

Critical Windows in Animal Development: Interactions Between Environment, Phenotype and Time

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Abstract

Observable phenotypic traits of an animal are a result of the interaction between the genome and environment. Differences in phenotypic traits between individuals induced by the environment, an indicator of phenotypic plasticity, may have immediate and long-term consequences for individuals, populations and species. During development, animals are often most responsive or susceptible to changes in their environment, and phenotypic plasticity can be particularly prevalent. It is increasingly apparent that the way in which the environment influences an animal's physiology may differ not just across a species' lifetime, but also within a species' ontogeny. Periods of development during which an animal may show greater likelihood of phenotypic changes are termed 'critical windows' or 'sensitive periods'. Across animal taxa, experiments utilize exposures to particular environmental, chemical or pharmacological stressors at certain time points of development to detect and understand critical windows during development. This chapter examines the emergence of critical windows as an important physiological concept using examples from the literature that span model and non-model invertebrates and vertebrates exposed to a range of environmental conditions. This chapter also outlines considerations for the continued search for critical windows. Critical window experimental designs can range in complexity, and variables such as the timing of exposures, if a single or multiple doses of a stressor are used and when endpoints are assessed should be considered. A continued focus on critical windows will no doubt contribute to our growing knowledge of the interaction between the environment and physiology during animal development.

1. Introduction

1.1 *Definition and History of Developmental Critical Windows*

Phenotype is the result of the interaction between the genome and environment. Differences in phenotypic traits between individuals induced by the environment, an indicator of phenotypic plasticity, may have immediate and long-term consequences for individuals, populations and species. During development, animals are often most responsive or susceptible to changes in their environment, and phenotypic plasticity can be particularly prevalent. Recognizing and understanding both the potential positive and negative consequences of plasticity is at the core of developmental physiological research. Chronic exposure to certain environmental conditions throughout development is the classic approach to understanding how environment and physiology interact. However, it is apparent that the way in which the environment influences an animal's physiology may differ not just across a species' lifetime, but also within a species' ontogeny. Periods of development during which an animal may show greater likelihood of phenotypic changes are termed developmental 'critical windows' or 'sensitive periods'.

The terms 'sensitive period' or 'critical period' first appeared in the medical literature in the 1940's and 1950's, and became prevalent in the 1970's and beyond, particularly in relation to the required developmental processes that occur at certain times during human development (Vito et al. 1979; Colombo 1982; Johnson and Newport 1989). The idea of sensitive periods has been discussed extensively in relation to sensory development, with critical windows defined as periods when developing neural circuits are particularly sensitive to stimuli and may need signals for normal development to occur (Rice and Barone Jr 2000; Andersen 2003; Hensch 2004; Knudsen 2004; Uylings 2006). Similarly, sensitive periods in cardiovascular, endocrine, reproductive, respiratory and immune development are discussed in relation to the developmental trajectories of these systems (Barr Jr et al. 2000; Dietert et al. 2000; Pryor et al. 2000; Selevan et al. 2000; Andersen et al. 2006).

Much of the earliest work on critical windows occurred in humans, or with a human focus, but critical windows have also become a fundamental concept of comparative physiology and animal toxicology. Across animal taxa, experiments that utilize exposure to particular environmental, chemical or pharmacological stressors at certain time points of development are used as a means to detect and understand critical windows during development. Detecting such periods is vital for understanding how the environment may influence the developmental phenotype both in the short-term (during embryonic or larval development) and long-term (mature life stages).

1.2 Critical Windows are Central to the Interaction Between Development, Physiology and Environment

The environment may exert larger impacts on physiological systems at particular time points due to the developmental status of the animal. Exposure to a stressor that itself may play a key role in developmental processes can be used to uncover the developmental trajectory of an animal or system. Retinoic acid, for example, plays a key role in axis formation and limb patterning, and thus its application during certain stages of development can be used to infer the series of developmental events that constitute these important developmental processes. Embryos of the African clawed frog (*Xenopus laevis*) are most sensitive to retinoic acid in early gastrulation stages, with significant truncation of the body axis. This sensitivity is related to the disruption of the expression of cement-gland-specific genes that are normally expressed in late gastrula and early neurula stages (Sive et al. 1990). Thus, retinoic acid exposure illustrates that early in progression of the body axis patterning the process is somewhat plastic, and this may be important for subsequent development.

Periods of developmental plasticity or susceptibility can be defined by piecing together the findings of numerous studies that cover multiple developmental stages. Again using the example of retinoic acid, separate studies in rodents have examined the production of morphological abnormalities following retinoic acid exposure at gastrulation (Vickers 1985; Sulik et al. 1995) and organogenesis (Kochhar et al. 1984). Assessment of exposures and doses of retinoic acid used in these studies, and the resultant effects, indicates that retinoic acid sensitivity decreases as development proceeds in rodents. In some instances, an individual researcher or research lab has pieced together changes in developmental responses to a stressor across various published works. An excellent example of this is in a series of papers from 1956 to 1971 that examined the hatchability of embryonic chickens (*Gallus gallus*) following exposure to hypoxia, hyperoxia and hypercapnia at particular development ages (Taylor et al. 1956; Taylor and Kreutziger 1965, 1966, 1969; Taylor et al. 1971). After examining these different exposure studies, a number of conclusions on the stage-specific effects of various respiratory gas exposures on chicken embryo hatchability can be made. Embryos show a general trend for increased tolerance with later hypercapnic exposures, but no significant sensitive period for the effect of hypoxia on hatchability (hypoxia does exert stage-specific morphological and physiology effects on chicken embryos, discussed in more detail in section 2.1). Hatchability is also sensitive to hyperoxia exposure during days 5-8 and particularly during days 17-21. Thus, the eventual hatchability of embryos is influenced most by early hypercapnia and late hyperoxia exposure.

With recognition of the importance of critical windows, a more systematic approach has emerged in which individual studies perform multiple exposures during particular, distinct periods of development to

assess variability in sensitivity across development. The timing of exposures is often determined based on significant developmental events, such as hatching, birth, metamorphosis and molting. Thus, many studies examine the sensitivity of an animal during the embryo versus larval period, for example (e.g. Fent and Meier 1994; Bridges 2000; Greulich and Pflugmacher 2003). Yet, within these periods differential susceptibility may also occur as an animal develops and their physiological status progresses so that the extent of their cellular differentiation, organogenesis and enzymatic activity influences how sensitive or responsive they may be to environmental stressors. For example, animals may show varied responses to environmental toxicants due to the stage of maturation of the immune system and the developmental status of immune cells and organs (Dietert et al. 2002). In light of this, developmental milestones within an individual physiological system are now being considered as a means for dividing up development into different windows of exposure (Dietert et al. 2000; Landreth 2002).

The majority of critical window studies use a design in which a subset of animals are raised in control conditions, a subset of animals are chronically exposed to the stressor of interest, and a subset of animals are exposed to the stressor during distinct, separate windows (Fig. 1). These windows may be chosen based on developmental events (Aronzon et al. 2011; Eme et al. 2015; Mueller et al. 2015c), or they may be arbitrary divisions of development (Dzialowski et al. 2002; Chan and Burggren 2005; Oxendine et al. 2006; Hanlon and Parris 2014). In either case, this approach is a tried and trusted method for detecting periods of susceptibility or plasticity in the physiology, morphology and biochemistry of an animal.

This chapter examines how critical windows are detected using examples from the literature that span model and non-model invertebrates and vertebrates exposed to a range of environmental conditions. The examples reflect areas of research in which critical windows have received the most attention, as well as areas in which there is an opportunity to undertake a search for critical windows. Exposures to important naturally occurring environment variables, such as hypoxia and temperature, during distinct developmental periods has been undertaken across animal groups. The field of environmental toxicology also has a focus on understanding periods of susceptibility, particularly exposures to heavy metals, pesticides and endocrine disrupting chemicals in developing invertebrates and aquatic vertebrates. The concept of critical windows is central to appreciating the importance of the environment during development and this chapter outlines considerations for the continued search for developmental periods of sensitivity or plasticity that will ensure critical window research remains central to the field of developmental biology.

2. Stage-specific Sensitivity to Naturally Occurring Environmental Stressors

2.1 Respiratory Gases

Hypoxia and hypercapnia are naturally occurring environmental stressors for many developing animals, both in aquatic and terrestrial environments. Experimental manipulation of oxygen and carbon dioxide levels can reveal ecological implications for animals (Petranka et al. 1982; Rombough 1988; Latham and Just 1989; Kam 1993; Mills and Barnhart 1999; Seymour et al. 2000; Mueller et al. 2011a), and are also very useful for assessing the physiology of the developing respiratory and cardiovascular systems (Tazawa 1981; Tazawa et al. 1992; Dzialowski et al. 2002; Bavis 2005; Crossley and Altimiras 2005; Liu et al. 2006; Bavis and Mitchell 2008; Ferner and Mortola 2009; Eme et al. 2011a; Eme et al. 2011c; Bavis et al. 2013; Eme et al. 2013; Eme et al. 2014).

Hypoxia has been used to examine the development of respiratory control in rats during the first three weeks of postnatal development (Wong-Riley and Liu 2005; Liu et al. 2006; Liu et al. 2009; Liu and Wong-Riley 2010). The hypoxic ventilatory response following 5 min of exposure to hypoxia (10% oxygen) is blunted on day 12-16, and particularly on day 13. Measurement of respiratory variables, including minute ventilation, breathing frequency and tidal volume throughout prenatal development, indicate that respiratory control undergoes a considerable shift at this time, with respiratory frequency peaking on day 13 (Liu et al. 2006). Additionally at this time, body temperature abruptly increases, metabolic rate is heightened in normoxia but comparatively reduced in hypoxia (Liu et al. 2009), the brain stem respiratory nuclei demonstrate a transient dominance of inhibitory over excitatory neurotransmission (Wong-Riley and Liu 2005), and serotonin transmission decreases (Liu and Wong-Riley 2010). In light of these findings, day 12-16 most likely represents a critical window during which numerous physiological and neurochemical changes occur simultaneously, which may result in a reduction in respiratory modulation that causes animals to be less responsive to respiratory stressors. This period in rats is comparable to 2-4 months postnatal development in humans, a time represented by the highest incidence of sudden infant death syndrome (SIDS) (Hakeem et al. 2015). Thus, understanding the developmental changes that occur in rodents during this time may shed light on the physiological mechanisms that lead to SIDS.

Avian and reptile embryos are often used as substitutes for mammalian fetuses for understanding developmental physiology, particularly as they are separate from maternal influences. *In ovo* hypoxia exposure is easy to undertake and ecologically relevant in many instances. Many chronic exposure studies in bird and reptile embryos have demonstrated changes in morphological and physiological phenotype of embryos, particularly in response to hypoxia (Wangensteen et al. 1974; McCutcheon et al. 1982; Crossley II

et al. 2003; Crossley and Altimiras 2005; Copeland and Dzialowski 2009; Eme et al. 2011a; Eme et al. 2011c; Eme et al. 2013; Eme et al. 2014). However, in recent years studies have examined if there are particular critical windows for hypoxia sensitivity (Dzialowski et al. 2002; Chan and Burggren 2005; Tate et al. 2015). The physiology of chicken embryos is generally more hypoxia sensitive as development progresses (Grabowski and Paar 1958), and hyperoxia during days 14-18 of the 21 day incubation period produces greater decreases in body mass, hematocrit and lung mass compared to embryos exposed during days 7-18 (Xu and Mortola 1989). Thus, later stage chicken embryos are more sensitive to both low and high environmental oxygen. This finding is not surprising considering the increase in metabolic activity as embryos approach hatching (Romanoff 1967).

Hypoxia exposure during distinct windows of chicken development reveals some interesting time-specific phenotypic changes. For example, embryos exposed to 15% oxygen during days 1-6 have reduced body mass and a lower oxygen consumption rate on day 12 compared to normoxic embryos, but body mass and metabolism recover by hatching. Likewise, embryos incubated in hypoxia during days 12-18 also have reduced mass, with only a lower dry mass persisting at hatch. Embryos exposed during days 6-12 also have reduced mass on day 12 and 18 but recover by hatching. However, these embryos show an altered respiratory phenotype that persists to hatching. They are initially able to cope well with hypoxia, but show an eventual decrease in oxygen consumption at hatching compared to normoxic embryos (Dzialowski et al. 2002). Thus, the middle third of embryonic development of chickens appears to be a critical window for oxygen consumption, however, hypoxic-induced alterations in organ and size occur throughout embryonic development, and in some instances hatchlings display normal morphological sizes following return to normoxia. As the chorioallantoic membrane, the main gas exchange organ of these embryos, increases to cover a significantly larger portion of the inner eggshell during this time (Ackerman and Rahn 1981), it is likely to be impacted by hypoxia and may alter the metabolic phenotype of the embryos. While the middle third of development is important for oxygen consumption, the last third of incubation is a critical window during which hypoxia blunts ventilation of chicken embryos. This is thought to be due to hypoxia influencing the normal development of the carotid bodies that become functional during this time (Ferner and Mortola 2009).

Different windows for hypoxia sensitivity for oxygen consumption and ventilation in chicken embryos indicate how different components of a system may show critical windows that correspond, overlap, or occur at separate times of development. Exposure to hypoxia (10% O₂) between 50% and 70% of development in the common snapping turtle (*Chelydra serpentina*) enlarges heart size relative to body size. Yet, the critical window for baseline mean arterial pressure is broader, with mean arterial pressure decreasing when hypoxia exposure occurs from 20% to 70% of development (Tate et al. 2015). In this

instance, the sensitivity of the physiological function of a system is more extensive, and morphological effects of a stressor are confined to a smaller proportion of development. It is not known if the critical windows of these two components influence each other.

2.2 Temperature

Temperature has pervasive effects on all biological processes. Animals that develop *in utero* (mammals) or with parental care (many birds) are somewhat protected from variations in environmental temperature. For ectothermic animals, however, including invertebrates, fishes, amphibians and reptiles, temperature can drive survival, development times, growth, metabolism and sex. These temperature effects have been assessed by incubating developing animals in different constant temperatures and measuring physiological functions such as growth rate (Sweeney and Schnack 1977; Angilletta et al. 2004), development rate (McLaren and Cooley 1972; Herzig and Winkler 1986; Rombough 2003) and oxygen consumption rate (Kuramoto 1975; Feder 1985; Kamler et al. 1998; Gillooly et al. 2001; Mueller et al. 2011b).

Periods of increased thermal sensitivity during fish development, when physiological variables are particularly plastic, is of increased interest. Atlantic salmon (*Salmo salar*) embryos raised to a larval feeding stage in water 4.6°C above ambient, display higher maximum growth rates compared to control fish when both groups are raised after feeding at common temperatures (Finstad and Jonsson 2012). Zebrafish (*Danio rerio*) raised from hatching to adulthood at 27°C, but incubated as embryos at 22°C, 27°C (control), or 32°C, show increased thermal sensitivity to exercise performance at temperatures different than respective embryonic incubation temperatures. Furthermore, both high and low temperature incubation groups display better exercise performance than control fish at 16°C (Scott and Johnston 2012). The effects of temperature changes during embryonic development are also of interest. Oxygen consumption, heart rate and survival of lake whitefish (*Coregonus clupeaformis*) embryos is reduced following a temperature shift at the end of gastrulation compared to embryos in constant temperatures. In comparison, when the temperature shift occurs at the end of organogenesis the embryos show no change in metabolism or heart rate (Eme et al. 2015; Mueller et al. 2015c). Thus, lake whitefish embryos show greater plasticity in these variables with a temperature change during organogenesis than later in development before hatch.

2.2.1 Temperature-dependent Sex Determination

An excellent example of the how genome-environment interaction can determine the phenotype of an animal is environmental sex determination. Temperature during embryogenesis or larval development is the

prevailing environmental factor that is involved in environmental sex determination in ectothermic vertebrates, such as reptiles, amphibians and fishes (Hillman 1977; Conover 1984; Korpelainen 1990; Baroiller and D'cotta 2001; Sarre et al. 2004).

The period during development in which temperature-dependent sex determination occurs in turtles provides some of the first examples of critical windows studies in comparative physiology (e.g. Yntema 1979; Bull and Vogt 1981; Pieau and Dorizzi 1981; Yntema and Mrosovsky 1982). The critical window for sex determination is assessed by shifting eggs between male and female producing temperatures during certain stages of development, and examining the resultant sex ratios. In the common snapping turtle, the male producing temperature is 26°C, while the female producing temperatures are 20 and 30°C (Yntema 1976). Yntema (1979) demonstrated that developmental stages 14-19 (stages defined by cranial, neck and forelimb formation; (Yntema 1968)) are temperature sensitive for female determination at 30°C, whereas the window for female determination at 20°C is during stages 14-16. Thus, while the critical window at 20°C covers less developmental time, the total chronological time for the critical window at 20°C (21 d) is greater than at 30°C (12 d) due to a relatively slower development rate at 20°C. Sensitive stages of male determination at 26°C in the common snapping turtle are influenced by incubation temperature prior and subsequent to the thermally sensitive windows (stages 14-19 or 14-16). When embryos are incubated at 30°C prior to stage 14, stages 14-19 are the male-producing critical window for embryos shifted to 26°C, whereas embryos incubated at 20°C prior to stage 14, stages 14-16 are the male-producing critical window for embryos shifted to 26°C (Yntema 1979). The critical thermal window for sex determination occurs between stages 14-20 in the loggerhead sea turtle (*Caretta caretta*), stages 16-20 in the red-eared slider (*Trachemys scripta elegans*), and stages 16-22 in the map turtle (*Graptemys ouachitensis*), painted turtle (*Chrysemys picta*) and European pond turtle (*Emys orbicularis*) (Bull and Vogt 1981; Pieau and Dorizzi 1981; Yntema and Mrosovsky 1982; Wibbels et al. 1991). These critical windows represent approximately 15-20% of total incubation (Pieau and Dorizzi 1981), and occur approximately during the middle third of incubation prior to sexual differentiation of the gonads (Wibbels et al. 1991). The similarity in the sensitive stages across turtles indicates that the window for temperature-dependent sex determination is conserved across species in this clade. All extant crocodylians studied also show temperature dependent sex determination, and in the American alligator (*Alligator mississippiensis*), similar to the turtles discussed above, the critical window for temperature-dependent sex determination covers 20% of incubation, during weeks 2 and 3 of the 10 week incubation period (Ferguson and Joanen 1982). This period is earlier in incubation compared to the turtles, but alligators are laid at a more advanced embryonic stage and so the critical window is quite similar (Ferguson and Joanen 1982).

Interestingly, Pieau and Dorizzi (1981) found the length of exposure depends upon the stage within the critical period at which the temperature shift occurs. That is, if the temperature shift is late in the critical window, a longer exposure to that temperature is required. Additionally, different incubation temperatures, irrespective of if male- or female-producing (Wibbels et al. 1991), can have differing influence on sex differentiation, and this may influence the exact width of the critical window. There is some suggestion that critical windows for sexual differentiation are not so important and instead temperature drives the expression of a certain genes that will produce variable ratios of one sex over another (Deeming et al. 1988). However, recent work has found that certain genes that may be involved in sex differentiation have temperature-dependent expression and this alteration in regulation often occurs during the critical window for sex determination (Kettlewell et al. 2000; Murdock and Wibbels 2002).

Fish also show temperature-dependent sex determination. The mangrove killifish (*Rivulus marmoratus*) is the only self-fertilizing hermaphrodite vertebrate, and hermaphrodites are the predominant form in nature (Turner et al. 1992). However, while hermaphrodites are produced at temperatures of 25°C or above, exposures to 20°C or under during a small window prior to hatching will produce males (Harrington 1968). As males are scarce in nature, however, it seems unlikely that killifish are often exposed to temperatures below 20°C during this critical window for male production. In Atlantic silverside (*Menidia menidia*), temperature-dependent sex determination is correlated with body size, and, as found for the common snapping turtle, the timing and length of the critical window is dependent on temperature (Conover and Fleisher 1986). The sex ratio of Atlantic silverside larvae shifted between 15°C and 21°C is intermediate between constant 15°C (1:1 male:female) and constant 21°C incubation (9:1 male:female) when they are shifted at lengths of 8-21 mm. At 21°C the window for sex determination is shorter both in terms of total development time but also in relation to the developmental stage of the larvae (Conover and Fleisher 1986). Thus, the sex ratio of silverside is determined by a combination of the overall incubation temperature and if larvae experience a temperature change during the critical window.

2.3 Salinity

Salinity is increasing in many freshwater systems as a result of clearance of native vegetation, agricultural irrigation and rising groundwater (Williams 2001), and this has lead to a focus on salinity tolerance for a range of freshwater aquatic organisms (Kefford et al. 2003; Karraker et al. 2010). However, whether these organisms are particularly sensitive to increased salinity at particular developmental time points is still largely unstudied. A study examining the effect of salinity during certain windows of exposure on survival, growth and development of the salt-adapted brine shrimp (*Artemia franciscana*) is an example of a search

for a critical window for salinity tolerance. Brine shrimp survival is highest following exposure to low salinity (10 and 20 ppt) compared to high salinity (40 and 50 ppt) early in development, but this difference disappears when animals are exposed later in development (Burggren and Mueller 2015). Similarly, differences in growth and maturation between salinities are greatest following exposure during days 1-6 of the 15 day development period (Mueller, Willis and Burggren, personal communication). Thus, the earliest brine shrimp instar stages are most sensitive to changes in salinity, but if this is related to an immature osmoregulatory ability is unknown.

Just as hypoxia tolerance is related to metabolic demands, salinity tolerance is related to osmoregulatory ability. Thus, if freshwater or marine developing animals are particularly sensitive to salinity changes during certain critical windows, it is likely that the sensitivity is due to the absence of, or undeveloped, osmoregulatory ability. Studies that relate tolerance to environmental stressors to physiological function provide an opportunity to understand the mechanism behind periods of sensitivity. Tolerance of low salinity in American lobster (*Homarus americanus*) and Japanese tiger shrimp (*Penaeus japonicus*) decreases during larval development, is lowest at metamorphosis and increases during post-larval development (Charmantier et al. 1988). It is possible that the late larval stages, and the process of metamorphosis, are most sensitive to salinity changes. Charmantier et al. (1988) correlated high salinity sensitivity to low osmoregulatory ability, particularly during metamorphosis, when both species shift from osmoconformers to adult type osmoregulators. Thus, the shift in osmoregulatory ability defines the critical window for salinity tolerance in these species.

2.4 Pathogens

The sensitivity of an animal to pathogens is related to its resistance (ability to resist infection following exposure) and tolerance (ability to survive damage following infection). Resistance and tolerance may change throughout development resulting in critical windows of high pathogen susceptibility. For example, rainbow trout (*Oncorhynchus mykiss*) fry challenged with the myxozoan parasite *Myxobolus cerebralis* exhibit at least a 95% infection rate following exposure at 1, 3, 5, 7 or 9 weeks post hatch. In comparison, the prevalence of infection in Chinook salmon (*O. tshawytscha*) decreases from 100% and 90% in 1 and 3 week old fry, respectively, to just 1.7% in 9 week old fry (Sollid et al. 2003). Despite differences in infection rates across fry development between the two species, the disease states, such as whirling behavior and blacktail, exhibited by both species decreases as fry development proceeds (Sollid et al. 2003). Thus, early fry stages have both low resistance and tolerance to disease, the tolerance of rainbow trout increases during development, and both resistance and tolerance increases in Chinook salmon.

Disease is a common cited causes of world-wide amphibian declines (Stuart et al. 2004). Thus, assessment of critical windows of disease susceptibility will contribute to understanding the effect of disease on amphibian populations, particularly as frog tadpoles demonstrate stage-specific sensitivity to pathogens. Mortality and abnormalities in the Pacific chorus frog (*Pseudacris regilla*) following infection with the nematode parasite (*Ribeiroia ondatrae*) is greatest in pre-limb (stages 23-24 (Gosner 1960)) and early limb (Gosner stages 26-30) tadpoles, and this sensitivity steadily decreases until metamorphosis (Johnson et al. 2011). Similarly, mortality of northern leopard frog (*Lithobates pipiens*) tadpoles exposed to the same parasite is also highest following exposure during pre-limb stages (Gosner stage 24-25), and abnormalities are highest following exposure during limb-bud stages (Gosner stage 27-28) (Schotthoefer et al. 2003). Additionally, the type of abnormalities displayed by the Pacific chorus frog is stage-specific. When infection occurs prior to limb development, the predominant abnormality is missing limbs while later exposure results in extra limbs or abnormal limb projections (Johnson et al. 2011). The abnormalities from such stage-specific exposures can be related to abnormalities seen in the wild, which may have implications for conservation efforts.

3. Critical Windows for Exposure to Environmental Contaminants

3.1 Ethanol

While not an environmental contaminant in the traditional sense, the effect of ethanol on mammalian development is of interest when examining the consequences of alcohol consumption during human pregnancy. Fetal alcohol syndrome (FAS) in mammals includes growth deficiency, cognitive impairment and craniofacial abnormalities following alcohol consumption by the mother (Mattson and Riley 1998). Whether FAS is stage-specific has been examined in rats and primates. Short, early exposures produce neurobehavioural and morphological abnormalities in rhesus monkeys (*Macaca mulatta*) and the southern pig-tail macaque (*Macaca nemestrina*), indicating early embryogenesis may represent a critical window for FAS (Clarren et al. 1992; Schneider et al. 2001). In contrast, hyperactivity of rats is highest following ethanol exposure during the later half of gestation (Tran et al. 2000), but learning does not appear to be affected by ethanol exposure at various timepoints throughout development (Cronise et al. 2001). Thus, specific critical windows for certain neural and morphological effects of FAS may be species-specific in mammals.

The search for critical windows of exposure for mammalian-relevant stressors, such as ethanol, are often undertaken in lower vertebrates in which exposure occurs *in ovo*. For example, 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 subsequent embryonic organogenesis (Ali et al. 2011). Likewise, different endpoints in Japanese medaka (*Oryzias latipes*) embryos display different potential critical windows for ethanol exposure. Embryos exposed to ethanol in the first and last third of development (0-3 and 6-9 dpf – ‘days post fertilization’) have the highest incidence of reduced head width and body length. However, the incidence of apoptosis decreases with early exposure and increases with late exposure, suggesting 6-9 dpf may be when medaka are most sensitive to ethanol (Oxendine et al. 2006).

3.2 Heavy Metals

The lethal and sub lethal effects of heavy metals in the environment have been studied across a range of animal groups (Pérez-Coll and Herkovits 1990; Mariño-Balsa et al. 2000; Bunn et al. 2001; Lee et al. 2001). Exposure of heavy metals during development of aquatic invertebrates in particular has implications not only for the invertebrates themselves but for higher trophic levels (Lavalpe et al. 2004). Metals such as mercury, copper, zinc and lead are toxic during development and alter developmental variables such as survival, growth, morphological development, swimming, feeding behavior and osmoregulation (DeCoursey and Vernberg 1972; Wong et al. 1993; Bambang et al. 1995; Itow et al. 1998; Lavalpe et al. 2004). Lead is most toxic during the first half of embryonic development of the estuarine crab (*Chasmagnathus granulatus*). However, exposure to copper and zinc during the second half of embryonic development of *C. granulatus* produces a higher incidence of eye abnormalities and chromophore hypopigmentation (Lavalpe et al. 2004). Likewise, exposure of embryos of the grass shrimp (*Palaemonetes pugio*) to copper late in embryonic development increases mortality and eye abnormalities compared to exposure during early embryo stages (López Greco et al. 2002). The increased sensitivity to copper and zinc in later embryonic development is thought to be related to the increased permeability of the egg envelope as the embryo approaches hatching (Glas et al. 1997; López Greco et al. 2002; Lavalpe et al. 2004). However, exposure during post hatching stages has not been undertaken in these species to assess if removal of the egg envelope increases sensitivity. Zinc exposure during gastrulation stages of the embryonic development of American horseshoe crabs (*Limulus polyphemus*) produces the highest percentage of malformations (17%), including double embryos and segment-defective embryos, with the lowest incidence of malformations (1-2%) occurring prior to hatch (Itow et al. 1998). Likewise, embryonic American horseshoe

crabs are also highly sensitive to an organotin compound, a tin-containing substance used in biocides and antifouling paints, with the incidence of abnormalities decreasing from 6-11% to 3% at the end of gastrulation. A second peak in abnormalities also occurred prior to first embryo molt before decreasing again prior to hatch (Itow et al. 1998). Japanese horseshoe crabs (*Tachypleus tridentatus*) also show the same two peaks in sensitivity to organotin compounds as their American counterparts (Itow et al. 1998). Thus, early embryonic development until gastrulation and the period around the first embryo molt are likely critical windows for toxicant sensitivity in this group of crabs. Metal toxicity also varies during marine invertebrate larval development. Larva of the common prawn (*Palaemon serratus*) and fiddler crab (*Uca pugilator*) are increasingly sensitive to mercury exposure (DeCoursey and Vernberg 1972; Mariño-Balsa et al. 2000), whereas copper toxicity decreases during larval development of the Japanese tiger shrimp (Bambang et al. 1995).

With the increasing occurrence of deformed individuals in the environment, developmental stages of amphibians are also used extensively to assess developmental toxicity of a number of aquatic contaminants (Degitz et al. 2000). These studies highlight that amphibians often exhibit stage-specific sensitivity to a range of toxicants. Embryos of the South American toad (*Rhinella arenarum*) have a reduced body size, curved bodies and tail, fin and gill deformities following lead exposure at any stage of embryogenesis. However, exposure in the middle of embryonic development during neuromuscular activity results in a significant decrease in survival and higher incidence of malformations than at other stages of exposure (Pérez-Coll and Herkovits 1990). Similarly, western-clawed frogs (*Xenopus tropicalis*) are most sensitive to organotin compounds in the middle stages of embryonic development. During this time, normal fin formation is disrupted and other abnormalities include an enlarged proctodaeum and pigment loss in melanophores (Yuan et al. 2011).

Both bird and mammal embryos show increased immunotoxicity in response to lead following exposure during particular periods *in ovo* or *in utero*. Rat offspring have higher levels of lead in their blood and bone after the mother is given water containing lead during gestational days 15-21 compared to days 3-6 (Bunn et al. 2001). Despite no differences in consumption, mothers have higher blood lead levels when exposed late in their pregnancy, which, together with a potential higher transfer between mother and fetus, leads to greater suppression of the cellular immune response in offspring following late gestation exposure. In chicken embryos exposed to sub lethal lead levels, stage-specific immunotoxic effects occur (Lee et al. 2001). Lead exposure on day 5, 7 and 9 of incubation affects primarily macrophages, while exposure on day 12 results in a loss of T-cell immune function. This pattern may be due to lead interrupting the migration of T-cells from the bone marrow to the thymus, a process that only occurs from day 12 (Lee et al. 2001).

3.3 *Endocrine Disrupting Chemicals*

Both natural and man-made chemicals that mimic hormones can disrupt normal development and reproduction in a range of animals (Tyler et al. 1998). This has been of particular focus in organisms that are often used as indicators of environmental health and in toxicological assays, such as aquatic invertebrates, amphibians and fish. Water fleas (*Daphnia magna*) are parthenogenetic and females will produce either all female broods during times of abundant resources, or male broods when resources become limited. Male offspring will sexually reproduce with females to produce eggs that can enter diapause until conditions improve. The switch from producing female to male broods is responsive to multiple environmental cues (Stross and Hill 1965; Carvalho and Hughes 1983) but is also under endocrine control by the natural crustacean hormone, methyl farnesoate (Olmstead and Leblanc 2002). Chronic exposure to methyl farnesoate causes male brood production, but exposure must occur during ovarian egg maturation, and the 60-72 h period of ovarian egg development in particular produces the most male broods (Olmstead and Leblanc 2002).

Endocrine disruption during development can also affect single sex dimorphisms, and help reveal how such dimorphisms are regulated. Two critical windows have been detected for the development of male dimorphism in dung beetles (*Onthophagus taurus*). Dung beetles exhibit male horn length dimorphism, in which larvae that experience favorable conditions grow large and produce horns while larvae in poor conditions do not (Hunt and Simmons 1997). Exposure to a juvenile hormone analog, methoprene, late in the third instar stage, once larvae have ceased feeding prior to metamorphosis, can switch the trajectory of male development. That is, exposure to methoprene during this window can lead to small males producing horns (Emlen and Nijhout 1999). Furthermore, exposure to methoprene earlier in development, during the late feeding stage, increases the body size threshold at which horns are developed so that fewer males develop horns (Emlen and Nijhout 2001). Exposure to methoprene during these two periods suggests the first period is the window during which the fate of the males, whether horned or hornless, is determined, while the second period represents the time during which horn growth is regulated so that it matches body size (Emlen and Nijhout 2001).

Fish in particular are often used to infer toxic effects in higher animals groups, as the molecular processes involved in responses to toxic substances are highly conserved across vertebrates (Ankley and Johnson 2004). The effects of endocrine-disrupting chemicals on fish development are of particular interest from both a toxicological perspective, especially considering the plasticity in fish gonadal development (van Aerle et al. 2002). The effects of hormones on developing stages is also useful for sexual reversal techniques used to optimize commercial production of farmed species (Hunter and Donaldson 1983;

Chiasson et al. 2008). Exposure to estrogens and androgens during early larval development of zebrafish, medaka, fathead minnow (*Pimephales promelas*), Mozambique tilapia (*Tilapia mossambica*), and Egyptian mouthbrooder (*Hemihaplochromis multicolor*) influences sexual differentiation and gonadal development during a period in which the reproductive system is particularly plastic (Nakamura and Takahashi 1973; Hackmann and Reinboth 1974; Koger et al. 2000; van Aerle et al. 2002; Ankley and Johnson 2004; Maack and Segner 2004). When exactly the critical window for endocrine disruption or sex differentiation occurs is species-specific. Fathead minnow are most sensitive to ethinylestradiol exposure during day 10-15 of their 100 day post hatch development (van Aerle et al. 2002). Likewise, zebrafish are most sensitive to ethinylestradiol during 43-71 dpf of the 190 dpf development, a time during which the hermaphrodite gonad differentiates into testes or ovary (Maack and Segner 2004). Another study found zebrafish are most sensitive to ethinylestradiol during a similar window from approximately 22-62 dpf (Andersen et al. 2003). In some instances, such as in the common carp (*Cyprinus carpio*), the period prior to sexual differentiation is also a sensitive period for endocrine disruption (Gimeno et al. 1997). In comparison, sexual differentiation in marine species occurs after metamorphosis during juvenile development (Hendry et al. 2002; Chiasson et al. 2008). Thus, these fish are likely to display a later critical window for hormone sensitivity.

Amphibians exhibit heightened sensitivity to endocrine disruptors during metamorphosis, a period that is characteristic of large changes in the synthesis and action of endogenous hormones, such as thyroid hormone (Kikuyama et al. 1993; Hayes 1997). The northern leopard frog is developmentally delayed following ethinylestradiol exposure during the middle of metamorphosis (Gosner stages 30-36 (Gosner 1960)), and this delayed development persists until metamorphic climax (Hogan et al. 2008). This sensitivity corresponds to the onset of thyroid gland function and a rise in thyroid hormone levels, thus estrogens may modulate thyroid action during this metamorphic critical window. In comparison, ethinylestradiol exposure during the early period of metamorphosis (stages 27-30) produces a female-biased sex ratio compared to unexposed tadpoles, and this is related to the period of gonadal differentiation (Hogan et al. 2008). Thus, ethinylestradiol can induce different windows of sensitivity for different physiological parameters, and this is dependent upon the trajectory of certain developmental processes. Examining critical windows for endocrine disruption during fish and amphibian development allows straightforward toxicological assays to be completed in a relatively short time frame. Such research can provide a basis for quantifying the physiological and ecological impacts of endocrine disrupters (Ankley and Johnson 2004).

3.4 Sensitivity of Embryo Versus Larval Stages

In light of the many environmental stressors, animal models, physiological systems and stages of development assessed across critical windows studies, it is difficult to make a broad statement about when during development animals are most sensitive to environmental stressors. However, critical windows have been frequently demonstrated across vertebrate and invertebrate taxa, suggesting that more complex experimental designs (Figs. 2-4) may better represent the effects of environmental stressors on physiological phenotypic plasticity, compared to classic experimental designs (Fig. 1). It can be argued that later developmental stages will show increased tolerance to stressors due to advancement of their physiological systems. Embryos, as the earliest developmental stage, may be considered the most sensitive to stress. For those species that develop *in ovo*, the egg itself may provide some protective benefits, resulting in embryos being more tolerant than post hatch stages (Van Leeuwen et al. 1985; Green et al. 1986; Fent and Meier 1994).

The egg chorion of invertebrates, fish and amphibians, also known as the capsule or envelope, is thought to serve as a potential barrier against environmental contaminants. For example, the chorion of fish eggs can absorb up to 98% of cadmium and 70% of zinc due to binding between the metals and proteins in the chorion containing sulfhydryl groups (Wedemeyer 1968; Blaxter 1969; Beattie and Pascoe 1978). The egg capsule of amphibians can also provide a protective barrier against pesticides. While early tadpole stages represent a critical window for parasite infection in the Pacific chorus frog, the embryos are unaffected by exposure, indicating the function of the egg capsule to prevent parasite infection (Johnson et al. 2011).

The protective nature of the egg, if present, may change during development. The insecticide cypermethrin is most lethal to medaka embryos late in embryonic development (stage 34 of 39 (Iwamatsu 2004)), however, a high incidence of edema occurs earlier in embryonic development (González-Doncel et al. 2004). As evident by the morphological abnormalities, cypermethrin must be able to enter medaka eggs at all stages of embryogenesis, but the higher lethality later in development suggests that changes in the egg chorion structure, a natural process of hatching, may decrease the protective nature of the chorion. In fact, when medaka embryos are exposed to the herbicide thiobencarb with and without the chorion intact, the LC_{50} (lethal concentration, where 50% of the population is killed by the contaminant) of dechorionated embryos is 2.0 mg/l of thiobencarb compared to 3.9 mg/l in chlorinated embryos, illustrating the protective nature of the egg structure

The chorion may also be directly affected by a toxicant. For example, the egg chorion of medaka develops fractures and holes following exposure to silver nanoparticles (Wu and Zhou 2012). The loss of chorion integrity compromises osmotic balance within the egg, resulting in edema and morphological

abnormalities of medaka embryos following silver nanoparticle exposure (Wu and Zhou 2012). In this instance the egg chorion may actually facilitate the toxicity of the nanoparticles (Villalobos et al. 2000).

4. Considerations for Critical Window Experimental Approaches

As our knowledge of developmental phenotypic plasticity and critical windows grows, how we approach their study must also evolve. Experiments can range from those that expose a developing animal to a single stressor dose during a few windows of development (Fig. 1) to those that utilize multiple exposure windows and stressor doses to examine the extent of phenotypic change (Figs 3-4). The latter designs increase in complexity, which can result in large treatment and animal numbers that are not always possible or manageable. Species that are relatively easy to obtain, house and care for in large numbers lend themselves to complex critical window studies. Irrespective of the animal model, there are a number of factors to consider when designing an experiment aimed at detecting critical windows, and they will be discussed below.

4.1 Length of Exposure and Overlapping Exposures

Often, the length of the exposure to an environmental stressor can impact the phenotypic changes induced in the developing animal. Furthermore, the length of exposure may vary considerably across species. For example, common prawn and spider crab (*Maja squinado*) larval mortality is limited following 24 h exposure to mercury, copper or cadmium, but increases following 72 h exposure. In comparison, mortality caused by heavy metal exposure in the larvae of lobster (*Homarus gammarus*) is evident after just 24 h (Mariño-Balsa et al. 2000). The way in which exposure times affect species is dependent upon how the length of exposure is related to overall development time. That is, the same exposure time in two species with different developmental trajectories will more than likely result in different effects in each. Within a species, an increase in an effect with a longer exposure time may indicate the accumulative effect of the stressor. However, it may also indicate that the longer exposure time covers more of a critical window of sensitivity during development. For Japanese tiger shrimp nauplii, the ammonia concentration that produces 50% mortality is much lower in late nauplii after 48 h of exposure compared to early nauplii after 24 h exposure (Lin et al. 1993). This is because post-molt late nauplii no longer relying on endogenous reserves and are feeding, increasing their sensitivity to the environment. Even in the more ammonia-tolerant zoea and mysis developmental stages, ammonia-induced mortality increases with exposure time due to increased

sensitivity during molting (Lin et al. 1993).

Whether the researcher is purposely investigating the presence of a critical window or not, the fact that experimental animals are constantly developing must be taken into account when designing exposure times. This is particularly relevant if comparing across species, as the exposure times must be relative to their developmental trajectories. Figure 2 demonstrates an experimental design in which exposures occur during well-defined periods of fish embryogenesis. This design is perhaps an improvement on experiments that use arbitrary exposures that divide development into even exposure windows (Fig. 1). In this instance, phenotypic changes that may occur during exposures can be more easily related to what is happening developmentally.

Furthermore, Figure 2 shows a design that incorporates overlapping exposures, which can be useful for defining the boundaries of a detected critical window. For example, using exposures 2, 4, 6 and 8 only in Figure 2, if a phenotypic change occurs following exposure 6 then the critical window may be defined as occurring during circulation and fin development. However, in an instance when all overlapping exposures in Figure 2 are used, a phenotypic change may be greatest following exposures 5 and 6, but not 7. This suggests that exposure must occur during the first half of circulation and fin development (as in exposure 5 and 6), and this period is the true critical window. A limited number of studies have employed the approach of overlapping exposure windows (Olmstead and Leblanc 2002; Hogan et al. 2008; Tate et al. 2015) and they demonstrate the usefulness of overlapping exposures. An example analogous to that described in Figure 2 is evident in an experiment using juvenile hormone exposure to find the critical window for sex determination in *Daphnia* (Olmstead and Leblanc 2002). Exposure to juvenile hormone during 36-60 h and 72-96 h of development produces a low percentage (<40%) of males, while exposure during 48-72 h and 60-84 h produces a high percentage (>80%) of males. Therefore, the critical window for producing male broods is defined as 60-72 h, as this is the common period shared by the two exposure windows that produce the highest percentage of males. In comparison, if exposures had only occurred during separate, non-overlapping 24 h exposures (0-24, 24-48, 48-72 and 72-96 h), the window would be defined as wider (48-72 h) than actually exists.

4.2 Stressor Doses

The dose or level of a stressor used is a vital consideration in critical window designs. In chronic studies, a very high dose of an environmental toxicant that causes mortality is useful for defining lethality only. A slightly lower dose, however, may provide more information about the morphological, physiological and biochemical effects of a toxicant, particularly if the dose is environmentally relevant. In critical window

studies, a dose that produces mortality following exposure during certain periods can be used to define a critical window for mortality. However, doses that are sub-lethal at all stages of development may provide more useful information about phenotypic traits that are affected by the stressor and if phenotypic changes are more prevalent during certain critical windows.

Another aspect of critical window experiments that requires attention is if a single dose or level of a stressor is used, or if multiple doses are used (as depicted in Figure 3). Of the critical window studies cited in this chapter, less than 40% use more than a single dose or level of a stressor. Many studies undertake preliminary tests to determine the best dose to use before undertaking exposures during multiple windows (e.g. López Greco et al. 2002; Ali et al. 2011) or select a single environmentally relevant dose (e.g. Clarren et al. 1992; Boone et al. 2013). However, this approach may limit the information that can be obtained from the study and may mask the subtleties of stressor-induced effects. This is particularly relevant when attempting to define the extent of a critical window. For example, a higher dose may induce a phenotypic change slightly earlier in development and it may persist slightly longer as the critical period closes. Thus, the critical window defined from the higher dose will be wider than the same window defined from a lower dose (Mueller et al. 2015a).

Experimental designs that employ multiple exposure windows and stressor doses are useful for defining the extent of the critical window. However, by examining the phenotypic modification induced by all possible combinations of exposure time and dose allows critical windows to be considered using a three-dimensional construct (Fig. 4a; (Burggren and Mueller 2015)). In this construct, in which the phenotypic change at each time point is dependent upon the dose, the effect of a stressor may be examined in significant detail. Studies employing multiple exposure windows and stressor doses that have presented data in a three-dimensional manner include examination of salinity tolerance in developing brine shrimp (Fig. 4b; Burggren and Mueller 2015) and pesticide sensitivity in the southern leopard frog (Bridges 2000). This approach may be particularly useful for assessing how a phenotypic trait is modified by different stressor doses *within* the critical window once the potential window has been detected using a more simple exposure design (such as depicted in Fig. 1). Visualizing the interaction between exposure time, dose and magnitude of phenotypic modification may also help to pinpoint the mechanism of stressor action.

4.3 *Systems Approach to Critical Windows*

Critical window studies often focus on changes or abnormalities in morphological characteristics, as they are often the easiest to observe and measure. Studies examining sex determination and endocrine disruption, for example, tend to examine the morphology of the developing gonads. Yet, the critical window for

endocrine disruption may be influenced by other physiological aspects, such as the onset of hormonal regulation due to the presence of endogenous hormones and receptors. For instance, the critical window for endocrine disruption in coho salmon (*Oncorhynchus kisutch*) is just after hatching (Piferrer and Donaldson 1989), and this occurs in concert with a peak in endogenous testosterone (Feist et al. 1990).

The absence of traditional indicators of phenotypic modification during critical window exposure to a stressor is not always indicative of a non-effect. For example, three day exposure to carbaryl, a broad-spectrum insecticide, late in tadpole development of the green frog (*Lithobates clamitans*) does not alter survival or time to and size metamorphosis, but alters mRNA levels of thyroid regulated genes in the brain four months later, following metamorphosis (Boone et al. 2013). Thus, assessing one or two aspects of a system, in this instance growth and development rate, may mask other potential stressor effects, such as transcriptome changes. Taking a system approach to studying critical windows, and understanding that different aspects of a system, be it morphological, physiological or biochemical, may have different periods of sensitivity is key to understanding the overall system effect of an environmental stressor.

4.4 Combined Stressors

Understanding the impacts of natural and anthropogenic environmental stressors on animals has moved from the study of individual stressors alone to combined effects, as this is more representative of what animals are likely to experience in their natural environment. The consequences of combined stressors are also worth consideration in the context of critical windows as the combination of stressors may influence the timing of susceptibility during development. The concept of combined stressors is particularly relevant in the context of environmental contamination in a situation in which a species is already under some natural environmental stress. Combined stressors may have an additive or synergistic effect (e.g. Relyea and Mills 2001; Jones et al. 2011), or one stressor may lessen the effect of the other. An example of the latter situation is pesticide exposure in frog tadpoles naturally exposed to pathogens. The effect of fungal infection on tadpoles, including reduced survival and growth, is mitigated by the broad-spectrum herbicide glyphosate in the gray treefrog (*Hyla versicolor*) and by the fungicide thiophanate-methyl in the southern leopard frog (*Lithobates sphenoccephalus*) (Hanlon et al. 2012; Hanlon and Parris 2014). Hanlon and Parris (2014) exposed gray treefrog tadpoles to glyphosate and the fungus during early, mid or late tadpole development but not find a stage-specific interaction between the two stressors. However, this study is an example of how the concept of critical windows can be incorporated when examining the potential effects of combined stressors in the environment.

4.5 *Timing of Endpoint Measurement*

The search for critical windows is often concerned with the immediate, or developmental, impacts on the animal. However, the long-term consequences of an environmental change or stressor exposure during a certain period of development are also of importance. Phenotypic changes induced during critical window exposure may persist into mature life stages, influencing the adult phenotype. In some instances, immediate developmental effects may not be evident, but may materialize at a later stage, such as the transcriptome changes that appear months after insecticide exposure in green frog tadpoles discussed in section 4.3. Often the appearance of an effect is specific to the endpoint. For example, transient morphological abnormalities, such as edema, occur in medaka embryos exposed to the insecticide cypermethrin early in embryonic development, with embryos recovering prior to hatch. However, following late embryonic exposures, mortality is high and longer term effects, such as spinal curvature, an inability to inflate the swim bladder and behavioral changes, manifest in the surviving larvae (González-Doncel et al. 2004).

While some effects may not appear until later in life, developmental effects may also disappear later in life, often evident of self-repair in the animal (Burggren and Reyna 2011). Morphological changes, such as decreased body mass, shorter toes and beaks, following hypoxia exposure during the windows of day 1-6, 6-12 or 12-18 of chicken embryo development are present on day 12 and 18, but almost all changes disappear by hatching (Dzialowski et al. 2002; Chan and Burggren 2005). Thus, given time back in normoxia, chicken embryos show a remarkable ability to recover to the normal morphological phenotype. Likewise, zebrafish exposed to estrogens during the period for sex differentiation show an initial skew in the sex ratio and kidney pathology, but after recovery in clean water until the adult stage the sex ratio, gonad morphology and kidney histology return to normal (Hill Jr and Janz 2003; Weber et al. 2003). However, the exposure may still have long-term ecological implications as viable egg production and hatchability is reduced. In comparison, exposure to estrogen alters sex ratios and increases the incidence of intersex in the northern leopard frog 2-3 months after tadpole exposure (Hogan et al. 2008). Thus, the ability to recover from endocrine disruption during critical windows of reproductive development is variable among fish and amphibian species and may be related to their mode of sex differentiation (Hogan et al. 2008).

The endpoints chosen in a critical window study will depend on the particular biological question of interest. If examining developmental toxicity of an environmental contaminant, then endpoints measured shortly after exposure during development should be the focus. However, if potential long-term consequences are of interest, then endpoints measured at the end of development or in an adult stage will be most useful. In many cases, multiple endpoints across time will provide the best picture of phenotypic

changes induced by an environmental stressor. Endpoints measured at different life stages can also shed light on the mechanism of self-repair and if such a process occurs following exposure during certain distinct windows of exposure. Beyond endpoints measured during the life history of a single individual, the potential transgenerational effect of exposure to a stressor during a critical window of development may also be of interest. Transgenerational transfer and epigenetic effects are of increasing focus in developmental physiology (Ho and Burggren 2010; Burggren 2014; Burggren and Crews 2014; Mueller et al. 2015a). The interplay between critical windows, the resultant phenotype of an individual at all life stages, and potential transgenerational effects should be of focus due to their ability to link the developmental environment to physiological traits at all life stages and across generations.

5. Conclusions

The concept of critical windows has become ubiquitous in the medical, comparative physiology and environmental toxicology fields, as it recognizes that the environment may influence the biology of an animal differently during ontogeny. The study of critical windows has the ability to inform us about normal developmental progression of different physiological systems while also demonstrating how animals may respond via phenotypic modification in response to environmental stressors. For example, exposure to hypoxia during distinct periods of development can inform us about the ontogeny of the cardiovascular and respiratory systems, while exposure to different salinities during specific time points can inform us about osmoregulation. At the same time, exposure to these environmental variables during specific windows of development can indicate how an animal may be able to respond to changes in their natural environment. This is particularly relevant for appreciating the potential future challenges, such as global climate change, faced by animals during development. Likewise, understanding if there are critical windows during which developing animals are particularly sensitive to environmental contaminants, and how that sensitivity relates to developmental status, can be used to understand the mechanism of toxicity and may be useful in conservation efforts.

As our knowledge of developmental phenotypic plasticity and critical windows grows, we must continue to assess and improve how we approach their study. A search for a critical window may begin broadly, covering as much of development as possible and using a relatively simple experimental design. Once a period of plasticity or susceptibility has been detected then that time period can be of closer focus, with more detailed experimental approaches used to define the window with as much accuracy as possible. The considerations for critical window experimental designs discussed in this chapter need not be

incorporated into every study, as experiments would no doubt become unmanageable in terms of the number of treatments and animals required. However, variables such as the timing of exposures and if a single or multiple doses of the stressor or combined stressors are used should be considered in the context of the study species and questions of interest. Likewise, the form of the endpoints examined, whether morphological, physiological or biochemical, and when those endpoints are measured are important decisions that can help understand how the developmental environment influences animals at all stages of life. A continued focus on critical windows, and advancements in the approaches used to detect and study them, will no doubt contribute to our growing knowledge of the interaction between development, environment and physiology.

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Figure Captions

Fig. 1 Schematic of a simple critical window experimental design. The design includes a control treatment, in which animals are exposed to control conditions throughout development, a chronic exposure treatment, in which animals are exposed to an environmental stressor throughout development, and treatments with exposures during distinct, separate periods of development. In this instance the windows of exposure cover the first, middle and last third of development

Fig. 2 Schematic of a critical window experimental design during the embryonic development of a fish, modeled on lake whitefish (*Coregonus clupeaformis*) (Price 1934a, b, 1935; Eme et al. 2015). Cleavage and gastrulation, during which division of early cells and formation of the germ layers occurs, covers approximately the first 15% of development. Early organogenesis, which represents the first formation of organs from the ectoderm, mesoderm and endoderm and ends at the onset of the heartbeat, covers approximately 16-30% of development. Circulation and fin development, which represents continued differentiation of tissues, covers 31-60% of development. Growth and pigmentation, during which pigmentation increases, embryos grow and become progressively active, occurs during 61%-hatch. The design includes a control and a chronic exposure to an environmental stressor (exposure 1). Windows of exposure also occur during each developmental phase (exposures 2, 4, 6 & 8) or overlap these phases (exposures 3, 5 & 7) to create a series of overlapping exposure windows

Fig. 3 Schematic of a critical window experimental design with multiple exposure windows and stressor doses. The design is similar to that presented in Figure 1 but includes chronic exposure treatments for each dose, and treatments with an exposure to each dose during three distinct, separate periods of development. This design can be used to build a 3-D critical window, which illustrates the interaction between exposure window, dose and phenotypic modification (Fig. 4)

Fig. 4 a) A critical window as a three-dimensional construct illustrating the interaction between exposure time, stressor dose and phenotypic modification. This three-dimensional window can be constructed using an experimental design as illustrated in Figure 3. **b)** Relationship between salinity (ppt), time of exposure in development (d), and mean survival (%) of developing brine shrimp. Modified from Burggren and Mueller (2015) with permission. © 2015 The University of Chicago Press

Figure 1

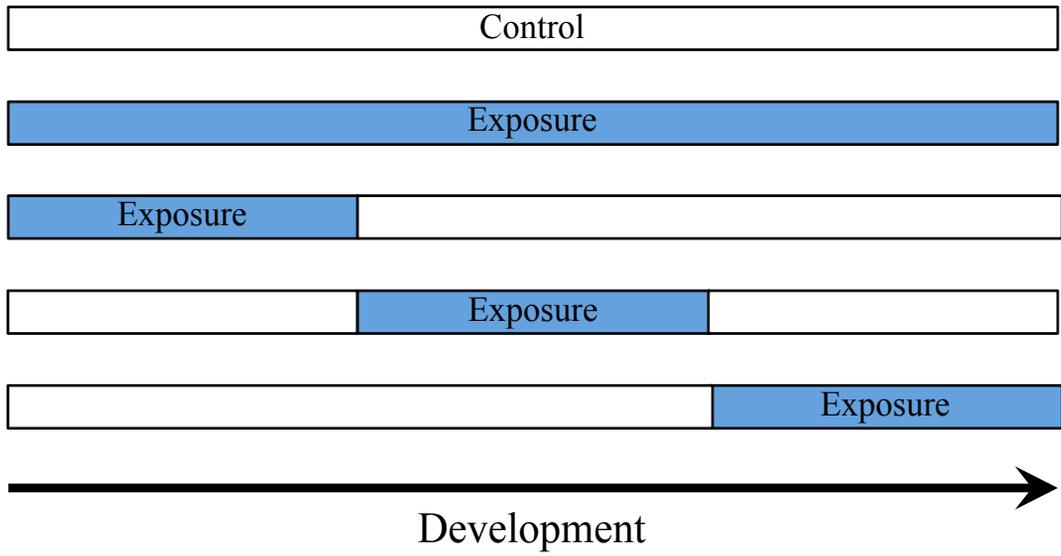


Figure 2

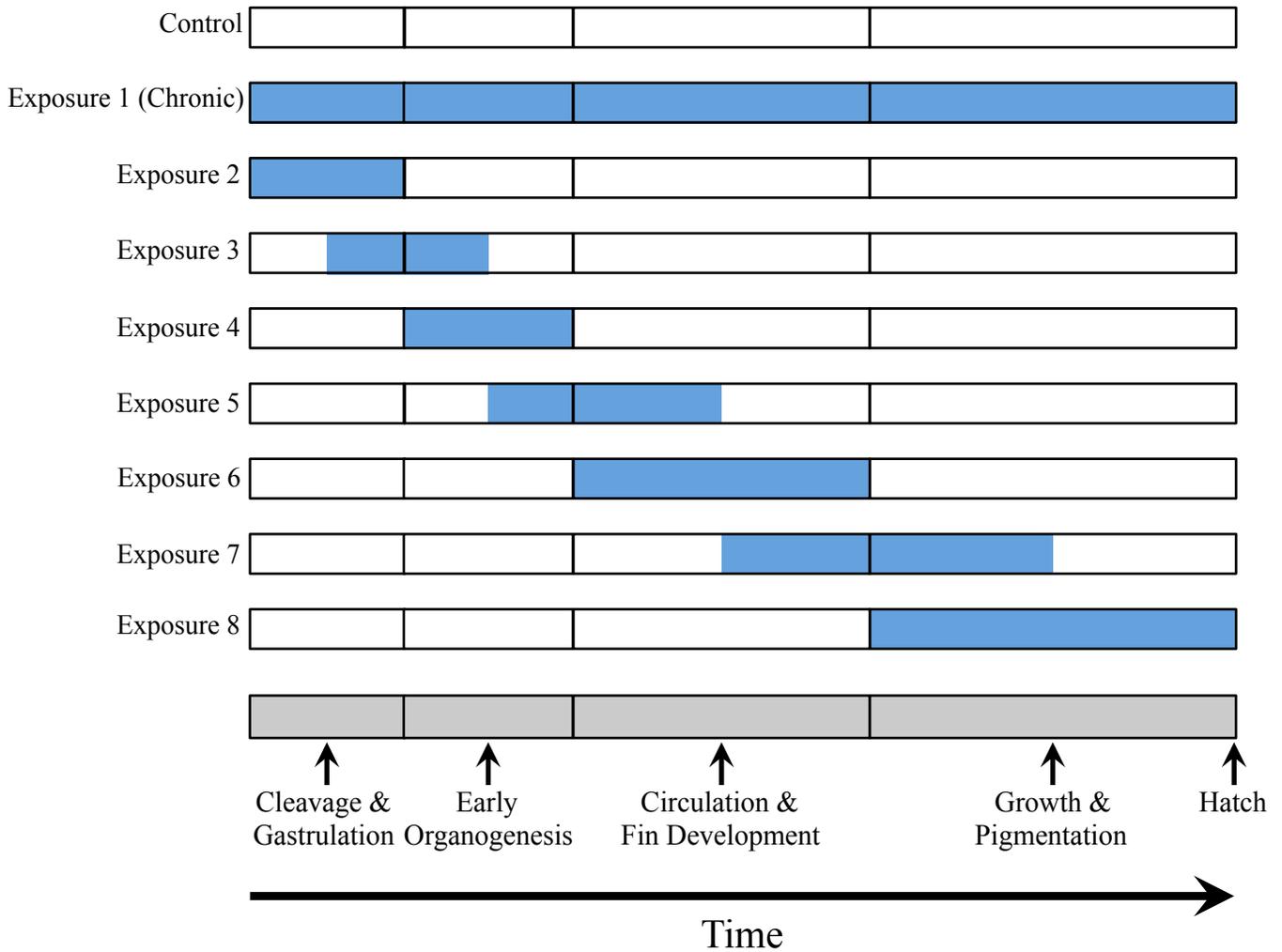


Figure 3

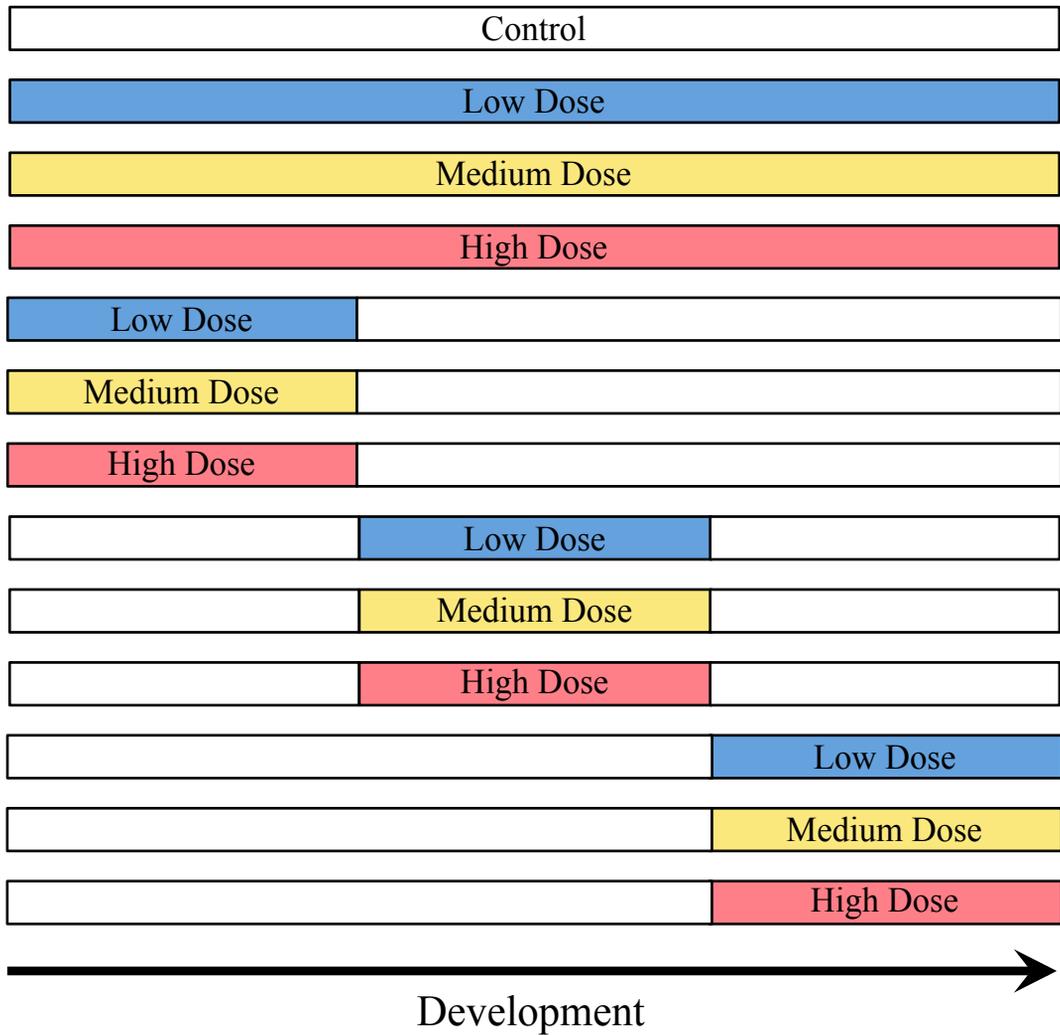
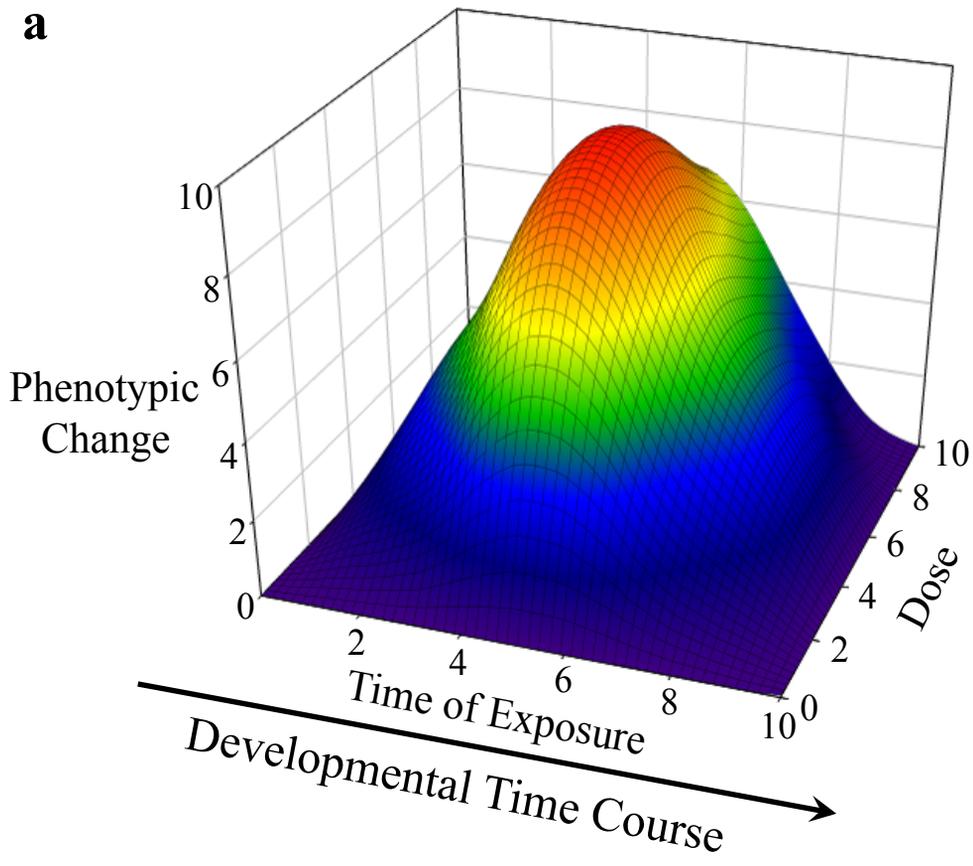


Figure 4

a



b

