INVITED PERSPECTIVE

# Developmental Critical Windows and Sensitive Periods as Three-Dimensional Constructs in Time and Space*

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## ABSTRACT

A critical window (sensitive period) represents a period during development when an organism's phenotype is responsive to intrinsic or extrinsic (environmental) factors. Such windows represent a form of developmental phenotypic plasticity and result from the interaction between genotype and environment. Critical windows have typically been defined as comprising discrete periods in development with a distinct starting time and end time, as identified by experiments following an on and an off protocol. Yet in reality, periods of responsiveness during development are likely more ambiguous that depicted. Our goal is to extend the concept of the developmental critical window by introducing a three-dimensional construct in which time during development, dose of the stressor applied, and the resultant phenotypic modification can be utilized to more realistically define a critical window. Using the example of survival of the brine shrimp (*Artemia franciscana*) during exposure to different salinity levels during development, we illustrate that it is not just stressor dose or exposure time but the interaction of these two factors that results in the measured phenotypic change, which itself may vary within a critical window. We additionally discuss a systems approach to critical windows, in which the components of a developing system—whether they be molecular, physiological, or morphological—may show differing responses with respect to time and dose. Thus, the plasticity of each component may contribute to a broader overall system response.

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## The Concept of the Developmental Critical Window

The concept of a critical window is a basic and venerable tenet of comparative developmental physiology and morphology. Also referred to as a sensitive period, especially in the psychological and behavioral literature (e.g., Penhune 2011; Skogen and Overland 2012; Trabulsi and Mennella 2012; Nelson et al. 2014), the critical window is a specific period during development in which an animal's emerging phenotype is especially plastic and can be shaped by a wide variety of intrinsic or extrinsic (environmental) factors. Phenotypic modification of a system during its critical window is a form of developmental phenotypic plasticity, the ability of an animal to modify its phenotype as its environment changes during development. Such plasticity is ultimately constrained by genotype-environment interactions, defining a family of reaction norms at the individual, population, and species levels (for a review, see Hutchings 2011). Phenotypic plasticity is most often viewed as resulting in phenotypic changes aiding survival (Burggren and Reyna 2011; Hutchings 2011) and, indeed, is selected for through so-called plasticity genes (e.g., Loebrich and Nedivi 2009; Zhang and Ho 2011; Hensch and Bilimoria 2012).

How critical windows/periods are viewed in the literature essentially revolves around whether the phenotypic change induced during the critical window/period is adaptive or maladaptive. In the medically based neurobiological, psychological, and linguistic literature, for example, critical periods are those periods in which sensory development, motor and language skills, higher cognition, and other neurological activities develop and mature (e.g., Rice and Barone 2000; Hensch 2004; Hensch and Bilimoria 2012). That is, phenotypic changes must happen during these specific periods for normal development to occur.

In the toxicological, teratogenic, and comparative physiological literature, however, critical windows are more often depicted as those specific periods when normal development is especially vulnerable to internal or external stressors, with the impact of such stressors being alterations away from the normal phenotype (fig. 1). Such phenotypic modification arises at least in the short term, and such changes are often permanent (see Burggren 1998; Nijland et al. 2008; Loebrich and Nedivi 2009). Significantly, the window is often viewed as an abrupt change in developmental sensitivity, a topic we return to below. The view that phenotypic changes during critical windows lead especially to maladaptive phenotypes is particularly prevalent in the medical literature as it pertains to human development (fig. 2). A graphic and tragic example of maladaptive phenotypic modification involving a critical

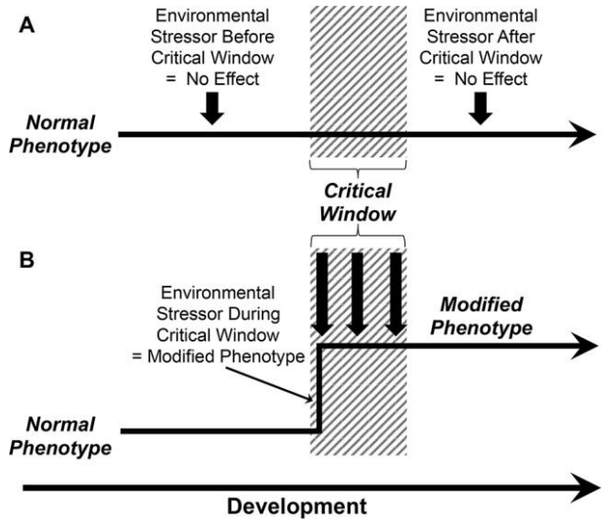


Figure 1. Traditional view of the critical window. Presence of a stressor before or after the critical window (*A*) results in little or no phenotypic modification, whereas presence of a stressor at any point during the critical window (*B*) results in phenotypic modification. In this depiction, the critical window is viewed as a discrete period and the effects as digital (i.e., off-on-off).

window is that of the drug thalidomide, α-(N-phthalimido) glutarimide. This effective sedative also reduces morning sickness in pregnant humans during the end of the first trimester of pregnancy (Knobloch and Rüther 2008). First released in West Germany in 1957 and prescribed to pregnant women primarily during the 1960s, it was soon discovered that even a single dose of thalidomide was also a potent disruptor of normal limb development, the critical window for which overlapped almost exactly with the period of morning sickness in mothers (fig. 2). The tragic result was the birth of so-called thalidomide babies born with limbs highly truncated or completely absent. Other examples of human disease resulting from stressors occurring during critical windows include atherosclerosis resulting from childhood malnutrition (Singhal 2009) and lung development abnormalities in response to specific environmental chemicals (Miller and Marty 2010).

In the comparative physiological literature, critical windows leading to modified phenotype have been demonstrated in invertebrates, fish, amphibian, and avian models. Examples from the invertebrate literature include changes in osmotic and ionic regulation as a sign of high ammonia toxicity during the early nauplii stage of the shrimp *Penaeus japonicus* (Lin et al. 1993) and a higher occurrence of body, eye, and pigment abnormalities after exposure to copper and zinc during the late embryonic stages of the estuarine crab (*Chasmagnathus granulates*; Lavolpe et al. 2004).

In ecotoxicology studies of fishes, exposure to a variety of contaminants has revealed developmental periods of greater susceptibility. For example, exposure to endocrine-disrupting chemicals during early larval development of fish species—such as zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), and fathead minnow (*Pimephales promelas*)—influences sexual differentiation and gonadal development during a critical window in which the reproductive system is particularly plastic (Koger et al. 2000; van Aerle et al. 2002; Ankley and Johnson 2004; Maack and Segner 2004). In another zebrafish study exploring the concept of fetal alcohol syndrome, the toxic effects of ethanol exposure are highly stage specific. Zebrafish mortality is highest after ethanol exposure during gastrulation, while morphological malformations and a reduction in larval swimming performance are most severe after ethanol exposure during embryonic organogenesis (stages *prim 6* and *prim 16*; Ali et al. 2011*a*).

In amphibians, stage-specific toxicity of xenobiotics that disrupt retinoid signaling pathways also occurs. In the Western clawed frog (*Xenopus tropicalis*), exposure to the algicide triphenyltin during the late tail bud to early tadpole stages results in increased fin defects compared with exposure earlier in development, indicative of a critical window for fin formation (Yuan et al. 2011). In comparison, embryos of the African clawed frog (*Xenopus laevis*), green frog (*Lithobathes clamitans*), mink frog (*Lithobathes septentrionalis*), and wood frog (*Lithobathes sylvaticus*) are most susceptible to retinoic acid–induced mor-

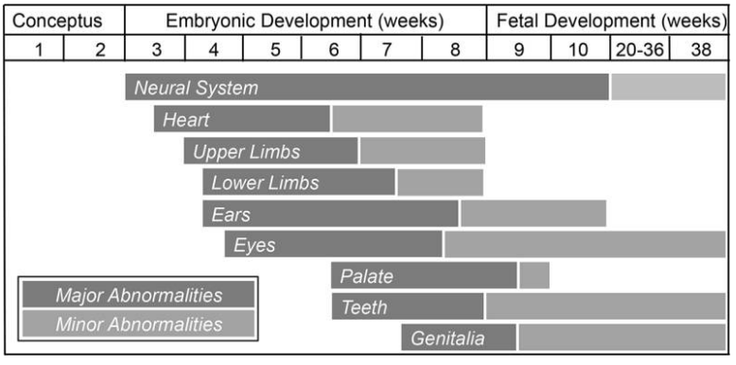


Figure 2. Critical windows for human development, depicting the periods for major and minor disruptions to normal morphological development.

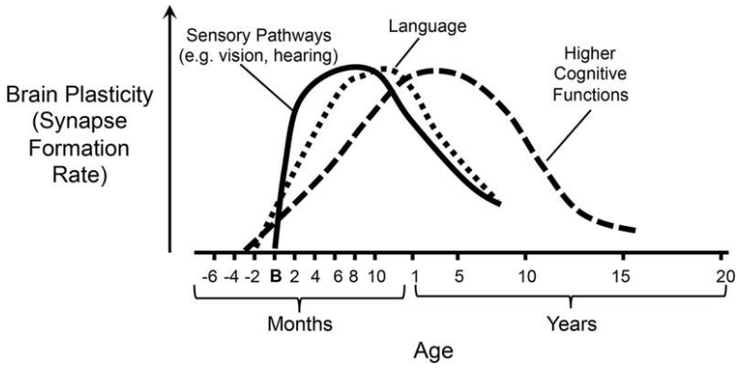


Figure 3. Critical periods for human brain plasticity, as measured by the rate of synapse formations. B, birth. After Nelson (2000).

tality and abnormalities during midblastula stages (Degitz et al. 2000).

Exposure of embryonic chickens (*Gallus gallus*) to hypoxia during certain windows results in altered morphological and metabolic phenotypes (Dzialowski et al. 2002; Chan and Burggren 2005; Ferner and Mortola 2009). In another avian model, the Northern bobwhite quail (*Colinus virginianus*), a preincubation critical window has been identified, with exposure to low constant, low fluctuating, high constant, or high fluctuating temperatures during this time influencing survival and oxygen consumption later in embryonic development (Reyna 2010).

A key question is whether existing differences between the responses of organisms in early development are merely species specific or whether they reflect subtle yet significant differences in experimental protocols, dosing, and exposure times. The aforementioned studies—and how to interpret them—highlight both the growing importance of critical windows in comparative physiology as well as the need to formulate a more sophisticated approach to their study.

## How Clearly Demarcated Are Critical Windows in Development?

Critical windows are typically presented as though they are clearly defined periods. That is, at any given time in development, a particular organ system of a developing animal is either outside its window or inside its window, as depicted in

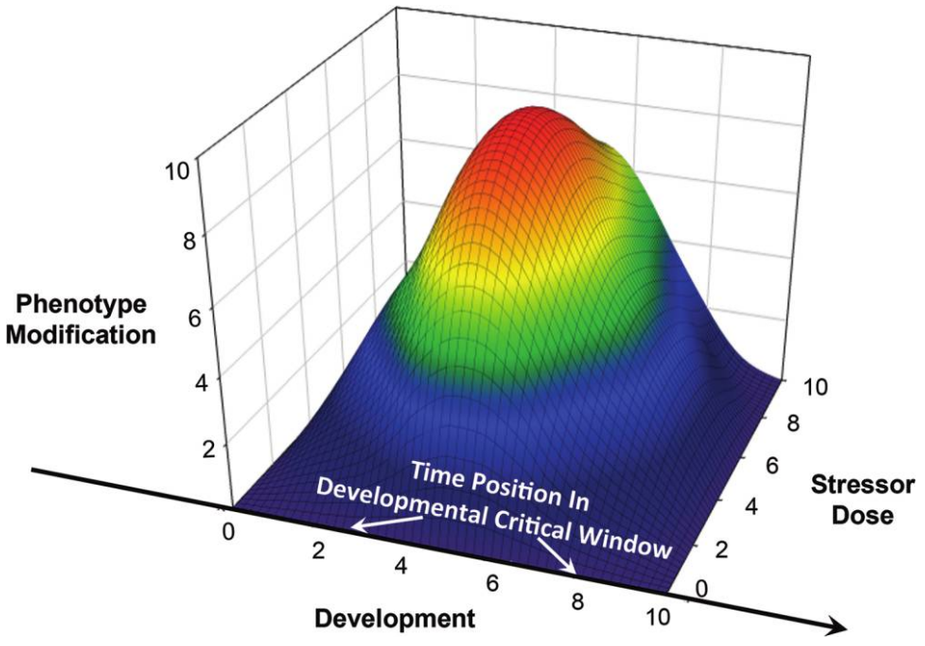


Figure 4. Critical window as a three-dimensional construct, defined by dose-time interactions in the developmental critical window. The consequence of these interactions is a large range of potential phenotypic modifications.

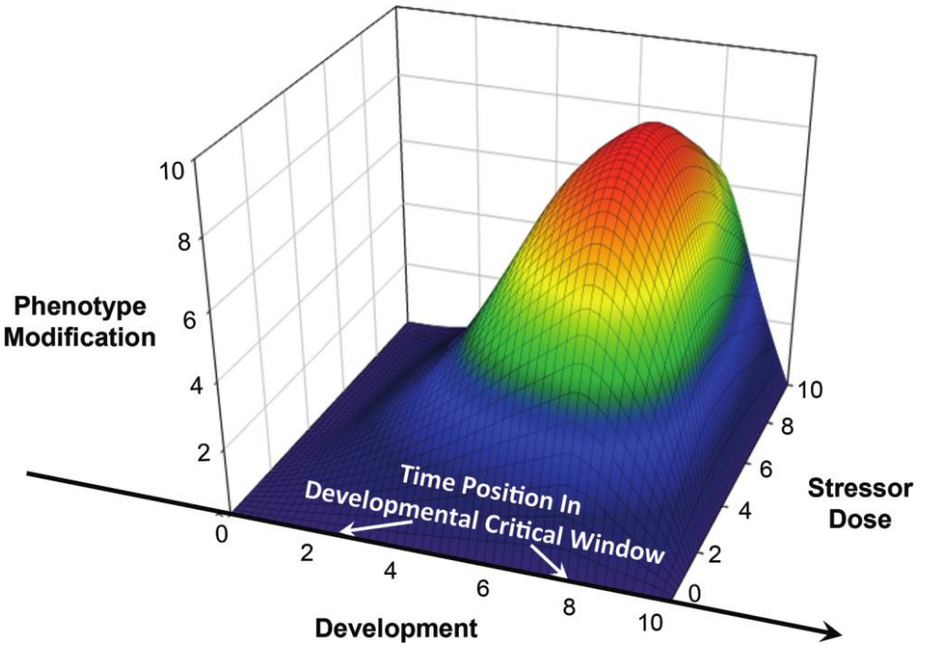


Figure 5. The three-dimensional critical window for phenotypic modification of an organ system may be skewed in complex ways, depending on specific susceptibility of phenotype to stressors at particular times in development. Contrast this critical window with the symmetrical critical window depicted in figure 4.

figures 1 and 2. Putting it differently, the critical window is viewed as having distinct edges, like a true window. The experimental protocols used in the search for critical windows generally involve the switching on and off of an environmental stressor during certain—often arbitrary—times in development. Then, if functional or structural changes are detected, a critical window is defined by the time of exposure to the stressor. For example, exposure to hypoxia from days 6–12 of chicken embryonic development alters the oxygen consumption of the resultant hatchlings; thus, days 6–12 are considered a vulnerable period for metabolic development (Dzialowski et al. 2002). Likewise, hypoxia during days 1–6 reduced beak growth, while hypoxia during days 12–18 increased the mass of chorioallantoic membrane, demonstrating variable timing of the critical windows of these organs (Chan and Burggren 2005). Days 1–6, 6–12, and 12–18 each represent approximately a third of chicken embryonic development. Likewise, amphibian development has been divided into thirds for critical windows studies (Hanlon and Parris 2014). These abrupt, arbitrary divisions are a good first approach to detecting critical windows, but they do not permit the full consideration of the developmental processes within each time period, nor do they reveal the width of the real critical window. While the precise developmental timing of critical windows is typically viewed as fixed, it has been recognized for some time that the specific parameter being measured within the fixed critical window waxes then wanes as development progresses. For example, the critical periods for brain plasticity related to sensory pathways, language, and higher cognitive functions are shown in figure 3. Each of these processes has a different critical period, and for each of them, there are changes in sensitivity over time.

The view of critical windows with varying sensitivity within the window indicated in figure 3, though still two dimensional, at least suggests a more sophisticated view of critical windows than depicted in figures 1 and 2, in that the developmental windows are not portrayed with sharp edges. Yet in this article, we advocate an even more nuanced view of critical windows, treating a critical window as a three-dimensional construct of time, phenotype effect, and effector dose. Why is this approach needed, since isn't it intuitively obvious that phenotypic effects vary with stressor doses? In fact, most studies of developmental critical windows per se focus on the timing of the phenotypic modification resulting from a single stressor dose. Indeed, our survey of the literature indicates that about two-thirds of studies examining critical windows employ only a single dose. A much less common approach involves measuring the potentially graded effects of dose within the critical window. Indeed, by taking dose into account as an additional variable, the actual shape of the critical window thus becomes defined not just by the particular point in developmental time—which remains, of course, as a fundamental variable—but also by the dose of the intrinsic or extrinsic stressor at any given point in development.

To illustrate this three-dimensional construct, consider in figure 4 a stressor dose of arbitrary level 4 at developmental time point 0; there is no measured phenotypic change because the developing organism has just entered its critical window. However, at developmental time point 2, there is some phe-

notypic modification, which increases at time points 4 and 6 as the organism moves through the middle of the critical window. As the organism begins to leave the critical window, at time point 8, the phenotypic modification is decreased and eventually becomes undetectable as we leave the critical window at time point 10. It is important to note that the measured phenotypic change at each time point is dependent on the dose used. Generally stated, then, the product of time × dose defines the magnitude of the phenotypic effect. Consequently, at lower stressor doses, the phenotypic effect may be much lower, to the point of being below the threshold for observation, while at higher stressor doses, measurable phenotypic effects occur early in the critical window, culminating in the greatest phenotypic modification at the highest effective dose well within the critical window.

The three-dimensional view of the critical window depicted in figure 4 suggests a symmetrical set of time-dose interactions. However, there is no reason a priori to anticipate that such time-dose reactions would peak at the center of the critical window. Figure 5 shows one of many alternative views of a hypothetical critical window, in which a given dose of a stressor certainly has modest effects early in development but exerts its greatest effect toward the end of the critical window.

Are these time-dose interactions hypothetical, and/or are they of merely abstract interest to comparative developmental physiologists? To address the first question, our laboratory has acquired preliminary data strongly supporting our proposed time-dose interaction construct (C. A. Mueller, C. Willis, and W. W. Burggren, unpublished manuscript). Brine shrimp (*Artemia franciscana*) were chosen as an animal model because of their short generation times (~15 days at 25°C) and the potential for phenotypic modification with exposure to different environmental salt levels (Engel and Angelovic 1968; Abatzopoulos et al. 2003; El-Bermawi et al. 2004; Pinto et al. 2013).

A necessarily complex experimental design was created that involved exposure of developing brine shrimp to a range of salinities at different periods during development, a design that generated the phenotype data required to populate the three-dimensional structure grid depicted in figures 4 and 5. Commercially available *A. franciscana* cysts (San Francisco Bay Brand, Newark, CA) were hatched in a salinity of 20 ppt at 25°C in an inverted 2-L plastic bottle. Using 20 ppt as a control (recommended salinity for rearing), *A. franciscana* were exposed to 10 (hyposaline), 30, 40, or 50 ppt (hypersaline) during days 1–6, 7–9, 10–12, or 13–15 of development (fig. 6). Solutions were created with Instant Ocean Sea Salt and buffered to pH 7 with sodium bicarbonate. On day 1, ~700 hatched nauplii were added to one of five 10-L tanks housed within a cooler thermostated to 25°C. Each tank was filled with 2 L of either 10, 20, 30, 40, or 50 ppt salinity and 15 mL food mix consisting of 10 mg $mL^{-1}$ spirulina powder (Ta

| Treatment Number | | Time Periods Tested for Critical Window Potential | | | |
|---|---|---|---|---|---|
| | | Days 1-6 | Days 7-9 | Days 10-12 | Days 13-15 |
| 1 | Hypo/ hyper salinity | 10 ppt | 20 ppt | 20 ppt | 20 ppt |
| 2 | | 30 ppt | 20 ppt | 20 ppt | 20 ppt |
| 3 | | 40 ppt | 20 ppt | 20 ppt | 20 ppt |
| 4 | | 50 ppt | 20 ppt | 20 ppt | 20 ppt |
| 5 | Control | 20 ppt | 10 ppt | 20 ppt | 20 ppt |
| 6 | | 20 ppt | 30 ppt | 20 ppt | 20 ppt |
| 7 | | 20 ppt | 40 ppt | 20 ppt | 20 ppt |
| 8 | | 20 ppt | 50 ppt | 20 ppt | 20 ppt |
| 9 | | 20 ppt | 20 ppt | 10 ppt | 20 ppt |
| 10 | | 20 ppt | 20 ppt | 30 ppt | 20 ppt |
| 11 | | 20 ppt | 20 ppt | 40 ppt | 20 ppt |
| 12 | | 20 ppt | 20 ppt | 50 ppt | 20 ppt |
| 13 | | 20 ppt | 20 ppt | 20 ppt | 10 ppt |
| 14 | | 20 ppt | 20 ppt | 20 ppt | 30 ppt |
| 15 | | 20 ppt | 20 ppt | 20 ppt | 40 ppt |
| 16 | | 20 ppt | 20 ppt | 20 ppt | 50 ppt |

Figure 6. Schematic of the experimental protocol for the onset, termination, timing, and dosage of exposure to a salinity stressor during development to map critical windows in the brine shrimp *Artemia franciscana* (C. A. Mueller, C. Willis, and W. W. Burggren, unpublished manuscript).

Aquaculture, Malta) and 2–3 drops of microalgae paste (Nutraplus Micro, Nutra-Kol, Mullaloo, Western Australia). On day 6, 40 brine shrimp were transferred via pipette to 250-mL round plastic containers containing 150 mL of water at the relevant salinity (as per fig. 6), 1 mL of food mix, and 0.5 mL of live *Dunaliella salina* (152160; Carolina Biological Supply Company, Burlington, NC). In both the tanks and the containers, water was aerated and brine shrimp were exposed to 12L : 12D light. At the end of days 9 and 12, brine shrimp were transferred to new containers with fresh water at the relevant salinity, irrespective of whether they were transferred directly to the same or different salinity. Twice daily, dead shrimp and waste were removed and remaining brine shrimp were fed ~0.25 mL of food mix and ~0.25 mL live *D. salina*. To replicate treatments, the entire experiment was repeated in the same manner four times.

Survival of *A. franciscana* was determined at the end of each window (days 6, 9, 12, and 15) and averaged across the four replicates (fig. 7). The presentation format of figure 7 can be considered a traditional way of presenting such data but is not ideal for visualizing the effect of salinity on survival. To map salinity-dependent phenotypic changes throughout development, mean survival is transferred to the three-dimensional form (fig. 8). The strong interaction between time and dose (in this case, salinity) is clearly evident. Early in development, survival is highest at the lowest salinity dose (i.e., 10 ppt) but falls to near 0 at the highest salinity dose of 50 ppt. Yet with hyposaline/hypersaline exposure later in development, survival decreases at the lowest salinity and increases at the highest salinity as the brine shrimp approach adult stage. Thus, our data underscores the complex three-dimensional interaction between time, dose, and the measurable phenotypic modification.

Previous studies examining survival of various *Artemia* strains raised chronically in different salinities (ranging from 5 to 180 ppt) during development found either little effect of salinity or reduced survival at higher salinity (Vanhaecke et al. 1984; Triantaphyllidis et al. 1995; El-Bermawi et al. 2004; Pinto et al. 2013). Salinity tolerance is variable and strain specific, but our study suggests that reduced survival under higher salinities after chronic exposure may be driven by stress during high salinity exposure in the first few days of development. Furthermore, we can hypothesize that *Artemia* strains that can survive extreme salinities early in development are likely to be those that show little effect of salinity during chronic exposure. The possible link between the results from chronic exposures and multiple-dose critical window experiments underscores the applicability of this experimental approach to understanding how tolerance to stressors changes during development.

Our survival data for *A. franciscana* is just an example of the first step in undertaking experiments that are geared toward addressing our three-dimensional concept of critical windows. To advance even further, the length of the exposure time should be varied, and exposure windows should overlap. In the first instance, varying the exposure window size would determine the size of the critical window. Using the brine shrimp as an example, exposure to a particular salinity during

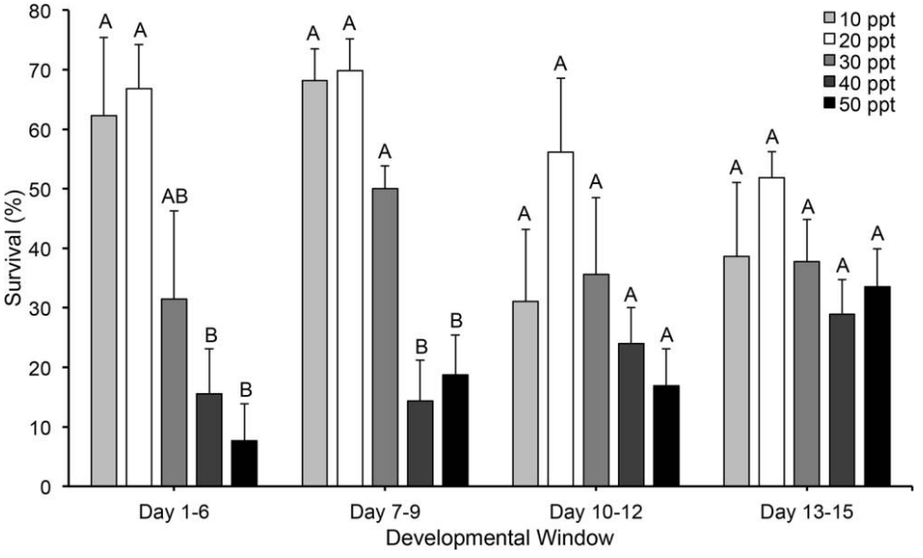


Figure 7. Mean survival (%) of developing brine shrimp (*Artemia franciscana*) at the end of each developmental window (d) after exposure to different salinities (ppt), with 20 ppt used as the control salinity. Mean survival for each treatment is averaged from four distinct, replicated experiments. Different letters indicate significant differences ($P < 0.05$) between salinities within each developmental window (one-way ANOVA, Tukey honest significant difference pairwise comparisons). Despite trends for altered survival within a single salinity across developmental windows, these were not significant.

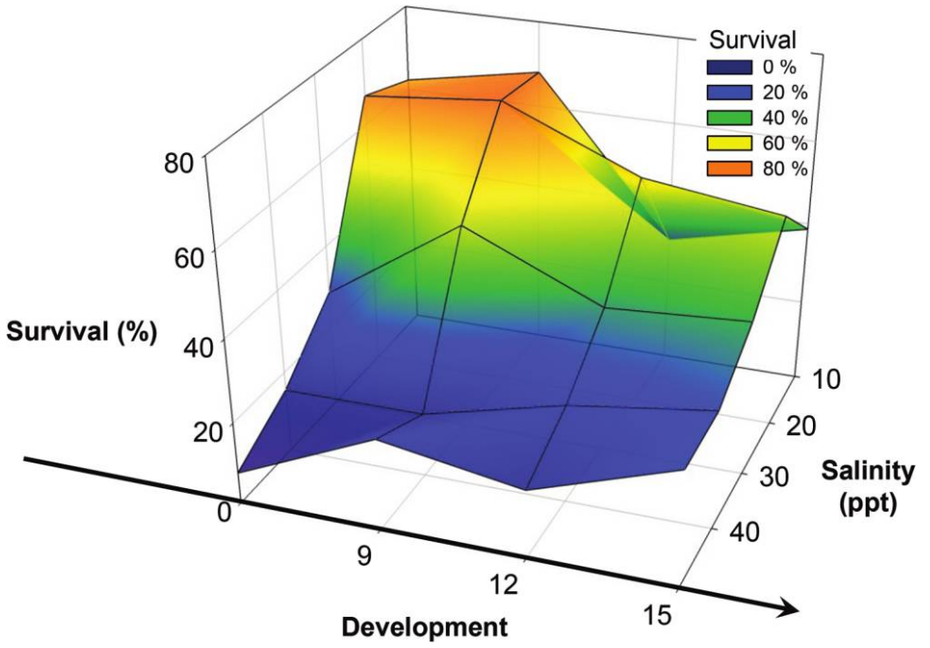


Figure 8. Relationship between salinity (ppt), mean survival (%), and developmental time (d) in brine shrimp (*Artemia franciscana*), based on data from four distinct, replicated experiments. Discrete points in the plot represent mean values for survival at that time. Note that the critical window for survival is defined by both the degree of salinity and the position within the overall critical window for development.

days 7–9 of development may produce the same results as when exposure occurred during days 6–10 or 5–11. Second, an overlap in the exposure windows will allow the outer limits of the window to be defined. Using the brine shrimp example again, the magnitude of phenotypic changes during exposure from days 7–9, 8–10, and 9–11 will define when the stressor effect diminishes and eventually disappears. These approaches, together with a range of doses, may necessitate a large number of treatments or several experiments but would tease apart the outer limits—and thus the size—of a critical window.

## Critical Windows in Comparative Developmental Physiology

### *Determination of Critical Windows*

Is a three-dimensional view of time-dose interactions that modify phenotype simply of abstract interest, or might this approach have significance to comparative developmental physiologists and the experiments they design? We believe that, just like the proverbial iceberg, only the tip of the possible suite of phenotypic effects of stressors during development has been revealed to date. In the context of three- rather than two-dimensional critical windows, the apparent first onset of the critical window for the structure or process of interest is, we speculate, often defined too late in development, and the end of the critical window is set too early. Moreover, some phenotypic modifications that may be of considerable physiological, ecological, or even evolutionary interest may be completely missed because only high doses of stressor were used or only highly modified phenotypes were assessed (fig. 9). Compounding this situation is the fact that there are numerous sources of variation, including experimental measurement error, plasticity in the timing of developmental events that may introduce interindividual variation (Spicer and Burggren 2003; Spicer et al. 2011), and epigenetic influences that affect physiological data (for a review, see Burggren 2014). Thus, determination of the critical onset period detected by physiological variation may be more and more difficult to identify as lower and lower doses are deployed or more subtle molecular, anatomical, or physiological effects are assessed.

As previously alluded to, approximately two-thirds of experiments that identify critical windows do so using just one dose of a stressor. For example, many toxicology studies in developing fish and amphibians use just a single dose of a chemical stressor to define periods of susceptibility (Koger et al. 2000; van Aerle et al. 2002; Hogan et al. 2008; Hanlon and Parris 2014). In some instances, multiple doses are used in pilot studies to determine an effective dose (Ali et al. 2011*b*), or multiple doses are used in just one window within development rather than at all time points under study (Maack and Segner 2004). The use of one suitable or effective dose ignores the vital information that a multiple dose approach may reveal about the critical window. Those studies that do utilize experimental protocols with a factorial design of exposure window and stressor dose demonstrate the usefulness of multiple stressor doses when assessing critical windows (Degitz et al. 2000; Hu et al. 2009; Aronzon et al. 2011; Yuan et al. 2011). For example, multiple doses of retinoic acid in three frog species revealed that the doses that are lethal early in development are

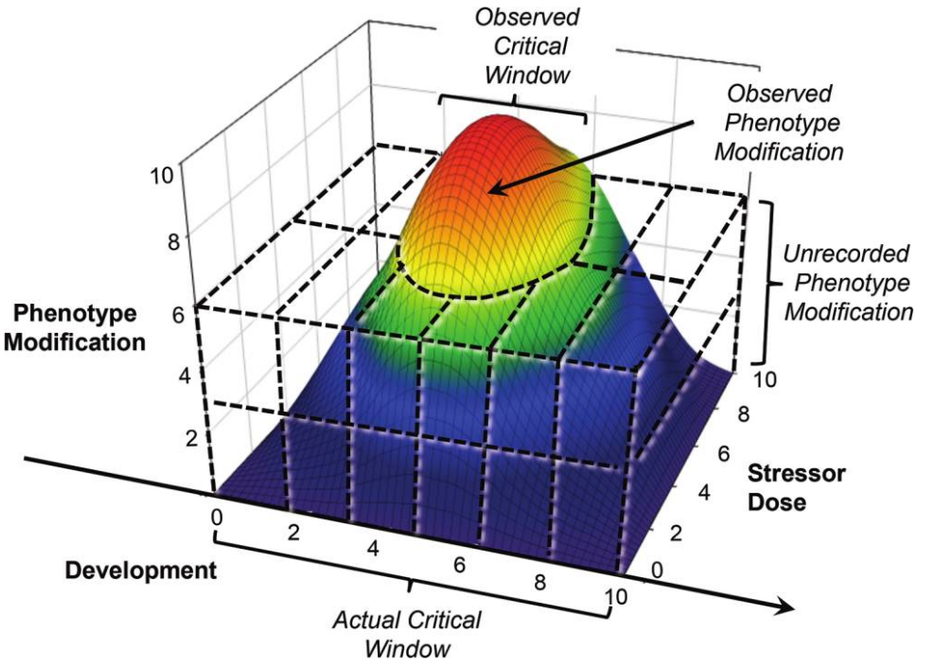


Figure 9. Critical windows may be greatly underestimated in developmental studies that use only high doses of stressor or look for only highly modified phenotypes. In this hypothetical example, potentially significant phenotypic modifications that would occur within the critical window below dose 4 escape observation, as do minor phenotypic modifications below level 6.

much lower than those that are necessary to cause limb abnormalities later in development (Degitz et al. 2000). Hence, frog embryos appear to have one critical window for survival and another critical window for limb malformations, and the detection of these windows are dose dependent.

### *An Organ System Approach to the Developmental Critical Window*

Historically, most of the literature on critical windows revolves around anatomical abnormalities, likely because such anatomical disruptions are most obvious to observe and are relatively easily measured. The classic view of critical windows for human anatomical development has already been shown in figure 2. Two aspects of this classic view are worth highlighting. First, the windows are typically depicted as distinct and clearly defined. Second, critical windows are shown to differ for different organ systems. In relation to the first aspect, we have discussed that a critical window may not be clearly defined by a distinct beginning and end, and to address the second aspect, we also suggest that an organ system approach be used to examine sensitive periods in development. It is important to recognize that critical windows—while most readily observed through their anatomical manifestations—can also be molecular, physiological, anatomical, behavioral, and so on in nature. Each organizational level or domain may have different sensitivities and different time courses through development (fig. 10). The overall system response to the stressor is an amalgam of all changes in all component parts. Thus, a hypoxic stressor, for example, might not only result in anatomical modification of the developing lungs (e.g., Lechner and Banchero 1980; Xu and Mortola 1989; Sekhon and Thurlbeck 1996) but also permanently reset chemoreceptor sensitivity and ventilation rates (Okubo and Mortola 1990), all of which would result in a collective modification of the respiratory system. Consequently, a more nuanced way to conceptualize a critical window is to contemplate a system critical window. Such a critical window for an organ system (e.g., respiratory, renal, skeletal, nervous) would then run from the earliest time point of sensitivity of the earliest component to be affected (hypothetically, the molecular critical window in fig. 10) to the last sensitivity of the last developing component (the anatomical critical window). Of course, there are numerous variations for how and when different system components may respond. An anatomical critical window, for example, may occur before—and have impact on—a subsequent physiological critical window, and this is likely to be dependent on the system and organism of interest.

Phenotypic modification of a system is most likely to involve modification at multiple levels because, simply put, molecular changes often underpin structural and/or physiological changes. This begs the question of whether a phenotypic change could involve only one of these levels of changes or whether by necessity all must be involved. If a critical window is narrow enough, might a system pass so rapidly through its critical window that there is time for only molecular phe-

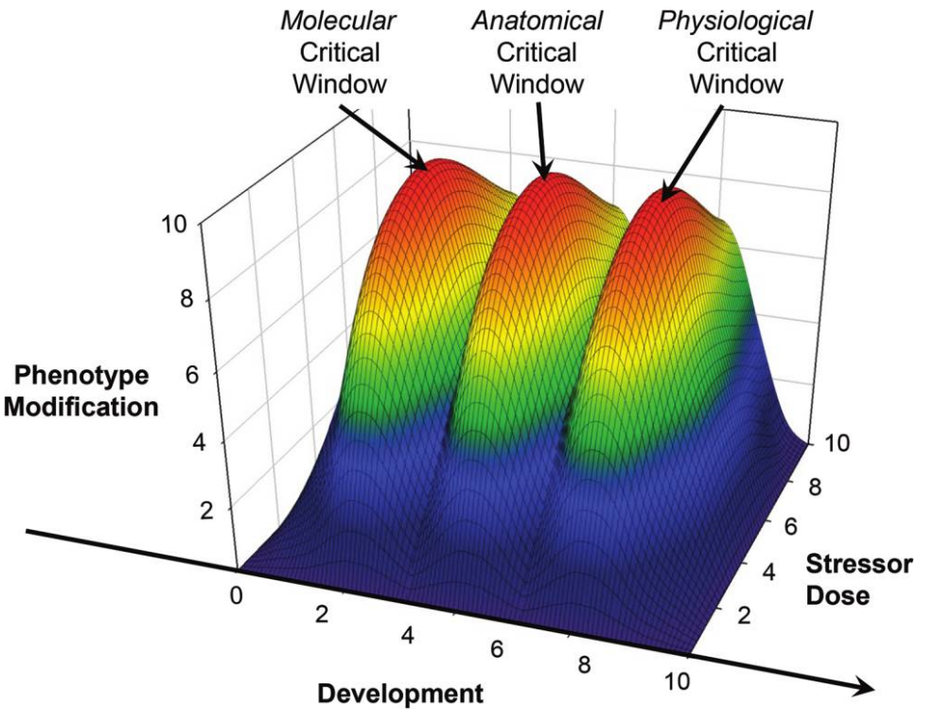


Figure 10. Each organ system, inherently composed of an array of phenotypic characteristics, can be thought of having an amalgamated critical window comprised of individual critical windows for anatomical, physiological, molecular, and other phenotypes representing different organizational levels of the organ system.

notypic changes to occur, without there being time for major morphological and/or physiological responses? Addressing such questions will be possible using only a systems approach to critical windows.

*Critical Windows and Self-Repair Capabilities*

Comparative developmental physiologists studying the disruption to normal development caused by environmental stressors in the context of critical windows sometimes go on to investigate any subsequent self-repair capabilities. For example, the change in phenotypic sex after developmental estrogen exposure in zebrafish was reversible once the fish were returned to clean water after exposure during the critical period for sex differentiation (Hill and Janz 2003). The same exposed fish showed repair of histopathological changes in the kidney and liver that were present immediately after estrogen exposure (Weber et al. 2003). The ability for self-repair can be thought of as dependent on the developmental processes that occur after exposure, but self-repair may also be related to when the actual exposure occurs. In other words, when phenotypic change occurs after exposure during a critical window, is there also a critical window of exposure in which self-repair following the phenotypic change is possible? To our knowledge, no studies have examined whether the ability to regain the normal developmental trajectory (potentially self-repair capabilities) are determined by the interaction between the window of exposure and the dose of the stressor given. We can hypothesize that an animal may be able to self-repair at lower doses but that higher doses may create modifications that are just too extreme to overcome, resulting in permanent phenotypic changes. The threshold dose for self-repair may differ depending on the individual system component and whether the alteration is molecular, morphological, or physiological.

*The Critical Onset Point*

In addition to identifying critical windows, many comparative physiological studies take a similar approach of using applied stressors at various points in development to identify the onset in development of regulatory mechanisms and their components. In the family of terms described above (critical window, critical period), we propose the term "critical onset point" to describe the first onset of physiological regulatory mechanisms. The neural regulation of cardiovascular function (Seidler and Slotkin 1979; Fritsche 1997; Unno et al. 1999; Crossley and Altimiras 2000; Crossley et al. 2003*a*, 2003*b*), thermoregulation (Tazawa et al. 2001; Khandoker et al. 2003; Dzialowski et al. 2007), or iono-osmoregulation (Doneen and Smith 1982*a*, 1982*b*; Bodinier et al. 2010) are among the many regulatory systems that have been examined for critical onset points in developing vertebrates. In such studies, typically the embryo at various points in development is exposed to one of a variety of environmental stressors, for example, low oxygen, abnormal

temperature, or elevated salt load. The first occurrence in development of the ability to regulate toward normal, homeostatic levels is then recorded, verified by pharmacological blockage of the presumed reflect, and that specific time point—the critical onset point—is reported as the morphological and functional maturational point of that regulatory system. An example of this approach has been employed to describe, for example, the onset of vagal involvement in the bradycardic response of the heart of the developing avian embryo (Tazawa et al. 1992; Crossley and Altimiras 2000; Chiba et al. 2004). The problem with almost all such comparative physiological experimental designs (including, out of fairness, almost all the studies of the current authors) is that these studies typically use only one or at most a few levels of stressor. Yet in the context of the paradigm of the three-dimensional time-dose-phenotype response described above, it is possible that without exploring a broad range of doses, the critical onset point is inaccurately estimated, most likely by indicating a point later in development than when the regulatory system is actually capable of exerting subtle regulation.

## Synthesis and Future Directions

Studies examining the influence of the environment on developmental processes continue to be carried out with often a rudimentary understanding of the concept of critical windows. When critical windows are, indeed, appreciated, they are typically investigated as windows with sharp rather than blurred edges (i.e., viewed as a digital phenomenon). Consequently, it is our belief that large numbers of subtle yet still highly significant effects in terms of survival, fitness, performance, and so on are being overlooked by biologists studying the interactions between the environment and organism development. We advocate a three-dimensional approach to investigate critical windows, in which the actual phenotypic modification is a function of time in development and dosage of the stressor.

Mapping of three-dimensional critical windows in any degree of granularity by necessity involves complex, factorial experimental designs. It also requires large numbers of experimental organisms and observations. Yet the rewards will be large in terms of enhanced understanding of developmental processes and how they are affected.

Several aspects of critical windows dimensionality warrant further exploration. For example, can exposure to stressors during the critical window for development affect just a subset of the total components of an organ system (e.g., only the alveoli but not the bronchi of developing lungs, or the atria but not the ventricles of the heart), or are all components affected? Related to this notion, can exposure during a critical window change the order of component appearance and/or development? This would, in essence, be an example of heterokairy (Spicer and Burggren 2003).

In this article, we have also discussed how different organizational levels (e.g., molecular, cellular, morphological, physiological, behavioral) could have different timing of critical windows in response to exposure to the same range of stressors. A variation on this theme of differential timing could also involve the differential responses of integrated systems that regulate a homeostatic function. Consider the integrated response of adult terrestrial vertebrates to acid-base challenge. In response to an $H^+$ load, for example, intracellular buffer systems react within seconds, $CO_2$ is blown off through hyperventilation within minutes, and renal excretion of $H^+$ occurs within hours. Yet all three actions occur in a coordinated fashion to mitigate acidosis. What might be the effect on the development of such a multifaceted regulatory system in an animal that passes through key developmental stages for the system in mere hours—would some parts of the system be modified but others developing afterward be unaffected, for example? Or would stressor-induced modification of the short-term component lead to modification of the longer-term component, even if it was not exposed to the stressor during its critical window? Answering these and additional developmental questions in the context of critical windows will contribute to a much deeper understanding of the relationship between development, stressor dose, and phenotype and the broader interaction between the environment and developmental trajectory.

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