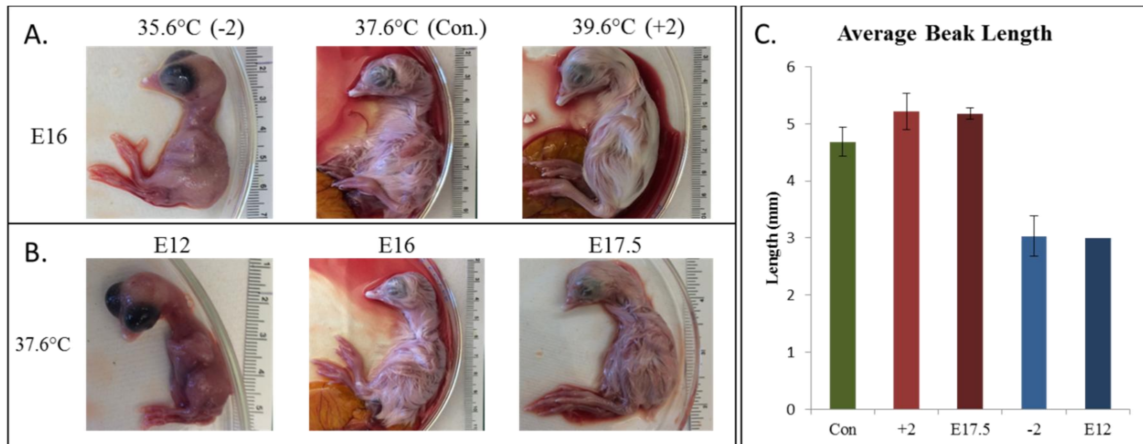


TABLE 1

Average Beak Lengths of developing chick embryos and associated statistics.

Average beak measurements in mm were compared via ANOVA with post-hoc Tukey-HSD. Predicted beak lengths are listed for comparison between Hamburger & Hamilton stages noted in their paper (Hamburger & Hamilton, 1951).

TABLE 1

Table 1: Average Beak Length & Statistics					
Condition	n	Average Beak length (in mm)	Standard Deviation	Predicted Beak Length (in mm)	HH Stage
Con	23	4.68	0.19	4.8	42
+2	19	5.21	0.25	5-5.35	43-44
E17.5	10	5.18	0.32		
-2	9	3.03	0.10	3.1	38
E12	18	3.00	0.35		

Beak Length Comparisons			
Comparison	p	Comparison	p
+2 vs -2	0.001	-2 vs Con	0.001
+2 vs Con	0.001	**-2 vs E12**	**0.899**
+2 vs E12	0.001	-2 vs E17.5	0.001
+2 vs E17.5	**0.899**		

Embryonic Mortality

Some embryos of each condition did not survive. While there may have been some incidences of non-fertilization as opposed to embryonic death, we examined the possibility that the changes in incubation temperature (either +2 or -2) could have resulted in an increase in embryonic morbidity. For this study, since we could not discount the possibility of unfertilized eggs, we included them in our calculations. Since the eggs were selected and grouped at random, we assume here that incidences of unfertilized eggs should be consistent across all groups and therefore should not significantly vary our measurements. Therefore, when we are considering “Embryonic mortality” we are considering rates of unfertilized eggs, embryonic death during development, and stoppage of development to fall under this variable. A representative example of an embryo that had died in vivo can be seen in Figure 2A.

We used Fisher’s exact test to determine whether or not embryonic mortality was due to temperature related changes. Expected frequencies were set to what was observed during the control condition (E16 at 37.6^{o}C) since the control group is at optimal temperature, thus, changes in temperature from optimal would be the only variable. There was a significant incidence of embryonic mortality in the -2 condition (Fig. 2B, $P < 0.0001$). The other conditions were non-significant (Fig. 2B, $P > 0.1$). This data indicates that a decrease from optimal temperature leads to significant increases in embryonic mortality

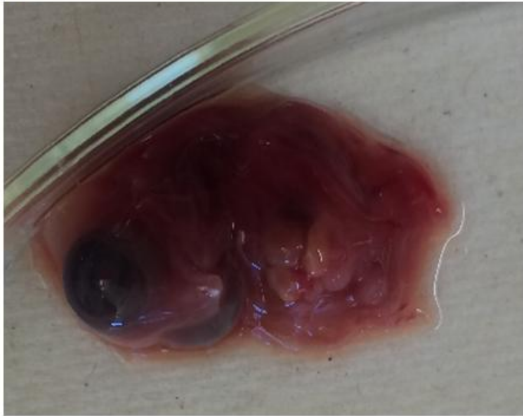
FIGURE 2:

Chicken embryonic mortality due to temperature.

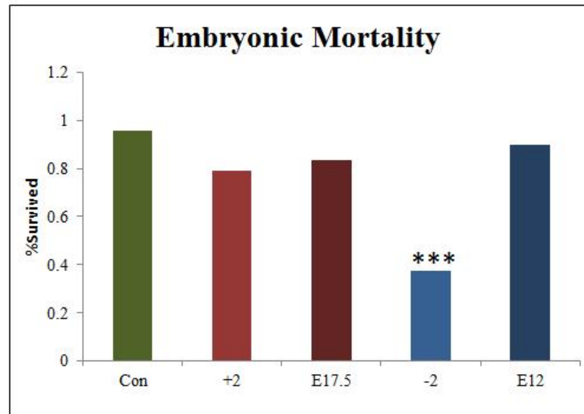
(A) A representative image of a non-viable chicken embryo. (B) The percent survival rate of chicken embryos in the control condition (E16, 37.6°C, Con), the +2 condition (E16, 39.6°C), the E17.5 condition (E17.5, 37.6°C), the -2 condition (E16, 35.6°C) and the E12 condition (E12, 37.6°C). Statistics obtained via Fischer's exact test. ***$p < 0.0001$

FIGURE 2

A.



B.



<u>TABLE 2</u>

Chicken embryonic mortality statistics.

This table lists all relevant statistics of the embryonic mortality analysis noted in Figure 2. The expected value of viable embryos used in Fischer's exact test was from the control condition (Con).

TABLE 2

Table 2: Embryonic Mortality					
Condition	Total Eggs	Viable Embryos	Non-Viable Embryos	%Survived	P-value
Con	24	23	1	95.83%	1
+2	24	19	5	79.17%	0.1882
E17.5	12	10	2	83.33%	0.2527
-2	24	9	15	37.50%	**<.0001**
E12	20	18	2	90%	0.5832

Western Blot Data

In order to examine whether cx43 protein expression was changed during chicken embryonic brain development due to changes in incubation temperature, Western Blot analysis was performed. Cx43 protein expression was found to be significantly increased after incubation in the increased temperature condition (+2 condition, 39.6^{o}C) when compared to all other conditions (Fig.3B, Table 3, $p<.05$, $n=6$, $F=10.41$). All other conditions when compared to each other were not significant. This data suggests that cx43 has a role in stress due to increased temperature in developing chicken embryonic brain.

Hsp70 was also found to be significantly increased in the +2 condition when compared to the -2 condition and the E12 condition (Fig .3C, Table 4, $p<0.01$, $n=6$, $F=7.21$). All other conditions when compared to each other were non-significant.

Hsp90 was not found to be significantly affected across any of the conditions (Fig. 3D, Table 5, $p>0.5$, $F=0.487$). This may indicate hsp90 has a conserved role in development that is independent of temperature and developmental stage.

FIGURE 3

Western blot analysis of chicken embryonic brain.

(A) representative images of blots with Cx43, Hsp90, Hsp70, and B-actin as a loading control using tissue from the E17.5 condition (E17.5, 37.6°C), the E12 condition (E12, 37.6°C), the control condition (E16, 37.6°C, C), the -2 condition (E16, 35.6°C), and the +2 condition (E16, 39.6°C). Western quantification for Cx43 (B), Hsp70 (C), and Hsp90 (D) ($n = 6$, * = $p < 0.01$, **= $p<0.01$ against all other conditions). Lines indicate changes from the beginning point of the line to the end of the line, not conditions in between.

FIGURE 3

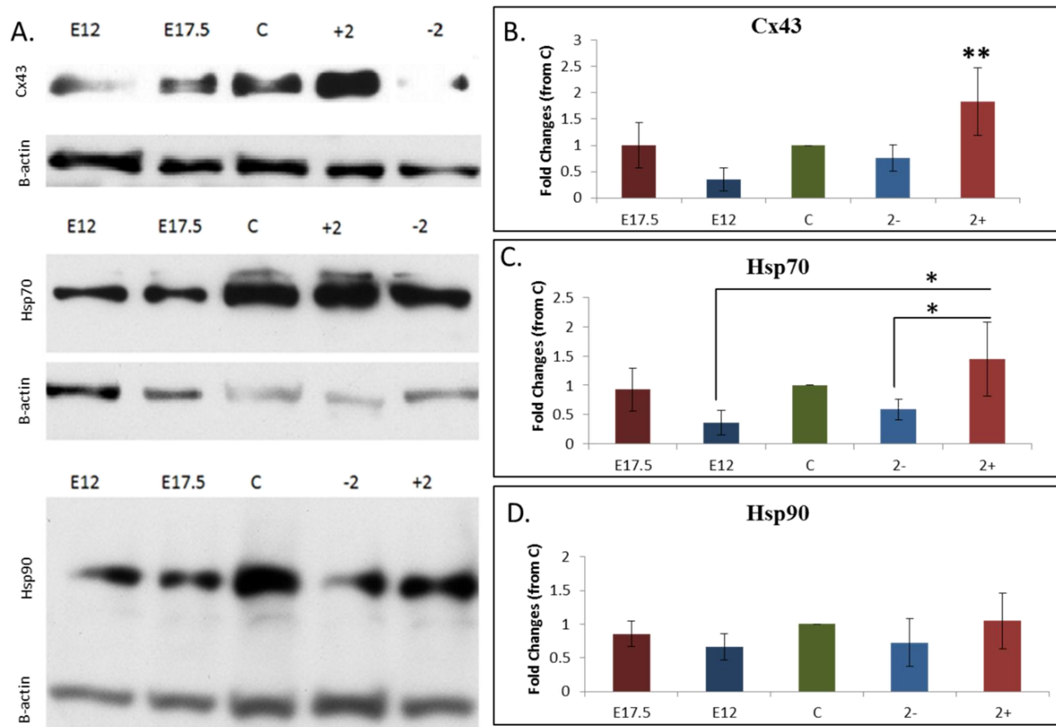


TABLE 3

Western Blotting Statistics; Cx43

This table lists data from the ANOVA and Tukey-HSD analysis of Cx43 bands for the +2 condition (E16, 39.6°C), the -2 condition (E16, 35.6°C), the control condition (E16, 37.6°C, Con), the E12 condition (E12, 37.6°C), and the E17.5 condition (E17.5, 37.6°C). (n = 6)

TABLE 3

Table 3: Western Blotting Statistics; Cx43						
Condition	Average Fold Change	F-Statistic	Comparison	Tukey-HSD p-value	Comparison	Tukey-HSD p-value
E17.5	1.00		**+2 vs C**	**0.0138**	-2 vs E17.5	0.42
E12	0.34		**+2 vs E12**	**0.0142**	C vs E12	0.9
C	1.00	**10.41**	**+2 vs -2**	**0.0012**	-2 vs E12	0.83
-2	0.76		**+2 vs E17.5**	**0.001**	C vs E17.5	0.074
+2	1.83		-2 vs C	0.84	E17.5 vs E12	0.072

TABLE 4

Western Blotting Statistics; Hsp70

This table lists the data from the ANOVA and Tukey-HSD analysis of Hsp70 bands for the +2 condition (E16, 39.6°C), the -2 condition (E16, 35.6°C), the control condition (E16, 37.6°C, Con), the E12 condition (E12, 37.6°C), and the E17.5 condition (E17.5, 37.6°C). (n = 6)

TABLE 4

Table 4: Western Blotting Statistics; Hsp70						
Condition	Average Fold Change	F-Statistic	Comparison	Tukey-HSD p-value	Comparison	Tukey-HSD p-value
E17.5	0.93		**+2 vs E17.5**	**0.001**	C vs E17.5	0.053
E12	0.36		-2 vs C	0.35	E17.5 vs E12	0.11
C	1.00	**7.2106**	+2 vs E12	0.15	C vs E12	0.9
-2	0.58		+2 vs C	0.27	-2 vs E17.5	0.82
+2	1.45		**+2 vs -2**	**0.005**	-2 vs E12	0.54

TABLE 5

Western Blotting Statistics; Hsp90

This table lists the data from the ANOVA and Tukey-HSD analysis of Hsp90 bands for the +2 condition (E16, 39.6°C), the -2 condition (E16, 35.6°C), the control condition (E16, 37.6°C, Con), the E12 condition (E12, 37.6°C), and the E17.5 condition (E17.5, 37.6°C). (n = 6)

TABLE 5

Table 5: Western Blotting Statistics; Hsp90						
Condition	Average Fold Change	F-Statistic	Comparison	Tukey-HSD p-value	Comparison	Tukey-HSD p-value
E17.5	0.85		**+2 vs E17.5**	0.9	C vs E17.5	0.9
E12	0.66		-2 vs C	0.9	E17.5 vs E12	0.79
C	1.00	0.487	**+2 vs E12**	0.9	C vs E12	0.9
-2	0.72		**+2 vs C**	0.9	-2 vs E17.5	0.69
+2	1.05		**+2 vs -2**	0.9	-2 vs E12	0.9

IV. DISCUSSION

Data presented in this thesis show that both increased and decreased incubation temperatures during chicken development significantly affect embryonic chicken development via acceleration or deceleration respectively. We have shown that embryonic mortality can be significantly altered as a result of either increased or decreased incubation temperatures. Furthermore, increasing or decreasing these incubation temperatures can specifically alter the protein expression of cx43, hsp70, and hsp90. Cx43 protein expression was increased as a result of increased (but not decreased) temperature. Interestingly, hsp70 did not follow the same trend as cx43, and hsp90 did not vary at all in response to temperature. This could either mean that hsp70 and hsp90 are unrelated to this temperature-related increase in cx43. Another explanation could be that temperature-induced alterations in hsp70 and hsp90 may be functional and not dependent upon their protein concentration. For example, the binding properties of hsp70 and 90 could change in response to temperature and/or the increased cx43 expression, which would alter their function in the cell without protein concentration changes. This thesis has shown that decreases or increases in incubation temperature also significantly delay or promote the progression of development, respectively. While this thesis does not make any mechanistic claims, the data suggests a possibility that this temperature-mediated increase in developmental rate may be related to cx43 expression.

In order to determine whether or not cx43 is directly related to these developmental stages, knocking down cx43 and measuring indicators of metabolic rate and general embryonic morphology may be a good future experiment due to cx43's noted involvement in the mitochondria (Contreras et. al, 2002, Ruiz-Meana et. al, 2008, Trudeau, Muto, Roy 2012). On the topic of cx43 expression, it is also interesting how cx43 is only increased in response to an increase in temperature, but no significant differences in either the optimal or decreased temperature. This may indicate that cx43 has a role that is specific to hyperthermal conditions. Since it has been established that cx43 is protective against mitochondrial cell death (Rodriguez-Sinovas et al, 2006), cx43 may be acting as a metabolic brake. This would reduce mitochondrial stress resulting from increased temperature. If cx43 acts as a metabolic break in the mitochondria, then it could down-regulate respiration (which creates heat in the cell). While this is only speculation at the moment, it would account for the odd unidirectional modulation of cx43 expression. A possible future experiment to test this could be to knock down cx43 in both the control optimal temperature condition and in embryos incubated at elevated temperatures (the +2 condition) and observe embryonic mortality and changes in the mitochondria. Since our data also implicates cx43 in temperature mediated changes in development, it would also be interesting to observe changes in HH stage using the same model.

However, it should be noted that a major weakness of this thesis is that it does not distinguish between male and female chicks in embryonic development. It is known that

gene expression is different in male and female embryos in brain development (Scholz et al, 2006) which could affect protein expression of our proteins of interest. This could account for some of the variability that was observed in our westerns. Therefore, future experiments in this model should also account for sexual-dimorphism.

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