Title

Observing the Application of Haber's Law in Developmental Toxicity Associated with Nickel Chloride and Trichostatin A in Strongylocentrotus purpuratus (Purple Sea Urchins)

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A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Environmental Health Sciences

by

Jessica Michelle Munson

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ABSTRACT OF THE THESIS

Observing the Application of Haber's Law in Developmental Toxicity Associated with Nickel Chloride and Trichostatin A in *Strongylocentrotus purpuratus* (Purple Sea Urchins)

by

Jessica Michelle Munson

Master of Science in Environmental Health Sciences

University of California, Los Angeles, 2025

Professor Michael D. Collins, Chair

A goal of this project was to demonstrate how sea urchins could be utilized as an animal model to assess aspects of developmental toxicity relevant to humans as well as other organisms. Haber's Law states that the toxic effect of a substance depends on the concentration and exposure time (C x t = k (constant)). Theoretically, if the product of concentration and exposure time are equivalent, there should be a constant toxic response. To test this theory, *Strongylocentrotus purpuratus* (purple sea urchins) embryos were exposed to nickel chloride ($NiCl_2$), zinc sulfate ($ZnSO_4$), and trichostatin A (TSA) with differing combinations of concentrations and exposure times having equivalent Haber's Law products. The toxic endpoint observed was oral radialization for nickel chloride, animalization for zinc sulfate and aboral

radialization for trichostatin A. Phenotypes were scored approximately 96 hours post fertilization (hpf). The results revealed the sensitive period was around 16-24 hours post-fertilization (hpf) for oral radialization and approximately 8-16 hpf for aboral radialization. Additionally, the importance of seemingly nontoxic periods of time was revealed to contribute to the overall toxicity when the exposure period is extended. Since exposure time impacts toxic effects, it's important to study chemicals at "safe concentrations" to better understand the risks associated with these chemicals.

The thesis of Jessica Michelle Munson is approved.

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2025

Introduction

In this thesis, purple sea urchins (*Strongylocentrotus purpuratus*) were used as the experimental organism. Sea urchins have been employed as model organisms in developmental biology for over 150 years (Adonin et al., 2021). This is due to a number of advantages of this species for scientific experimentation. One of which is that they operate through external fertilization which makes it easier to observe this process as well as subsequent development in a controlled environment (Vacquier, 2011). They have a predictable developmental timeline enabling consistent observations at different life stages (Formery et al., 2022). A single adult sea urchin produces a large number of gametes (females produce millions of eggs while males produce hundreds of billions of sperm). "The first sequenced sea urchin genome, that of the Pacific sea urchin *Strongylocentrotus purpuratus*, revealed the extensive conservation of its gene repertoire with that of the human genome" (Marlétaz et al., 2023). This genome was shown to be approximately one-fourth the size of the human genome (814 million bases), but it is predicted to have a similar number of genes (23,300) compared to the human genome (19,500), as well as representative genes of nearly all vertebrate gene families (Amaral et al., 2023; Sea Urchin Genome Sequencing Consor, 2006). Sea urchins are closer to humans in terms of evolutionary distance than either flies or worms, two organisms that are standard models of development (Davidson, 2006), as indicated by the fact that they share more orthologous genes with humans than with these other species (Sea Urchin Genome Sequencing Consor, 2006). Although the sea urchin shares many of the same genes with the human (Ashburner et al., 2000), it is unclear to what extent the functions of those genes are conserved. Additionally, the sea urchin has an experimentally determined gene regulatory network (GRN) for early development (Ben-Tabou

de-Leon et al., 2013; Davidson et al., 2002; Li et al., 2013; Peter & Davidson, 2011; Shashikant et al., 2018).

"A GRN includes mainly regulatory genes encoding transcription factors and signaling molecules, and most importantly it makes explicit the instructions for spatial and temporal expression of regulatory genes embedded in their cis-regulatory modules." (Li & Davidson, 2009). GRNs can show the timing and location of gene expression, illustrating when, where, and how genes are turned on or off in an organism (Oliveri & Davidson, 2004). Davidson et al., (2002) developed an experimentally-determined GRN for *S. purpuratus* showing how gene interactions influence embryonic development by identifying which genes are active at specific times. This allows researchers to link gene interactions with developmental outcomes, making the GRN a valuable tool when deciphering the mechanisms of toxicity for different chemicals (Davidson et al., 2002; Oliveri & Davidson, 2004). There are subsets of the GRN, called kernels of the network, which, because of their developmental role and their particular internal structure, are most impervious to evolutionary change (Davidson & Erwin, 2006). Thus, these kernels are maintained in many species.

This thesis examines aspects of abnormal development in sea urchin embryos following exposure to three chemical compounds, namely zinc sulfate, nickel chloride, and trichostatin A. These three compounds have been selected for study not because they are necessarily thought to be human teratogenic agents, but because they induce dramatic phenotypes in the sea urchin embryo following early developmental exposure. The agents are thought to interfere with the formation of the primary axes of the sea urchin embryo, thus causing severe dysmorphogenesis of the developing organism. Since the gene products for these fundamental embryonic processes are predominantly conserved in the human, it would be of scientific interest to determine why

these chemical compounds are not human teratogens. This does not mean that these agents are not human teratogens, in fact it would be surprising if none of the three were teratogenic in the human, only that there was an alternative explanation for the selection in this thesis.

Toxicity in this study was assessed when embryos reached specific toxic endpoints: animalization for $ZnSO_4$, oral radialization for $NiCl_2$, and aboral radialization for TSA. Chemicals that are deemed animalizing agents either promote the development of cells found in the animal half of the embryo or prevent the differentiation of cells into various endodermal and mesodermal types which are normally located at the vegetal pole of the embryo. Radialization occurs when there is a disruption in the specification of the oral-aboral axis, which is critical for proper mouth formation (Bergeron et al., 2011). During oral radialization, the stomodeal invagination constricts in a circular pattern and it eventually pinches off as the tip of the archenteron fuses with it to produce a mouth (Hardin et al., 1992). The primary mesenchyme cells form a ring in the ectoderm and the spicule elements form a radial pattern (Hardin et al., 1992), as opposed to forming a pair (right and left) of primary spicules as occurs in normal sea urchin development. The specification of the oral-aboral axis happens early in development, during the cleavage stage, when a type of mesodermal cells, called pigment cells which are red at the 60-cell stage, and small micromeres (cells originating from the vegetal pole) become specified (Warner et al., 2012). The oral-aboral axis in sea urchins is like the dorsoventral axis in more complex animals. The molecular signals that control this axis in sea urchins are similar to the ones that guide dorsoventral development in higher organisms, showing that some developmental processes are shared across different species. The dorsal-ventral axis becomes fixed during early development. As early as the 2-cell stage, dorsoventral polarity is established. By the 32-cell stage, dorsal and ventral founder cells have migrated to their respective sides, and

once settled, they remain in place, unable to cross the boundary (the ciliary band) between the oral (equivalent of the ventral axis in vertebrates) and aboral (equivalent of dorsal axis in vertebrates) sides (Hardin et al., 1992). This means that disruptions to this process are irreversible once the cells have travelled to a specific side of the gastrulating embryo. Oral radialization incorporates the incomplete development of the gastrointestinal tract, multiple skeletal spicules, (Bergeron et al., 2011) and a lack of pigment cells.

These three phenotypes are easily delineable by observation if the particular pluteus has all of the characteristics of any specific dysmorphic phenotype, but there are instances where the developmental alteration may be incomplete. If the abnormal phenotype is complete, then the data collection process becomes Boolean from the perspective that a particular organism is either malformed or not, and the decision in the data collection process is relatively simple. However, in cases where the dose of a chemical may be excessively large or excessively small, there may be gradations of the phenotype (where a particular pluteus has a different phenotype or some portion of the characteristics of one of the specific phenotypes but not all of them). In such cases, it is more difficult to be definitive regarding the characterization of the pluteus.

This thesis initiated with the hypothesis that zinc sulfate exposure to sea urchin embryos caused an inhibition of the Wnt signaling pathway simply because exposure to both zinc sulfate and the Wnt pathway inhibitor Wnt C-59 induce a similar phenotype, animalization, in the embryos as they develop (Cui et al., 2014; Lallier, 1955, 1975; Timourian, 1968). This is not a novel discovery as there are a number of chemical agents that are able to induce this phenotype (e.g. A23187, Zn^{2+}, Cd^{2+}, azo dyes, sulfonated aniline blue dyes, antimetabolites, Evans blue, trypsin, monoiodoacetate) (Fujiwara & Yasumasu, 1991; GUSTAFSON & HORSTADIUS, 1955; Hörstadius et al., 1966; Immers & Runnström, 1965; Lallier, 1955; O'Melia, 1971, 1973).

A normal sea urchin embryo can be characterized as having an animal pole, which consists of ectodermal cells with long, immotile cilia or their precursors, and a vegetal pole, which consists of endodermal or mesodermal cells or their precursors. The animal-vegetal axis is established at the time of fertilization. Animalization of embryos consists of increasing the specification of ectodermal cells and reducing the specification of mesenchymal or endodermal cells (Lallier, 1955, 1975; Matsunaga & Honji, 1983; Nemer et al., 1985; Timourian, 1968). In its most severe form, an animalized embryo appears as a blastula (a dauerblastula) with an ectodermal layer of cells surrounding a blastocele that contains no archenteron, skeleton or pigment cells. The previously mentioned hypothesis provided a framework for determining the molecular mechanism of action of a dysmorphogenic dose of zinc in the sea urchin embryo. However, it was not a particularly strong hypothesis because there are a large number of chemical agents that may induce this endpoint. Although this hypothesis may be true, it was abandoned as the basis for this thesis because despite the similarity of the phenotype induced by the two compounds at high doses, it was determined in preliminary experiments that the phenotypes induced by low doses of these particular chemical agents were different. Zinc exposure at low doses produced radialization in the embryos which was previously detected by Lallier (1975) and was considered consistent with the animalization induced at high doses of zinc. However, this phenotype was not detected in embryos exposed to Wnt C-59 at low doses for unknown reasons. Thus, despite the commonality of the phenotype produced at high doses of the compounds, the two chemical agents may function via distinct pathways as indicated by the different phenotypes at low doses.

A key signaling pathway present in humans and sea urchins is the Wnt pathway (Adonin et al., 2021). The canonical Wnt pathway is a critical, highly conserved pathway that regulates cell proliferation and helps maintain homeostasis in organisms (Liu et al., 2022). This pathway

also regulates the animal-vegetal axis in sea urchins (as well as a large number of species), thus perturbations of this pathway can result in animalization or vegetalization of the sea urchin embryos (Adonin et al., 2021). Wnt-C59 is a small molecule porcupine (PORCN) inhibitor (Motono et al., 2016; Proffitt et al., 2013). PORCN is an endoplasmic reticulum transmembrane protein involved in processing wingless proteins such as WNT7A. It functions to O-palmitoleoylate these proteins (Caricasole et al., 2002) .

In a previous study (reported herein), the chemical trichostatin A (TSA) was tested to assess its adherence to Haber's Law. TSA was administered during sea urchin development at equivalent doses as defined by the product of concentration (C) and duration (T) to evaluate whether the primary dysmorphic effect was consistent with Haber's Law predictions. The primary dysmorphic effect produced by TSA was aboral radialization. When the TC_{50} (toxic concentration that impacts 50 % of the exposed population) values were plotted on a dose-response curve, they aligned closely with the predictions of Haber's Law. This finding that Haber's Law applied to a developmental outcome was unanticipated because in contrast to chemical lethality assessed in an adult organism where the exposure to a chemical at one time would be anticipated to be somewhat equivalent to exposure to the chemical at a different time because the adult is considered to have constant susceptibility (homeostasis) of physiological systems over time, the developing organism is perceived to be dynamic in such a manner that exposure at any particular time during development could be considered unique because of the constant maturation of the organism. However, since it was determined that Haber's Law seemed to apply to a particular developmental effect following exposure to trichostatin A, it was questioned whether it would apply to other dysmorphic outcomes produced by other chemical compounds. It was proposed that examining the same sea urchin developmental system for

applicability of Haber's Law following exposure to $NiCl_2$, which induces oral radialization, would determine if this concept was more generally applicable. In limited experimentation, it was found that ZnSO4 yielded the phenotype of animalization, however, evidence of ZnSO4-induced animalization following Haber's Law was inconclusive.

The second hypothesis of this thesis was that developing sea urchin embryos exposed to toxic chemicals will follow a limited version of Haber's Law. Haber's Law was developed by German chemist, Fritz Haber. Haber is best known for designing a process whereby nitrogen gas and hydrogen can be catabolized to form ammonia (a biologically usable form of nitrogen), thus providing a chemical reaction that can produce fertilizer for which he won the Nobel Prize in 1918. Among many other scientific exploits, he was known for development of gaseous chemical weapons. Haber's Law was first published as a lecture footnote stating: "For each war gas, the amount (c) present in one cubic meter of air is expressed in milligrams and multiplied by the time (t) in minutes necessary for the experimental animal inhaling this air to obtain a lethal effect. (Haber, 1924)." Thus, Haber's Law states that the concentration of a chemical (C) times the duration of exposure (t) equals a constant (denoted as $C \times t = k$) (Hoyle et al., 2010). The constant in this equation represents the quantity of damage induced by a toxic exposure. Since the exponent on both the concentration variable and the time variable are one, this equation states that concentration and time are equally responsible for the outcome. From this Law, it can be mathematically determined as to what chemical concentration at low doses over longer durations are equivalent to higher chemical concentrations over shorter durations of time. For example, a chemical administered at 10µM for 24-hours should yield an equivalent toxic outcome to administering a chemical at 30µM for 8-hours since the product, C x t, are equivalent. A more general form of Haber's Law has been suggested in which the formula is $(C-C_0)^a \times t^b = k$ where

C_0 is the threshold dose (Miller et al., 2000). This version would indicate that although the concentration of the toxicant and the duration of exposure are both important in the outcome, the impact of each variable may be different.

The idea that Haber's Law would have relevance to the field of developmental toxicity is not intuitive. When Haber originally exposed animals to the toxic nerve gases to produce the toxic (or more generally lethal) outcomes, the susceptibility of the animals would be thought to be relatively constant. Thus, it might be anticipated that a mouse of 6 months, or 9 months, or 12 months or 15 months of age would be relatively similar in susceptibility because the organs of the mouse have all reached a homeostatic size (approximately equal number of cells) and basically have similar physiological functions. Therefore, exposure of a mouse to a quantity of a toxic compound for a certain defined period of time at 6 months of age or at 15 months of age may be anticipated to lead to somewhat similar levels of toxicity. Alternatively, a developing organism is a dynamic system where an exposure to a chemical at one developmental time may have a completely different morphological impact than exposure to the same chemical on a subsequent gestational day. This idea of differential susceptibility according to developmental timing is well known in the field of teratology where there are sensitive periods during organogenesis for chemical agents that correspond to the developmental timing when specific organs are forming. To some extent, this difference between exposure to a pseudostatic adult organism and a dynamic developing organism is profound and that is why it is difficult to believe that this Law would apply to developing organisms because in a relatively short time following fertilization a developing sea urchin changes the nature of it axes, cells and tissues. Thus, it is not hard to imagine that exposure of a 6-hour post-fertilization embryo would be drastically different than exposure at 12 hours.

Sensitive periods play a critical role in developmental toxicity. A sensitive period is a phase of rapid development in an organism when it is especially vulnerable to external factors (Burggren & Mueller, 2015; Knudsen, 2004). Depending on the type of disruption or exposure during this time, the effects can significantly influence development, either positively or negatively (Colombo et al., 2020). The sensitive period corresponds to a specific phenotype and thus different organ systems in a particular organism have different sensitive periods because the temporal developmental profile for organs differs depending on embryological parameters. For example, in mammalian species the eyes develop early in embryogenesis whereas the urogenital system develops later in development. Thus, chemical exposures that induce teratogenesis of the eyes may have an early sensitive period compared to chemical exposures that impact the urogenital system. The sensitive period was recently described as a defined duration during which the susceptibility is variable over time and the outcome may be reversible (Colombo et al., 2020). The sensitivity to perturbation has a gradient such that the perturbation is greatest during the midpoint of the period but has reduced susceptibility at the initiation and termination of the sensitive period. Alternatively, the critical period was characterized as a defined duration during which the susceptibility to perturbation is constant over time and the developmental alteration is irreversible (Colombo et al., 2020). Based on the critical period having constant susceptibility and the sensitive period having variable susceptibility, it may be hypothesized that the critical period is a subset of the sensitive period, however, it is difficult to imagine how the reversibility of developmental alteration is dependent on the time of administration as opposed to the administered dose.

Generally, early developmental exposures appear to have more widespread and severe impacts, while later exposures tend to target specific biological systems, resulting in less

extensive morphological effects (Colombo et al., 2020). It has been proposed that the most sensitive periods in embryogenesis are during transitions from an important developmental event to another such as the transition from proliferation to differentiation (Salimi & Jamali, 2024). The sensitive period for disrupting a particular organ or tissue may occur prior to the appearance of the anlagen for the organ or tissue. For example, renal anomalies were induced by irradiating rat embryos on day 9 of gestation, but the earliest primordium of the mesonephros does not appear until day 12 (Wilson et al., 1953).

Originally, Haber's Law was used to measure the acute lethality of inhaled gases during WWI (Haber, 1924, p. 19). Today, it's being used in risk assessment and to set guidelines for toxic substances (Gaylor, 2000). However, there is uncertainty regarding applicability of Haber's Law. Haber derived the relationship by examining severe outcomes such as lethality in specific laboratory animals that were exposed to highly toxic chemicals through the inhalation route of administration. Questions have arisen such as: does the Law apply to all varieties of toxic endpoints (e.g. carcinogenesis, chronic neurotoxicity, teratogenicity)? Does it apply to all types of chemicals irrespective of whether the toxicokinetic parameter that defines the toxic outcome is the area-under-the-curve or the concentration maximum (C_{max})? To understand the extent of the applicability of Haber's Law there is a need to perform additional studies.

Gelzleichter et al., (1992) tested Haber's Law by exposing rats to ozone and nitrogen dioxide (NO_2), using acute lung damage as the toxic endpoint. The rats were exposed to ozone (0.2-0.8ppm), NO_2 (3.6-14.4ppm), and a mixture of the two. The highest doses (0.8, 0.6, 0.4 ppm for ozone and 14.4, 10.8, and 7.2 ppm for NO_2) were administered over 6, 8 and 12 hours a day, respectively. The lowest doses (0.2 ppm for ozone and 3.6 ppm for NO_2) were administered over 24-hours. This resulted in CxT products of 4.8 for ozone and 86.4 for NO_2. The greatest lung

injury was observed during the highest dosage periods, while the least severe damage occurred at the lowest doses. When a mixture of ozone and NO_2 was administered, lung damage was more severe at the highest concentrations, suggesting that the mixture operates under Cmax conditions—being driven more by peak concentration than cumulative exposure. The study's results demonstrated that while Haber's Law applied to individual compounds at medium to high doses, it failed to predict toxicity at lower dose rates and when the compounds were combined. This demonstrated that Haber's Law was followed at some doses, but not at all doses tested.

Zwart & Woutersen, (1988) looked at the acute inhalation toxicity of chlorine gas in rats and mice. Rats and mice were exposed to varying concentrations ranging from 935- 16,801 mg/m^3 of chlorine for 5, 10, 30, or 60 minutes. The toxic endpoint was mortality. Data analysis performed by Miller et al., (2000) showed that mortality data from this experiment fit Haber's Law power law function ($X^{\alpha} *Y=k$), where $\alpha=1$, X is concentration, and Y is time, for the chlorine LC 50 values. The data showed LC 50 values of chlorine (1321, 2033, 5642, and 15949 mg/m^3, respectively) over brief exposure periods (60, 30, 10 and 5 minutes, respectively) seemed to follow Haber's Law. The results of this study showed that chlorine followed Haber's Law at four specific concentrations and times but did not at the rest of the concentrations and times tested. Both studies showed that Haber's Law might not apply across all doses but is followed at certain specific concentrations.

In essence, the goal of the experimental work in this thesis was to explore the relationship of concentration and duration of exposure to abnormal developmental outcomes. Unfortunately, there were limitations to these experiments as performed. One limitation was that the concentration of the chemical was established in the external milieu of the sea water in which the embryos were maintained. However, the critical concentration for determining this relationship

is undoubtedly the internal concentration in the sea urchin cells. Although the cellular internal concentration was not determined in these experiments, the presumption is that these compounds were relatively permeable to the sea urchin cells because experiments where the compounds were removed from the sea water showed that abnormal outcomes that would be induced by later exposures were not detected. A second limitation of these studies was that transcriptome experiments to look at gene expression following chemical exposures were not performed which may have provided a molecular explanation for the various results. Such experiments were initially planned, but because of difficulties throughout the project that extended the time to accomplish various goals, there was insufficient time to perform these experiments.

Materials and Methods:

Trichostatin A, nickel(II) chloride hexahydrate, and zinc (II) sulfate heptahydrate were purchased from Sigma Aldrich (St. Louis, Missouri).

A colony of sea urchins (*Strongylocentrotus purpuratus*) were collected off the coast of San Diego, California. They were kept in sea water (collected from the Pacific Ocean) in a Sea Water Visions tank maintained at 13℃ with constant aeration. The sea urchins were fed dried kelp 2-3 times a week.

Gametes were collected by injecting 0.55M KCl (Fisher Scientific) into the soft tissues surrounding the oral cavity, using a 23- to 26-gauge needle. Sperm was collected using a glass pasteur pipette and transferred into a 1.5mL Eppendorf Microcentrifuge Tube. The tube was then put on ice until fertilization which generally occurred on the same day. Eggs were collected by placing the aboral side of the sea urchin over a 50mL beaker of sea water. After collection, the eggs were settled and rinsed three times with sea water. For fertilization, about 10μL of sperm

was diluted with 500µL of sea water using a 9-inch Fisher glass pasteur pipette. About 1 to 3 drops of the diluted sperm was added to the beaker of rinsed eggs depending on the quantity of eggs collected. Sea water was maintained at 16°C or colder during the gamete collection process. The fertilization time was recorded as the time when the sperm were added to the eggs. An incubation period of 90 seconds was maintained for fertilization to occur and then an aliquot of the eggs were checked for fertilization. Fertilization was determined by observation of the fertilization membrane under a dissecting microscope. Fertilization rates were always greater than 95 %. After fertilization, the settled eggs were suspended evenly by gentle agitation, and an aliquot of 1 mL of embryos was diluted (at 1:20 to 1:100 in filtered sea water depending on the density of the culture). From the diluted culture, which was suspended evenly, a sample was collected in a 200 µL glass pipette and counted. The aliquot collection and counting were repeated for a total of 8 total counts. The average number of embryos from eight counts was used to determine the number of sea urchin embryos per mL of the fertilized egg culture, and then a calculation was performed to determine what dilution (in sea water at 16°C) would produce a fertilized eggs culture with a density of approximately 1500 fertilized eggs per mL. 1 mL of the 1500 embryos/ mL solution was added to each 125 mL Erlenmeyer flask which contained 74 mL of filtered sea water (minus the volume of chemical to be added to the flask to achieve the desired concentration). Filtered sea water was removed from the 74 ml to account for the volume of the chemical being added. For example, if 3µL of chemical was being added to the flask then 3µL of seawater was removed from the 74 mL to ensure that the final volume of the culture was 75 mL. Thus, the final volume of the sea urchin culture in the flask was 75 mL which consisted of the 1 mL aliquot of 1500 sea urchin embryos, the generally small volume (usually less than 200 µL) of chemical to be added to the flask to achieve the desired concentration, plus the

amount of filtered sea water to obtain a total volume of 75 mL. The density of the sea urchin cultures were consequently approximately 20 eggs/embryos per mL of filtered sea water. After addition of the chemical to the culture flask, the flask was placed in an incubator (Model IS28SSD, Powers Scientific, Inc., Pipersville, Pennsylvania) at 17° C on an orbital shaker (Southwest Science SBT3000, Roebling, New Jersey) at 120 revolutions per minute. At various time points post-incubation, the various chemicals were removed from culture flasks by filtering the embryos with a 50 μm sieve and rinsing them (for a duration of 5 minutes or longer) prior to placing them back in the rinsed Erlenmeyer flask with fresh filtered sea water (that did not contain chemical). The culture flasks were then returned to the 17° C incubator where they were rotated at 120 rpm on the orbital shaker. At approximately 96 hours post-fertilization, the flasks were placed on ice, and aliquots from each culture were taken to allow at least 100 plutei (of the total 1500 plutei in the flask) to be individually examined under a dissecting microscope for phenotype assessment. In cases where it was deemed appropriate to document the phenotype, photographs of the plutei were taken on an inverted Zeiss microscope (Axiovert S100 TV, Carl Zeiss, White Plains, New York) with an AxioCam Hre camera (Carl Zeiss) with AxioVision 4 (Special Addition 64 bit) 4.9.1 software (Carl Zeiss).

Results

A

Agent:	Control	TsA	TsA	TsA	TsA	TsA	TsA	TsA	TsA	TsA	TsA	TsA
Concentration:	(no addition	1 nM	3 nM	5 nM	7 nM	10 nM	1 nM	2 nM	6 nM	10 nM	14 nM	20 nM
Exposure duration (a)		1-24 hpf	1-24 hpf	1-24 hpf	1-24 hpf	1-24 hpf	6-18 hpf	6-18 hpf	6-18 hpf	6-18 hpf	6-18 hpf	6-18 hpf
Phenotypes:												
Normal	113	93	1	2	0	0	107	97	37	24	7	7
Missing both anterolateral arms	0	5	34	21(b)	7	2	4	8	7	8	10	4
Missing a single anterolateral arm (side of absence) ©	1R	1R	2LL	1L	1L	0	0	1R	1L	1R	1L	1R
Missing an anterolateral arm and ipsilateral postoral arm @ 90° angle	0	0	0	0	0	0	0	0	1L	0	0	0
Missing a anterolateral and postoral arms on ipsilateral side	0	1L	0	0	0	1L	0	0	0	0	0	0
Missing both anterolateral and one postoral arm	0	0	0	0	0	1	0	0	0	0	0	0
Missing all four arms:												
with 3 primary spicules	0	1	0	0	0	0	0	0	0	0	0	0
with 2 primary spicules	0	2	0	1	2	2	2	5	4	8	7	1
with 0 primary spicules	1	1	0	0	0	0	0	0	1	1	1	0
with aboral radialization	0	8	75	92	103	110	0	6	64	78	84	93
Pregaastrula embryo/egg	1	2	0	3	5	1	3	3	2	2	7	1
Σ (overall number of post-gastrula & pre-gastrula plutei)	115 & 1 pg	112 & 2pg	112	117 & 3pg	113 & 5pg	116 & 1pg	113 & 3pg	117 & 3pg	115 & 2pg	120 & 2pg	110 & 7pg	106 & 1pg

(a) Time period of exposure in hours post-fertilization (hpf)
(b) One of the plutei had exogastrulation
© Side of absence is indicated as right (R) or left (L)

B

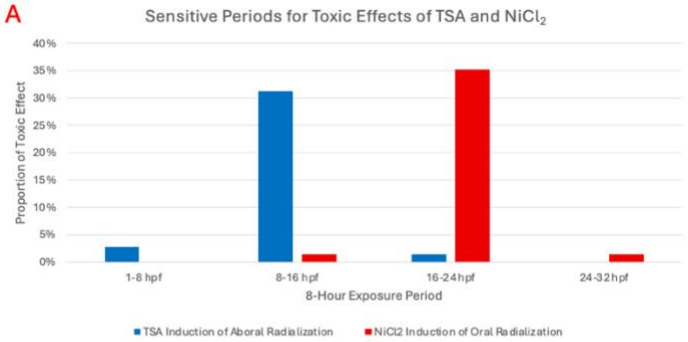
			Radialized	Missing all Four Arms						Missing Both 2° Arms								
Dose	Duration	Normal	Oral	0 Spicules	1 Spicule	2 Spicules	3 Spicules	Animalized	Double Spicule	Missing (1) 1° Ar	(3) 1° Spicules	inverse Apex	Missing both 1° arms	Missing a single 1° ar	Missing a single 2° ar	Missing (1) 1° arm and	Opaque Cavity v	Pre- gastrula
0	96	1500	0	4	0	25	0	0	0	2	0	3	1	1	5	0	0	23
3.33	1-24 hpf	110	174	24	0	361	43	0	17	8	7	3	0	0	1	1	0	32
3.33	1-32 hpf	10	286	8	0	240	32	3	60	5	0	5	0	0	2	2	0	17
3.33	1-96 hpf	17	120	0	0	180	28	0	45	4	1	24	0	0	3	0	0	19
3.33	16-20 hpf	186	21	2	0	98	3	0	0	5	0	1	0	0	10	1	0	3
3.33	16-24 hpf	267	30	56	0	100	1	0	4	9	0	6	0	1	28	5	0	17
3.33	24-32 hpf	356	6	2	1	1	0	0	0	0	0	0	0	0	2	0	0	3
3.33	8-16 hpf	298	5	12	0	53	0	0	0	0	0	0	0	0	3	0	0	10
3.33	8-32 hpf	12	306	1	0	254	32	0	29	0	0	2	0	0	0	0	0	20
3.33	8-96 hpf	16	123	1	0	186	16	2	39	5	0	25	0	0	1	4	0	15
10	16-20 hpf	180	15	7	0	88	1	0	0	1	1	0	0	2	9	0	0	7
10	16-24 hpf	200	88	25	0	108	2	0	9	12	0	0	1	3	23	9	0	9
10	24-32 hpf	332	2	6	0	8	0	0	1	1	2	0	0	1	5	0	0	3
10	8-16 hpf	332	2	6	0	8	0	0	1	1	2	0	0	1	5	0	0	3
30	16-20 hpf	147	22	4	1	111	16	0	3	13	0	7	1	3	11	0	0	1
30	16-24 hpf	78	130	20	1	164	25	0	12	7	0	7	0	2	3	1	0	13
30	24-32 hpf	317	4	8	1	16	0	0	0	1	2	0	0	1	4	0	0	2
30	8-16 hpf	280	5	9	2	62	2	0	0	1	1	0	0	1	2	1	0	13

Figure 1: **A.** Phenotypes used to categorize TSA exposed embryos **B.** Phenotypes used to categorize $NiCl_2$ exposed embryos

The raw data results were collected by examining each individual embryo under a microscope and then categorizing the embryo as a certain phenotype. An example of the extensive phenotype categories is seen in Figure 1a and 1b when observing embryos exposed to TSA and $NiCl_2$, respectively. Data categories were condensed to make the data more readable. A goal of this project was to demonstrate how sea urchins could be utilized as an animal model to assess aspects of developmental toxicity relevant to humans as well as other organisms. The data shows that there are a variety of phenotypes that are induced by the chemical exposures. These phenotypes can be categorized as either chemical specific phenotypes (e.g oral radialization for nickel chloride or aboral radialization for trichostatin A) or non-specific phenotypes that are induced by various chemical exposures (e.g. missing a single anterolateral arm, missing all four arms with 2 spicules). In Figure 1B, references to 1° arms are to postoral arms and references to 2° arms are to anterolateral arms of the sea urchin pluteus. In subsequent

references to the phenotype produced by various chemicals, such statements will refer to a single chemical specific phenotype which has been determined from each of the three chemical agents analylyzed, namely animalization for zinc sulfate, oral radialization for nickel chloride and aboral radialization for trichostatin A.

A



B

NiCl2				
	n (# obs.)	Normal	Oral radial	oral prop
1-8 hpf	336	285	0	0
8-16 hpf	1071	866	15	0.0140056
16-24 hpf	2474	596	872	0.35246564
24-32 hpf	1065	1005	14	0.01314554
TSA				
	n(#obs.)	Normal	aboral rad	aboral prop
1-8 hpf	1412	1331	38	0.02691218
8-16 hpf	3369	2162	1052	0.31225883
16-24 hpf	1323	1267	18	0.01360544

Figure 2: **A.** Sensitive periods shown through 8-hour exposure windows for TSA and $NiCl_2$ **B.** Tabular data used to generate Fig 2A table

The results of this study were intriguing, particularly with regard to the sensitive period of development. As mentioned earlier, a sensitive period refers to the time frame during which teratogenic (abnormal developmental) stressors are most likely to disrupt normal development (Burggren & Mueller, 2015; Tzimas et al., 1997). The phenotype is the parameter that is most critical to determine the sensitive period and the principal chemical specific phenotype produced by each of the chemicals was different. The findings revealed that the approximate sensitive period for $NiCl_2$ exposure causing oral radialization occurred between 16-24 hpf, while the sensitive period for TSA producing aboral radialization was approximately between 8-16 hpf (Fig. 2A). There was insufficient evidence to conclusively determine the sensitive period for $ZnSO_4$ exposure. While the study did provide some insights into the potential timing of toxic effects, the data was not robust enough to pinpoint a specific sensitive period for $ZnSO_4$. It would be anticipated that the sensitive period for producing animalization in the sea urchin would be earlier than the periods of induction of oral and aboral radialization because the animal-vegetal axis is the first to be embryonically determined. It is proposed that the primary

determinant of the sensitive period is the specific phenotype induced because the specific embryonic events that lead to the phenotype are critical parameters for the induction of the phenotype. A second determinant would be the chemical agent and the mechanism by which it induces the specific phenotype.

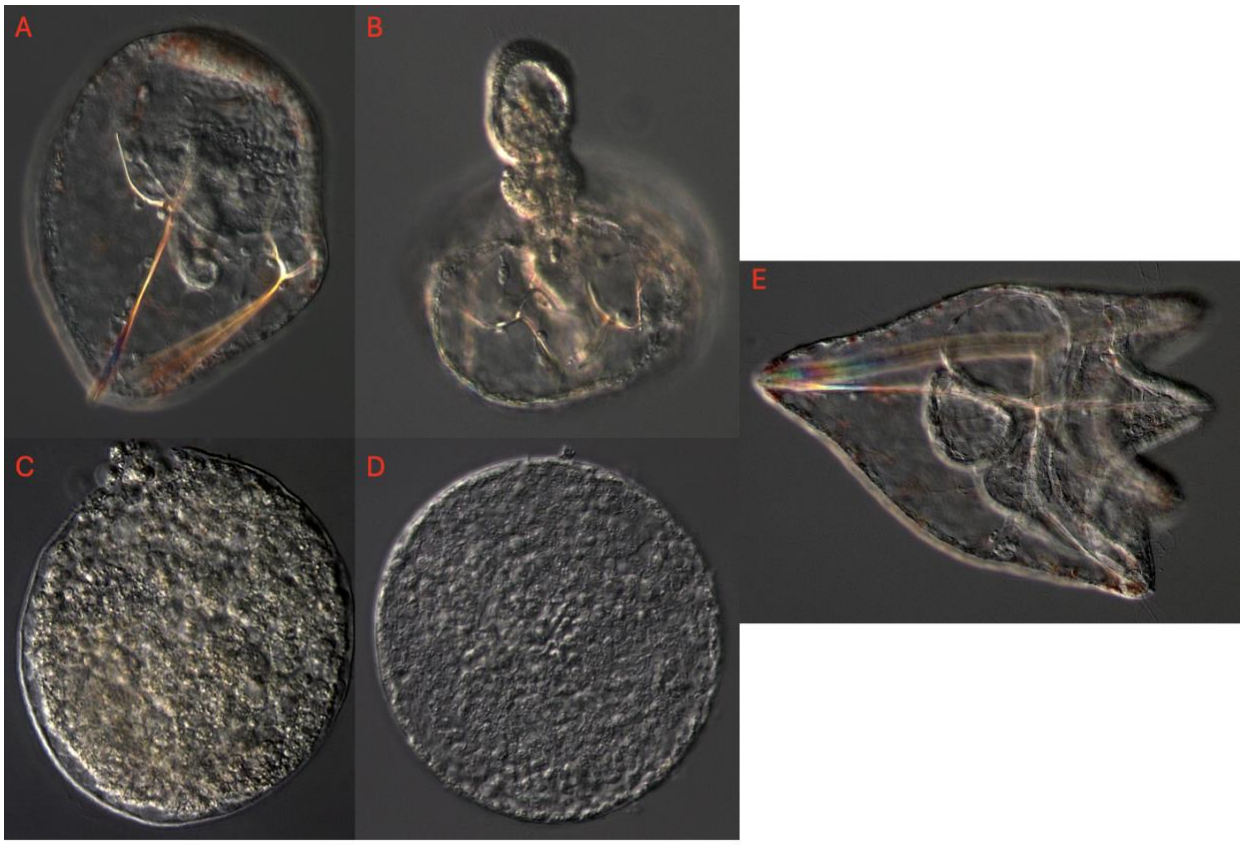


Figure 3 A: Low dose Wnt-C59 **B:** Low dose $ZnSO_4$ **C:** High dose Wnt-C59 **D:** High Dose ZnSO4 **E:** Control

Moving on to the results pertaining to $ZnSO_4$. Initially it was hypothesized that Wnt-C59 and $ZnSO_4$ disrupted the Wnt pathway through the same mechanism. However, experimental findings were inconclusive and could not determine sufficiently if Wnt-C59 and $ZnSO_4$ disrupted the Wnt pathway through the same mechanism or not. At lower doses (100nM, 200nM, 300nM, 400nM for Wnt-C59, and 10µM and 30µM for $ZnSO_4$), the two compounds did not produce the same phenotypes, indicating they do not share the same mechanism of toxicity (Fig. 3A & 3B). At higher doses (500nM for Wnt-C59 and 100µM for $ZnSO_4$), they did produce similar

phenotypes, that being animalization which is characterized by a pile-up of mesenchyme cells in the vegetal half of the embryo, lack of pigment cells, and no skeletal spicules (Fig. 4C & 4D), which suggests they might share a common mechanism at these elevated concentrations, or alternatively that each of the agents perturbs various developmental events that share a final common pathway thus producing the same endpoint. However, without further molecular analysis, it is impossible to definitively conclude if the mechanisms of these two agents overlap.

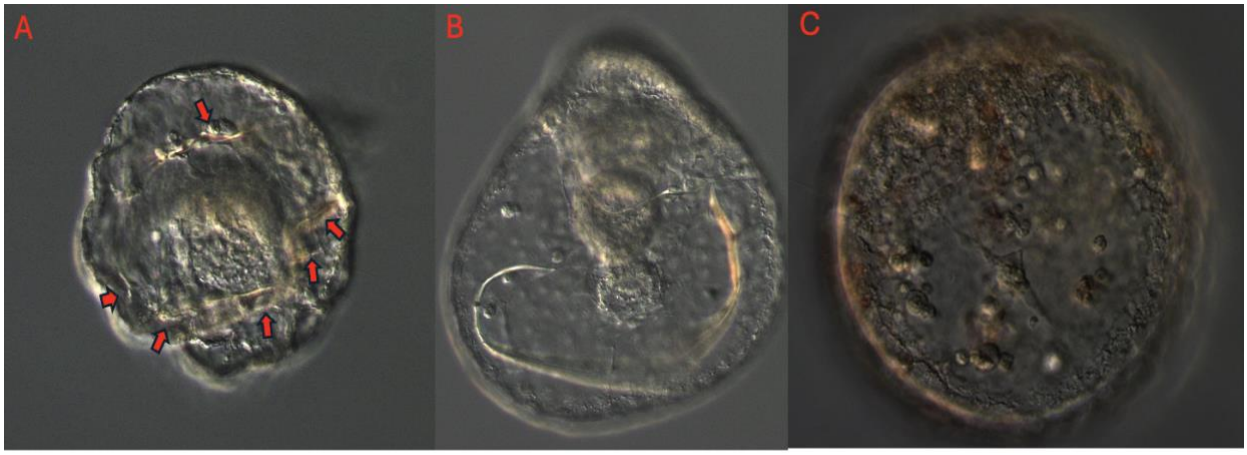
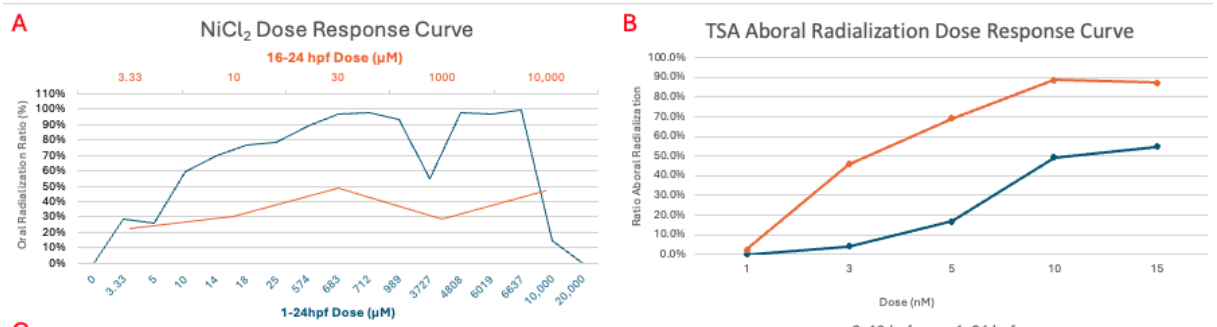


Figure 4: A. Oral Radialization in Sea Urchin embryo. No pigment cells present. Red arrows are pointing to partial skeletal elements that are indicative oral radialization has occurred **B.** Different angle of an embryo exhibiting oral radialization. It is not the same embryo as shown in 3A. It is another angle to show the GI tract of an embryo exhibiting oral radialization. **C.** Aboral radialization in Sea Urchin embryo. Pigment cells present and some skeletal elements present.

Next are the results concerning $NiCl_2$. As previously mentioned, the sensitive period for induction of oral radialization in purple sea urchins when exposed to $NiCl_2$ is approximately 16-24 hpf (Fig. 2A). Oral radialization is characterized by a non-specific oral field lacking pigment cells, incomplete gastrulation, a spherically-shaped pluteus, and the formation of multiple skeletal spicule rudiments (Bergeron et al., 2011) (Fig. 4A, 4B). This phenotype was used as the primary indicator of developmental toxicity produced by NiCl2. The dose-response curve suggests that $NiCl_2$ does not adhere to Haber's Law in the induction of oral radialization at any dose interval (Fig. 5A, 5D). Additionally, the rates of oral radialization increased a statistically

significant amount (p-value= 0.030) (Fig. 7) when the exposure period was extended from 1-24 hpf to 32 hpf (Fig. 6). Notably, when embryos were only exposed to $NiCl_2$ from 24-32 hpf, excluding the 16-24 hpf sensitive period, the severity of toxic effects was reduced substantially to a value close to 0. If the 24-32 hpf period is not included in the sensitive period, then it is enigmatic why exposure during this period would contribute to an increase in the oral radialization rate that was produced by exposure during the sensitive period of 16-24 hpf. This indicates that the 16-24 hpf window is essentially crucial for the onset of oral radialization.



C

1-24hpf 16-24hpf

Dose (nM)	Duration	Number Observed	Number observed -	Normal	Aboral radialization	Aboral Rad. Ratio (ab. Rad./ (#ob.- 0 spic. & death))
1	8-16 hpf	477	468	439	0	0.0%
3	8-16 hpf	858	830	642	34	4.1%
5	8-16 hpf	747	732	489	122	16.7%
10	8-16 hpf	859	850	323	420	49.4%
15	8-16 hpf	479	476	137	261	54.8%

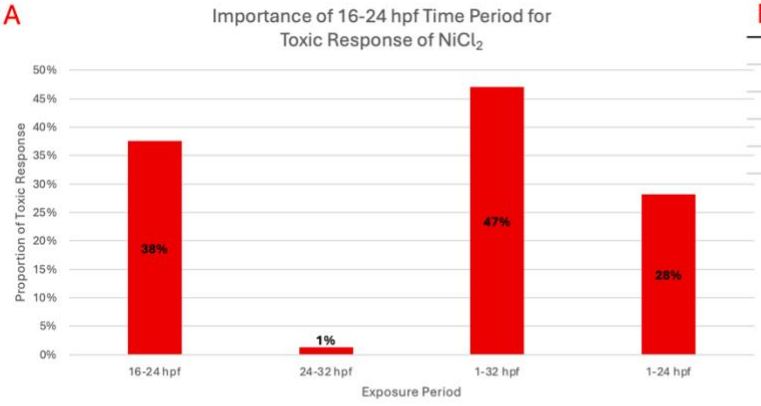
Dose (nM)	Duration	Number Observed	Number observed -	Normal	Aboral radialization	Ratio
1	1-24 hpf	1555	1516	1071	39	2.6%
3	1-24 hpf	3845	3246	649	1489	45.9%
5	1-24 hpf	1754	1626	145	1124	69.1%
7	1-24 hpf	934	921	31	816	88.6%
10	1-24 hpf	1679	1536	28	1343	87.4%

D

Dose (µM)	Observed	obs-PG-0	Normal	Oral Rad.	oral rad. Ratio
0	200	195	190	0	0%
3.33	800	744	110	210	28%
5	284	279	13	73	26%
10	327	323	5	192	59%
14	128	127	1	89	70%
18	337	335	0	258	77%
25	185	173	1	136	79%
574	223	217	1	193	89%
683	135	129	0	125	97%
712	53	49	0	48	98%
989	30	29	0	27	93%
3727	28	22	0	12	55%
4808	126	84	0	82	98%
6019	111	57	0	55	96%
6637	244	123	0	122	99%
10,000	200	7	0	1	14%
20,000	200	1	0	0	0%

Figure 5: Dose response curve for **A.** Nickel Chloride ($NiCl_2$) and **B.** Trichostatin A (TSA). TSA demonstrates following the rules of Haber's Law in the dose response curve. $NiCl_2$ demonstrates it does not follow the rules of Haber's Law. It may operate under another toxicokinetic parameter **C.** Tabular data used to generate Fig 5B **D.** Tabular data used to generate Fig 5A.

A



B

Duration	# Obs.	Oral Rad.	Oral Rad. Prop.
16-24 hpf	3017	1133	38%
24-32 hpf	1065	14	1%
1-24 hpf	744	210	28%
1-32 hpf	652	353	54%
1-96 hpf	426	169	40%

Figure 6: A. This graph is showing the importance of the 16-24 hpf exposure time period. Toxic effects increase when the time period is increased to 32 hpf from to 24 hpf. However, when 16-24 hpf is not included in the exposure window the toxic effect drastically decreases. **B.** Tabular data used to generate Fig. 6A and Fig. 8

The percentage of aboral radialization induced by TSA followed Haber's Law in certain circumstances. When a concentration-response relationship was graphed for aboral radialization induced by trichostatin A, the concentration that produced a 50 % induction of the abnormal phenotype (TC_{50}) could be linearly interpolated from the data found in Figure 5B. Such interpolations indicate that the 50 % radialization dose (TC_{50}) is 3.8 ± 0.3 nM when the exposure duration was 1-24 hpf, and the same parameter was 11.7 ± 3.8 nM when the exposure duration was 8-16 hpf. The Haber constant (k) for the 23 hour exposure would be 87.4 nM·hr compared to 93.6 nM·hr for the 8 hour exposure. These values are considered to be approximately

equivalent given the relatively crude calculations in the analysis. This makes TSA the only chemical examined in this study that successfully modeled the predictions of Haber's Law. However, in order to fully understand how these chemicals exert their toxic effects, more research needs to be done.

Expos. periods compare	p-value
1-32 hpf & 1-24 hpf	0.030
16-24 hpf & 1-24 hpf	0.317
1-32 hpf & 1-96 hpf	0.20118464
1-32 hpf & 16-24 hpf	0.121
24-32 hpf & 1-32 hpf	0.00222
24-32 hpf & 1-24 hpf	0.007
24-32 hpf & 16-24 hpf	3.0798E-05

Figure 7: Statistics and p-value when comparing various exposure periods using a 2-Tailed t-test analysis. P-value < 0.05 is considered statistically significant

Discussion:

The primary goal of this thesis was to determine if various forms of toxicity produced by different chemical agents after exposure during sea urchin development followed the predictions of Haber's Law. According to Haber's Law, the toxic effect is determined by the product of concentration and time (C x T) or various mathematical variations of this relationship.

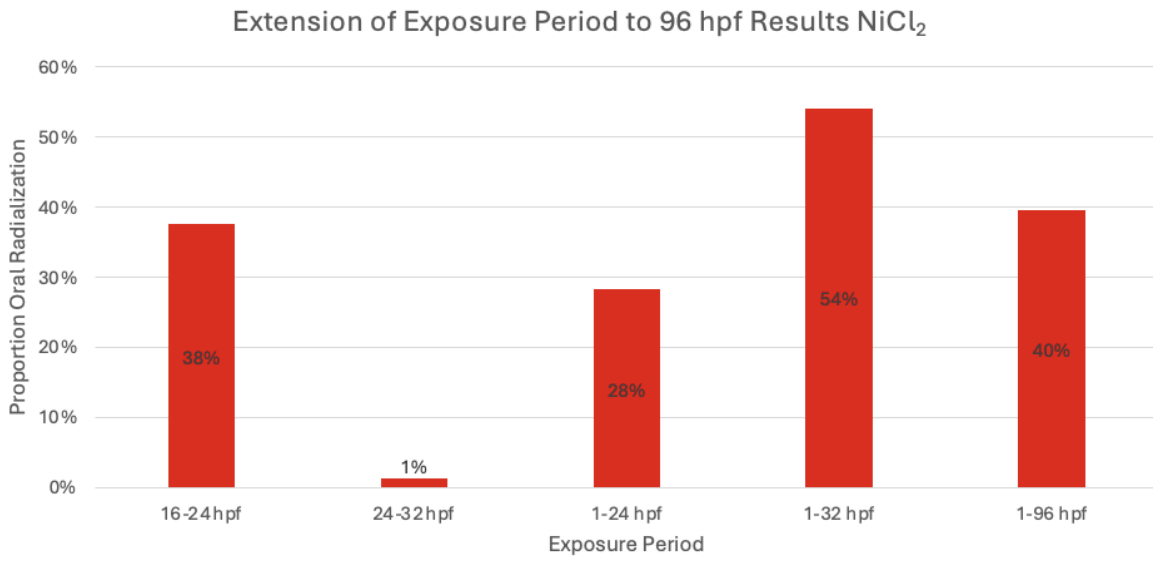


Figure 8: Data for $NiCl_2$ showing how toxic effects do not further exacerbate with increased exposure periods. It also shows the sensitive period of 16-24 hpf is critical for observing toxicity

One of the most interesting findings from this study was that sensitive period for induction of oral radialization by exposure to NiCl2 was approximately 16-24 hpf. This was determined by exposing embryos to 8-hour time intervals within a 24-hour period (1-8 hpf, 8-16 hpf, and 16-24 hpf) and assessing the proportion of orally radialized plutei. For the remainder of the exposure period the plutei were grown in clean sea water. The highest rate of oral radialization was observed during 16-24 hpf consistently (around 23 experiments and 2474 embryos observed), causing it to be determined the sensitive period. An additional exposure window of 24-32 hpf was added to test whether an extension beyond the sensitive period to 32 hpf would produce increased rates of oral radialization. The rates of oral radialization when exposed to a constant concentration of nickel chloride (3.33 μM) were 0 when the exposure occurred at 1-8 hpf, 1.4 % when exposure occurred between 8 and 16 hpf, 35.2 % when exposure was from 16 to 24 hpf, and 1.3 % when exposure occurred from 24 to 32 hpf (Figure 2B). Since the 8-16 and 24-32 hpf durations caused such low levels of oral radialization, the conclusion is that essentially the sensitive period for this form of dysmorphology is 16 – 24 hpf, but the fact

that there are some plutei that develop the aberrant phenotype when exposed outside this duration may indicate that the sensitive period may be somewhat greater than the 16 to 24 hpf duration (e.g. 15 to 25 hpf). This same phenomenon occurs with trichostatin A exposure where the sensitive period is predominantly 8-16 hpf, but there are small percentages of plutei with aboral radialization in the contiguous time periods (1-8 and 16-24 hpf). This issue was not examined in this study for either compound. Alternatively, in a large batch of embryos it may be that not all of the embryos are of exactly the same developmental stage. In this case, there may be a small percentage of embryos that have prematurely developed to what is considered the 16 hpf stage for the majority of the embryos by the 15 hpf time point. Alternatively, there may some small percentage of embryos that are developmentally delayed so that they reach the normal 24 hpf developmental stage at 25 hpf.

Interestingly, when the exposure period was extended to 32 hours and included the 16-24 hpf window (1-32 hpf), the rates of oral radialization increased compared to the 1-24 hpf time period, p-value= 0.030 (Fig 7). This means there is a statistically significant increase in rates of oral radialization in the 1-32 hpf exposure period compared to the 1-24 hpf exposure period. This is an interesting finding since the 24-32 hpf time period was essentially not part of the sensitive period Fig. 8). Since the 1-32 hpf exposure period produced a statistically significant higher rate of oral radialization than the 1-24 hpf exposure period it can be concluded that exposure during the 24-32 hpf time period is significant in terms of the impact on the percentage of dysmorphic plutei despite being a time period outside the sensitive period. Additionally, when the exposure period was extended past the 32 hpf (up to 96 hpf), the percentage of oral radialization did not increase (p-value= 0.20) (Fig. 7). This suggests that prolonged exposure beyond 32 hpf does not exacerbate toxicity. In the current literature there is not a known specific term found that

describes this 24-32 hpf exposure period where it seems to contribute to the overall toxicity but on its own essentially does not produce toxicity itself. However, it may be that this period relates to the period after exposure to a stress where some percentage of the damaged progeny undergo embryonic recovery. It has been shown that some percentage of the damage caused to embryos as a result of stress is subject to recovery during the gestational period. This has been demonstrated by showing the percentage of malformed offspring detected at times shortly after a stress may be significantly higher than the percentage that are observed at birth (Wilson 1954; Crowley et al. 1978; D'Amato 1982; Terry et al. 1996). Further research may be needed in order to characterize this specific time period and assign a definitive term to describe this time interval.

The raw data was collected by manually counting and categorizing sea urchin embryos with each of their unique phenotypes. As seen from Figure 1A and 1B, there is a broad spectrum of phenotypes observed when scoring the embryos. This brings up the issue of compound-specific outcomes versus non-specific outcomes. In 2009, a study in sea urchin embryos demonstrated that exposure to the classic teratogen thalidomide caused reduction of sea urchin arms (Reichard-Brown et al. 2009). This was considered a highly unanticipated finding because the specific limb reduction malformation phocomelia was induced in humans, non-human primates and rabbits, but was not detected in a variety of species including rodents which are the most popular species for testing potential teratogens (Neubert and Neubert 1997). Given the stringent species-specificity of this teratogenic agent, it was considered highly unlikely that a species as evolutionarily distant from human as the sea urchin would mimic the limb phenotype. Such a finding could advance the use of sea urchins in developmental toxicology testing. Furthermore, since the mechanism thalidomide embryopathy was unknown at the time and the

gene regulatory network of the sea urchin skeleton had recently been published (Oliveri et al. 2008), it was considered possible that elucidation of the mechanism in the sea urchin would provide clues regarding the mechanism in humans. However, the three chemical agents utilized in this thesis all produce this same developmental abnormality of absent arms in the sea urchin, and it is possible that all chemical substances at a sufficiently high dose may induce this phenotype. Thus, this phenotype is a non-specific developmental outcome of chemical stresses. It is therefore unlikely that the mechanism of this dysmorphogenesis in the sea urchin has any relationship to the mechanism of thalidomide malformations in humans.

There were some aspects of this study that were not performed due to the limitation of time. One limitation to this study was not varying the concentrations when looking at the different exposure periods. When testing how various exposure times affect $NiCl_2$ toxicity, the concentration was kept constant at 3.33µM, however, other concentrations were not tested. Our results would have been better supported had a range of doses been tested instead of just one. For future experiments, one could test how longer exposure times affects Haber's Law with varying NiCl2 concentrations.

Another limitation was the absence of molecular analysis to validate the results. Molecular analysis would have been useful to confirm that all embryos reached the same level of toxicity when scored manually. Additionally, it would have provided a deeper understanding of their toxicological profiles. Without molecular analysis, it is difficult to ensure that all embryos experienced toxicity to the same extent.

Conclusions:

The findings revealed that the sensitive period for induction of oral radialization in sea urchin embryos exposed to $NiCl_2$ occurs approximately between 16-24 hpf. The sensitive period may vary for different developmental phenotypes which may be determined by the chemical to which the developing urchins are exposed. For example, TSA induces aboral radialization in sea urchin embryos with a sensitive period that was determined to be approximately 8-16 hpf. Thus, the two chemicals cause different developmental abnormalities and therefore have different sensitive periods that include the developmental events crucial for the phenotype to be induced. Furthermore, this study demonstrates that exposures presumably outside the sensitive period for a particular developmental abnormality can impact the prevalence of the abnormality. Specifically, although the 24-32 hpf exposure period in the induction of oral radialization by nickel chloride was determined to be outside the sensitive period for this particular phenotype, it was a duration that was determined to contribute to an exacerbation of the percentage of plutei with the developmental toxicity. The highest rate of radialized embryos occurred at 1-32 hpf, but the proportion did not increase if you extended the exposure time beyond 32 hpf (up to 96 hours). Additionally, $NiCl_2$ did not appear to follow Haber's Law, $ZnSO_4$'s adherence remained inconclusive, and TSA aligned with Haber's Law when TC_{50} values were linearly interpolated from concentration-response curves. Molecular testing will be needed to confirm the findings from this study.

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