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Integrative and comparative reproductive biology: From alligators to xenobiotics

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ABSTRACT

Dr. Louis J. Guillette Jr. thought of himself as a reproductive biologist. However, his interest in reproductive biology transcended organ systems, life history stages, species, and environmental contexts. His integrative and collaborative nature led to diverse and fascinating research projects conducted all over the world. He doesn't leave us with a single legacy. Instead, he entrusts us with several. The purpose of this review is to highlight those legacies, in both breadth and diversity, and to illustrate Dr. Guillette's grand contributions to the field of reproductive biology. He has challenged the field to reconsider how we think about our data, championed development of novel and innovative techniques to measure endocrine function, helped define the field of endocrine disruption, and lead projects to characterize new endocrine disrupting chemicals. He significantly influenced our understanding of evolution, and took bold and important steps to translate all that he has learned into advances in human reproductive health. We hope that after reading this manuscript our audience will appreciate and continue Dr. Guillette's practice of open-minded and passionate collaboration to understand the basic mechanisms driving reproductive physiology and to ultimately apply those findings to protect and improve wildlife and human health.

1. Introduction

To leave a legacy is a great achievement for any person, but Dr. Lou Guillette was such a scientific force and a true collaborator that he leaves us several diverse legacies in the field of reproductive biology. Dr. Guillette regularly challenged dogma, and promoted the idea that conducting basic research to understand "normal" was essential for defining "abnormal". He also helped pioneer the use of cutting-edge technologies to investigate the effects of endocrine disrupting chemicals (EDCs) and to discover emerging contaminants.

One of Dr. Guillette's legacies is the idea that measures of variation in the response of an animal or population to environmental change are as important, or potentially more important, than measures of mean responses. This insight likely originated from his deep understanding of the dynamic nature of the endocrine system and importance of natural hormonal fluctuations for controlling normal reproduction. Indeed, his interest in reproductive biology facilitated vast contributions to the field of endocrinology. He contributed to our understanding of normal seasonal variation in hormone profiles of many different species and conducted diverse studies to understand basic reproductive physiology. He is also well known for helping to define the field of endocrine disruption, having contributed significantly to our appreciation of the diverse ways in which EDCs affect wildlife and human health.

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Dr. Guillette also made broad contributions to the field of evolutionary biology. He increased our understanding of temperature-dependent sex determination and showed that the endocrine chorioallantois is not unique to mammals. He worked tirelessly to translate his knowledge into advances in human reproductive health. In addition to his creative scientific pursuits, Dr. Guillette brought together scientists of multiple disciplines to compare and integrate perspectives. Ultimately, his creativity, deep knowledge, collaborative skill, and willingness to share credit and ideas in the building of scientific relationships are what lead to the breadth of his contributions.

2. Variation should be explored, not ignored

Living systems are inherently variable, and this variation allows organisms to respond to environmental change at both individual and population levels. In many studies, variance is viewed as an inconvenient feature of imperfect systems, and attempts are made to minimize or equalize variance among groups so that it can be ignored and attention can be focused on comparing measurements of central tendency. One of Dr. Guillette's enduring legacies will be his recognition that measures of central tendency often do not provide sufficient information about the response of an animal or population to environmental change. He and his colleagues argued that the distribution and variance of a dataset can provide insights into mechanisms and responses that are not apparent in a simple comparison of means (Orlando and Guillette, 2001). In other words, variance should not be viewed as an undesired, unfortunate attribute of a dataset. Rather, variance can be used to identify, predict, and explain the responses of organisms to environmental change.

In many studies, variance increases in populations exposed to environmental disturbance, even when measures of central tendency are similar among groups. For example, in mosquitofish (*Gambusia holbrooki*) exposed to pulp mill effluent, which contains a mixture of androgenic chemicals, mean hormone concentrations within a sex do not differ significantly between the exposed and reference mosquitofish populations. However, males and females exposed to pulp mill effluent demonstrate more variable testosterone (T) and 17β-estradiol (E_2) concentrations, respectively, indicating altered hormone regulation in a subset of individuals within the exposed population (Toft et al., 2004). Similarly, a small percentage of females in the contaminated population also develop markedly enlarged gonopodia (anal fin rays that function as an intromittent organ in males). However, the frequency of these masculinized females is small enough that mean fin size does not differ significantly between exposed and reference populations (Orlando and Guillette, 2001). Dr. Guillette realized that such differences in variance induced by a subset of the population could be biologically significant. Indeed, when a low potentially non-statistically significant percentage (e.g., ~1%) of human children display morphological abnormalities (e.g., congenital heart defects and hypospadias), we consider them devastatingly common birth defects.

Exposure to environmental contaminants is also associated with increased variance (without concomitant changes in mean) of body and organ sizes in gar (*Lepisosteus platyrhincus*) and fathead minnows (*Pimephales promelas*) (Orlando and Guillette, 2001). Similarly, female mosquitofish from a contaminated lake demonstrate greater seasonal variation in muscular E_2 than females from a reference lake (Edwards et al., 2010). In white ibises (*Eudocimus albus*), fecal corticosterone are more variable as MeHg exposure increases (Adams et al., 2009). Increased variance is thought to result from spatial or temporal heterogeneity of the environment (Maurer and Holt, 1996), as can be seen in environments impacted by pulsatile release of effluent or in habitats characterized by pollution gradients. Increased variance may also result from heterogeneity in xenobiotic metabolism among individuals (Silva et al., 2003). For example, organisms with diminished capacity for hepatic detoxification (the "responders") can be severely impacted by environmental toxicants even if the same toxicants have no discernable effects on the majority of individuals in a population (reviewed in Dorn, 2010). These negative outcomes can be innately difficult to detect via measures of central tendency if data from a sufficiently large number of "non-responders" are included in a dataset.

In other cases, contaminant exposure is associated with decreased variation in measured endpoints. For example, female mosquitofish from a contaminated lake demonstrate decreased variation in the timing of ovarian recrudescence, leading to increased synchronicity of this process (Edwards et al., 2010). Similarly, male phallus size and female hormone concentrations are less variable in American alligators (*Alligator mississippiensis*) from a highly contaminated lake than in their counterparts from a less contaminated site (Gunderson et al., 2004), and juvenile alligators from a contaminated lake demonstrate less seasonal variability in circulating sex steroids (Rooney et al., 2004). Less variation in T concentrations is also seen in male giant toads (*Bufo marinus*, now *Rhinella marina*) from highly agricultural areas in South Florida (McCoy et al., 2008). In summary, increases and decreases in the variance of measured outcomes within a population occur in response to contaminant exposure, indicating that variance is a parameter that should be explored rather than ignored in studies of environmental disturbance. Dr. Guillette believed that to truly understand how the environment affects wildlife and human health we must change our perspective from focusing on determining *if* an environmental insult can change the mean response, to understanding how the occurrence and severity of abnormalities scale along environmental gradients.

3. Contributions to endocrinology

Variation in hormone signaling is known to be essential for proper endocrine function, and abnormal hormone fluctuation can be an indication of disruption. Hormones drive much of reproduction and development, thus hormone assessments remain a key tool in studies of endocrine function. Understanding the particular hormones produced, the diel or seasonal cyclicity of these hormones, and the ways in which hormones are regulated, is often the first objective addressed when studying the reproductive biology of a species. It is not surprising that altered hormone regulation and signaling are hallmarks of studies of endocrine disruption, and forms the cornerstone of many assessments of reproductive dysregulation. The Guillette laboratory has employed hormone evaluations to greatly increase our understanding of reproductive biology as well as the ways in which environmental contaminants contribute to reproductive impairment.

Establishing seasonal variation in reproductive hormone profiles allows us to identify the breeding season for most species studied to date and lays the foundation for many studies of reproductive function. Dr. Guillette and colleagues have identified seasonal reproductive hormone profiles for a variety of species including mosquitofish, Florida gar, loggerhead turtle, white ibis and every life stage of the American alligator from a number of locales (Bermudez et al., 2005; Edwards et al., 2006b, 2013, 2010; Guillette et al., 1991, 1997; Hamlin et al., 2011, 2014; Heath et al., 2003; Kristensen et al., 2007; Orlando et al., 2007, 2003; Rooney et al., 2004). His laboratory has used hormone assessments to answer questions fundamental to reproductive physiology, including the endocrine control of parturition and oviposition, gonadotropin regulation of hormone synthesis, gene

regulation of steroidogenesis, and has used this information to address concerns related to human health (Crain et al., 1997; Edwards et al., 2004; Guillette et al., 1991; Hamlin and Guillette, 2010; Jones and Guillette, 1982). His laboratory was the first to discover the presence of dehydroepiandrosterone (DHEA) in a crocodilian, the patterns of which strongly parallel reproductive activity. Plasma DHEA concentrations are considerably higher than T during the non-breeding season. DHEA increases non-breeding aggression in birds, which are closely related archosaurs. Leading to the idea that, this hormone could play a role in maintaining aggression in crocodilians during the non-breeding season (Hamlin and Guillette, 2011; Hamlin et al., 2014).

Moreover, hormone assessments have also been used by the Guillette laboratory to understand responses to stress (Anderson et al., 2011; Gregory et al., 1996; Morris et al., 2011). These studies have increased basic understanding of the stress axis and improved animal welfare. For example, seahorses exposed to chronic ambient noise showed elevated cortisol and other stress indicators, suggesting that implementation of soundproofing in aquariums could decrease stress and reduce susceptibility to disease (Anderson et al., 2011).

Another key theme of Dr. Guillette's research was the use of altered hormone production to understand the mechanisms by which contaminated environments cause reproductive dysregulation. The widespread use and recalcitrant nature of many environmental pollutants has led to considerable concern for the health of both wildlife and humans. Lake Apopka, Florida, was the site of a major chemical spill in the early 1980s and was declared an Environmental Protection Agency (EPA) superfund site in 1983. Dr. Guillette identified significant developmental and reproductive dysfunction in resident wildlife, especially alligators. Resident alligators displayed altered production of sex-steroids and other hormones, which was suspected to have derailed proper development. Further, many abnormities persisted into later life stages (Crain et al., 1998, 1997; Gregory et al., 1996; Guillette et al., 1999a, 1994, 1996; Guillette et al., 1999b; Gunderson et al., 2001; Milnes et al., 2002; Pickford et al., 2000). Although Lake Apopka was a principal study site, Dr. Guillette and colleagues investigated a number of other contaminated locales including cattle feedlots, munitions sites, Florida Springs, the Florida Everglades, agricultural areas, Kennedy Space Center, Merritt Island National Wildlife Refuge, and sites near Kraft paper mills or sewage treatment plants (Bowden et al., 2014; Edwards and Guillette, 2006; Edwards et al., 2006a; Folmar et al., 2001, 1996; Hamlin et al., 2010; McCoy et al., 2008; Nilsen et al., 2016; Orlando et al., 2004; Soto et al., 2004). While field studies play an important role in scientific discovery, determining the specific factors that contribute to the etiology of a problem can be difficult, given the dynamic interplay of complex factors within the natural ecosystem. Laboratory studies are particularly useful complements to field studies in that they create a controlled, stable, and reproducible environment to isolate parameters of interest. In addition to decades of field research, the Guillette laboratory has conducted numerous laboratory studies to identify the effects of specific pollutants on the reproductive endocrinology of wildlife (Adams et al., 2009; Crain et al., 1997; Edwards et al., 2006a; Milnes et al., 2004, 2005).

Along with his work on steroid hormones, Dr. Guillette and colleagues were preeminent expert on thyroid development, hormones, disruption, and regulation in the American alligator. He recognized the importance of understanding general endocrinology such as alligator thyroid hormone cycles (Bermudez et al., 2005; Boggs et al., 2011; Gunderson et al., 2002) in order to more thoroughly investigate endocrine disruption. Dr. Guillette understood the interconnectedness of the endocrine system and showed that steroid hormones and thyroid hormones were linked, as evidenced by the discovery of estrogen receptors in the thyroid gland (Bermudez et al., 2011). Importantly, the Guillette laboratory explored thyroid disruption, induced not only by contaminants (Bermudez et al., 2006; Crain et al., 1998; Cruze et al., 2015; Hewitt et al., 2002; Milnes et al., 2004, 2008), but also by increased sensitivity to those contaminants under certain environmental conditions. For example, environmental iodine from coastal environments likely contributed to thyrotoxicosis in neonatal alligators and generally elevated plasma thyroxine concentrations among coastal juveniles compared to freshwater populations (Boggs et al., 2012). Dr. Guillette's emphasis on a holistic exploration of ecosystem – organism – contaminant interactions was a strength of his research on endocrine disruption in real-world environments.

Dr. Guillette believed in staying on the cutting edge of technology to improve our ability to monitor endocrine disruption. The Guillette laboratory pioneered the high-throughput proximity scintillation radioimmunoassay (RIA) for the measurement of thyroid and steroid hormones in alligators, producing the largest data sets on alligator endocrinology to date (Hamlin et al., 2011, 2014). However, he understood that in order to advance science, one had to continually embrace novelty and technology. That vision led him to partner with chemists at the National Institute of Standards and Technology (NIST) to develop comprehensive steroid profiling methods. His idea was to measure many hormones in a single sample using mass spectrometry methods rather than measure a single hormone per sample using antibodies and RIA. In collaboration with NIST scientists, Dr. Guillette's laboratory developed a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for steroid hormone cascade measurements. It was Dr. Guillette's ability to bring together scientists of multiple disciplines that made him so successful and it is this vision of collaboration that he instilled in his students and colleagues that will continue to contribute to his legacy.

4. Defining novel endocrine disruptors

In addition to integrating multiple perspectives, resources, and researchers to study physiology and endocrine disruption, Dr. Guillette was open-minded about what chemicals and environmental contexts could disrupt endocrine function. In 2005, Guillette and Edwards (2005) published a review proposing nitrate as an endocrine disruptor. Nitrate (NO_3) and its *in vivo* metabolites, nitrite (NO_2) and nitric oxide (NO), can reduce steroidogenesis (Panesar and Chan, 2000) and impede activity of steroidogenic enzymes, such as cholesterol side-chain cleavage enzyme (P450scc) and aromatase (CYP19) by binding the heme groups that characterize all cytochrome P450 enzymes (Delaforge et al., 1995; Hanke et al., 1998; Masuda et al., 1997; Yi et al., 2011). NO_3, NO_2, and NO can also reduce synthesis of thyroid hormones, induce goiter, or delay amphibian metamorphosis by competitively inhibiting iodine uptake by the sodium-iodine symporter in the thyroid (De Groef et al., 2006; Tonacchera et al., 2004). Since Dr. Guillette's original insight, the anti-thyroid effects of NO_3 have now been shown in several taxa including humans, bulls, rodents, teleosts, sharks, and amphibians (Edwards et al., 2006a; El-Wakf et al., 2015; Guillette and Edwards, 2005; Morris et al., 2011, 2012; Pearce and Braverman, 2009).

Identification of NO_3 as an endocrine disruptor has crucial implications because nitrogen pollution of surface and ground waters is so widespread (Kaiser, 2001). Background levels of NO_3 are typically below 1 mg/L NO_3–N, but contamination from fertilizers, septic tanks, waste water treatment, manure, and atmospheric deposition can elevate NO_3 concentrations in surface or ground waters to 100 mg/L NO_3–N or more. To protect infants from methemoglobinemia (blue baby syndrome), the U.S. drinking water limit

for NO_3 is 10 mg/L NO_3–N. Even so, at least 22% of private wells in the U.S. produce drinking water that exceeds this limit (Dubrovsky et al., 2010). The endocrine disrupting effects of NO_3 and its metabolites include inhibition of P450 steroidogenic enzyme activity leading to reductions in steroid hormone concentrations. Dr. Guillette's laboratory showed that *ex vivo* ovarian tissue from adult *Xenopus laevis* frogs produced significantly less E_2 and T when cultured in the presence of 24 or 49 mg/L NO_3–N, compared with controls (Barbeau and Guillette, 2007). Similarly, serum E_2 and T levels were reduced 93% and 97% respectively in freshwater carp (*Labeo rohita*) exposed to 2 mg/L NO_2–N in their tank water for 45 days (Ciji et al., 2013). However, the prediction that steroid levels will always decline with NO_3 exposure is not supported by all studies.

Several projects from the Guillette laboratory and others have found either increased steroidogenesis following exposure to NO_3 or no significant effects. Indeed, these differences are likely due to the concentration, timing of exposure, variance in responses among individuals, and environmental context in which the studies were carried out (Edwards et al., 2006a). For example, Freitag et al. (2015) observed increased plasma T levels among male and female juvenile salmon exposed to 10 mg/L NO_3–N, compared to those exposed to a lower dose of 5 mg/L (control) and a higher dose of 101 mg/L. Likewise, adult female Siberian sturgeon exhibited elevated plasma T, 11-ketotestosterone (11-KT) and E_2 concentrations following a 30-day exposure to 57 mg/L NO_3–N, compared to controls (Hamlin et al., 2008). Hatchling female alligators also exhibited elevated plasma T levels after 5 weeks and 5 months of exposure to 100 mg/L NO_3–N in tank water, compared with animals raised in water with 1 or 10 mg/L NO_3–N (Hamlin et al., unpublished). Finally, mosquitofish collected from eight Florida springs representing a range of NO_3 doses between 0 and 5 mg/L NO_3–N exhibited no clear association between NO_3 and steroid hormone levels. However, increasing NO_3 was correlated with altered hormone-dependent responses, including decreased sperm counts, increased testis weights, increased gonopodial lengths (androgen dependent), and decreased pregnancy rates (Edwards and Guillette, 2007; Edwards et al., 2006b).

The lack of concordance among NO_3 studies suggests multiple pathways by which NO_3 and its metabolites influence steroid hormone abundance and again point to the difficulties of analyzing dynamic responses such as endogenous hormone concentrations using population means. For instance, in addition to reducing steroidogenesis by inhibiting steroidogenic enzymes, NO_3 may increase steroidogenesis by competitively depleting extracellular chloride. Chloride depletion causes a chloride current across the membrane, which can enhance G-protein and cyclic AMP (cAMP)-dependent steroidogenesis, particularly when gonadotropin levels are low, as in juvenile animals (Choi and Cooke, 1990; Cooke, 1999; Cooke et al., 1999; Jensen, 2003; Panesar, 1999; Stocco et al., 2005). Furthermore, one study suggests that NO_2 can directly activate estrogen receptor alpha (ERα; ESR1) through the ligand-binding domain (Veselik et al., 2008). This latter effect could induce both negative and positive feedback control of steroidogenesis depending on other physiological parameters. As with other areas of investigation, Dr. Guillette's open-minded and holistic approach to studying the effects of environmental contaminants has helped shape our understanding of nitrate's diverse effects. As with many other contaminants, the relative influence of NO_3 on an organism's physiology depends on exposure timing, dose, and environmental and physiological context. Dr. Guillette recognized that environmental context determines the responses to environmental toxicants further supporting his idea that variation in effects is biologically interesting.

5. Contributions to evolutionary biology

5.1. Viviparity and the endocrine chorioallantois are not unique to mammals

Viviparity (or live birth) is a reproductive strategy that is most commonly associated with eutherian mammals, although it evolved independently in other amniote lineages. Among non-mammalian amniotes, viviparity is best represented in the squamates (Shine, 1985; Shine and Bull, 1979), in which embryonic development of viviparous species is supported by both a chorioallantoic placenta and a yolk sac placenta (Stewart and Blackburn, 1988). These structures form by apposition of the chorioallantoic or yolk sac membranes with the oviduct or uterus and participate in maternal-embryonic exchange of gases, water, nutrients, and wastes (Guillette, 1982).

It was once believed that viviparous squamates evolved from ovoviviparous species, which retain shelled embryos that are nourished exclusively with yolk within the reproductive tract until hatching (reviewed in Guillette, 1982). However, Guillette and colleagues recognized that internal development of truly independent, shell-bound embryos is unlikely. In the absence of placentae, which facilitate gas and water exchange, such embryos cannot survive the constraints imposed by thick eggshells and pronounced glandular epithelia of the oviducts and uterus (Albergotti and Guillette, 2011; Guillette, 1993). Guillette and colleagues suggested instead that viviparous squamates evolved directly from oviparous (egg-laying) species that retained their eggs for a period of time after shell formation. As the duration of egg retention increased, the need for efficient gas exchange acted as a strong selective force driving the co-evolution of placental viviparity, particularly in lineages living at high altitudes where oxygen partial pressures are lower (Guillette, 1982; Guillette et al., 1980). Traits that evolved under this selective pressure include reduced oviductal and uterine glands; thinner shells and shell membranes; and thinner but hypervascularized extraembryonic, oviductal, and uterine membranes (Guillette, 1982, 1987, 1991, 1992; Guillette and Jones, 1985; Guillette et al., 1980). All of these traits facilitate diffusion and thus delivery of oxygen to and removal of carbon dioxide from shelled embryos and may also facilitate maternal recognition of pregnancy (Guillette, 1989, 1993).

Dr. Guillette played a pivotal role in elucidating the endocrine functions of reptilian placentae and extraembryonic membranes. His laboratory provided the first indirect evidence that the chorioallantoic placenta of viviparous squamates is capable of progesterone synthesis (Guillette et al., 1981), as is the case in eutherians. In addition, the group contributed to a re-evaluation of the endocrine activity of extraembryonic membranes in other amniotes. In oviparous reptiles (including birds), the chorioallantoic membrane (CAM) supports embryonic development and survival. Despite morphological and functional similarities to the mammalian placenta, the CAM, until recently, was not considered an endocrine organ due to its presumed inability to synthesize and regulate hormones. Rather, maternally deposited yolk hormones were assumed to be responsible for directing embryonic development. This paradigm shifted after Guillette and colleagues discovered that the CAMs of chicken (*Gallus gallus*), *A. mississippiensis*, and turtle (*Pseudemys nelsoni*), synthesize and metabolize hormones and participate in steroid hormone signaling (Albergotti et al., 2009; Cruze et al., 2013, 2012). This work suggests that endocrine activity of the CAM is not unique to eutherians and steroidogenic extraembryonic tissues are a shared characteristic among amniotes.

5.2. Temperature-dependent sex determination

Dr. Guillette appreciated that temperature-dependent sex determination (TSD) was a unique framework through which to understand the evolution of sex determination systems, sex chromosomes, sexual plasticity, and fragility of the sexes. Since the discovery of TSD in the African lizard (*Agama agama*) in 1966 (Charnier, 1966), this sex determination system has been investigated by many researchers from many different perspectives (review Valenzuela and Lance, 2004). Dr. Guillette and colleagues made large contributions to our understanding of the molecular mechanisms of TSD and sensitivity of TSD to endogenous and exogenous estrogenic signals, which are reviewed here.

For each species that exhibits TSD, specific temperatures that will produce females (female-producing temperature, FPT) or males (male-producing temperature, MPT), and a pivotal temperature (PVT) produces an even ratio of males and females. However, Dr. Guillette's laboratory showed that the process of TSD at the PVT can be heavily influenced by incubation temperatures earlier than the known temperature sensitive period (TSP) in *A. mississippiensis* (McCoy et al., 2015). Since much of the work on TSD has been conducted in the laboratory where egg-incubation temperatures do not fluctuate as they naturally do in the field (Georges et al., 2004), further investigations into this earlier TSP, temperature fluctuation, and the molecular mechanisms driving TSD are required in *A. mississippiensis*, as well as other TSD species.

Dr. Guillette's laboratory has contributed to our understanding of the epigenetic modifications that might be associated with TSD. For example, the sexually dimorphic pattern of gonadal DNA methylation at the *aromatase* and *SOX9* (*sex determining region Y-box 9*) promoter region has been identified in *A. mississippiensis*, as well as in *Trachemys scripta* (Matsumoto et al., 2013; Parrott et al., 2014). DNA methylation of *CYP19* aromatase and *SOX9* promoter region could be involved in the stabilization of the sexes in TSD species, although it has not been evaluated whether this is a specific mechanism in TSD species or a general mechanism to commit sexual fate.

The genetic network of reptilian TSD is conserved, as seen in mammalian sex determination and differentiation. Many of the molecular mechanistic approaches used to study TSD have been borrowed from advanced investigations of sex determination and differentiation systems in mammals and have not specifically addressed the thermosensitive trigger due to technical difficulties such as a lack of molecular tools (Kohno and Guillette, 2013; Morrish and Sinclair, 2002; Shoemaker-Daly et al., 2010; Smith and Sinclair, 2004). However, Dr. Guillette and his collaborators recognized that investigating thermosensitive genes and proteins is critical to understand the initial trigger and specific molecules of TSD. One of the well-known thermosensitive protein families includes heat-shock proteins (HSP). Gonadally-derived mRNA of two HSPs showed sexually dimorphic patterns (*HSP27*, males > females; *HSP70A*, males < females) at one month of age in *A. mississippiensis* (Kohno et al., 2010). Importantly, these proteins modulate steroid hormone signals in mammals (Chen et al., 2008; Pratt and Toft, 1997; Wu et al., 2002) and thus could play a role in TSD in reptiles.

Although the HSP protein family is thermosensitive and has potential roles in TSD, these proteins are not thermosensor proteins. One thermosensor candidate is the TRPV4 ion channel which is activated by moderate heat (>30 °C) in mammals and induces *Sox9* expression in a murine chondrogenic cell line (Muramatsu et al., 2007; Nilius and Voets, 2013; Vay et al., 2012). Dr. Guillette and colleagues found that developmental exposure to a TRPV4 antagonist during TSP suppressed testicular differentiation in gonadal morphology and mRNA expression such as *AMH* and *SOX9* in *A. mississippiensis* (Yatsu et al., 2015). These results indicated that TRPV4 could be a strong candidate for the trigger protein for TSD. Further investigations into similar proteins and other species will elucidate the details of the trigger in the thermosensitive mechanism of TSD in reptiles.

Dr. Guillette's laboratory played a foundational role in describing how endogenous and environmental estrogens affect TSD. Exposure to E_2 *in ovo* induces ovarian differentiation at MPT with suppression of SOX9 expression in a wide variety of reptiles (Barske and Capel, 2010; Bull et al., 1988; Crews et al., 1989; Gutzke and Bull, 1986; Pieau, 1974; Stoker et al., 2003). Moreover, developmental exposure to estrogenic environmental contaminants such as dichlorodiphenyldichloroethylene (*p,p′*-DDE), bisphenol A (BPA), and polychlorinated biphenyl (PCB) *in ovo* also induced ovarian differentiation in several species (Bergeron et al., 1994; Milnes et al., 2005; Stoker et al., 2003; Willingham and Crews, 1999). This work aided in defining the "E_2-sensitive period" of induced ovarian differentiation at MPT and explicitly compared mechanistic differences between temperature and estrogenic effects. For example, in *A. mississippiensis*, E_2-induced ovaries at MPT exhibited a different mRNA expression pattern than the control ovaries produced at FPT, revealing that temperature and estrogens induce different gonadal mRNA expression patterns in this species (Kohno et al., 2015).

Dr. Guillette appreciated the importance of cloning and characterizing reptilian ESRs (including ESR1, ERα and ESR2, ERβ) and was involved with characterizing these receptors in several species (Katsu et al., 2004, 2008, 2010, 2006; Kohno et al., 2008). These *in vitro* characterization projects have revealed important insight into the function and sensitivity of these receptors across various species and have also provided a framework in which to screen chemicals for receptor interaction prior to further *in ovo* experiments (Kohno and Guillette, 2013). *In ovo* experiments provide information regarding the potential roles for ESRs in the sex determination process. For example, in *A. mississippiensis*, exposure to a selective ESR1 agonist induced ovarian differentiation at MPT whereas exposure to an ESR2 agonist did not (Kohno et al., 2015).

Genomic information and high-throughput sequencing analyses will make the molecular mechanisms of TSD, such as epigenetic modification, more accessible and may lead to better understanding of the plasticity and stability of the sexes, which are sensitive to estrogen and estrogenic contaminants in species with TSD and those with genotypic sex determination species.

6. Contributions to translational medicine

In 2010, Dr. Guillette made the bold decision to leave the University of Florida, where he spent over 25 years building a reputation as one of the leaders in comparative reproductive biology and endocrine disruption research, and to accept a position at the Medical University of South Carolina (MUSC). In addition to continuing his established environmental reproductive biology research with American alligators as a sentinel species, Dr. Guillette was given the unique opportunity to work with the vast resources at Hollings Marine Laboratory (HML), and formed collaborations with NIST, the National Ocean and Atmospheric Administration (NOAA), the South Carolina Department of Natural Resources (SC-DNR), and the College of Charleston. Another of Dr. Guillette's legacies is the idea that collaboration rather than competition leads to stronger and more interesting transformative science.

A growing theme in Dr. Guillette's research, since he arrived at MUSC, was to translate his work with the American alligator to human reproductive issues, especially those of women and children. In recent years, new collaborations have facilitated projects aimed at assessing impacts of EDC exposures on pregnant women

as well as those seeking assisted reproductive therapies. Over 400 pregnant women are actively enrolled in a clinical study analyzing the effects of prenatal phthalate exposure on reproductive development by assessing genital measurements both *in utero* using ultrasound technologies as well as postnatally. This study provides unique opportunities to identify distinct populations of women who have elevated phthalate exposure during pregnancy, so that exposure-reducing strategies can be implemented.

Dr. Guillette also advocated the use of innovative analytical techniques to assess contaminant profiles in ovarian follicular fluid, urine, and plasma in patients seeking *in vitro* fertilization with the ultimate aim of establishing measures predictive of fertilization success (McCoy, unpublished). The impacts of these assessments are far reaching and will provide novel insights regarding the ability of particular contaminants to compartmentalize in the ovary and alter ovarian cell signaling pathways. Dr. Guillette's team will continue this work and is attempting to develop intervention measures to increase fertility success. Together, these new human based research foci represent one of the next major chapters for the field of reproductive biology and will expand Dr. Guillette's substantial research legacy.

In addition to pursuing human health research at MUSC, Dr. Guillette was an advocate for wildlife and comparative reproductive biology. Within his tenure at MUSC, he continued his collaboration with researchers at National Aeronautics and Space Administration (NASA) (Bowden et al., 2014) and collaborated with international scientists at Kruger National Park, the University of Pretoria, University of Limpopo, and the Mpumalanga Parks and Tourism Agency to investigate the impact of EDCs on the health status of the Nile crocodile (*Crocodylus niloticus*) and other aquatic organisms, such as the sharptooth catfish (*Clarias gariepinus*) and Mozambique tilapia (*Oreochromis mossambicus*), within the Olifants River region in South Africa. In association with NIST scientists, he helped develop novel analytical techniques aimed at exploring the disease pansteatitis and the relationship between the lipidome and EDCs in aquatic organisms within the region. Dr. Guillette constantly challenged dogma and inspired his students and colleagues to develop novel techniques and questions aimed at generally understanding reproductive biology as well as the effects of EDCs across taxa.

Dr. Guillette was also a strong advocate of moving away from the traditional practice of investigating EDCs (and their effects) one-at-a-time to investigating real-world EDC mixtures and the effect of these mixtures on environmental and human health. He directed a collaboration with NIST scientists to explore contaminants that he often described as being, "not immediately visible by the light under a lamppost." He argued that "we want to search for the chemicals we cannot currently see or do not even know exist". Over the past five years, this impetus has generated projects focused on developing new analytical approaches to measure these emerging chemicals of concern. Dr. Guillette was also driven to find biomarkers capable of indicating health status and/or EDC exposure using novel analytical chemical strategies. Taking approaches commonly used in the detection of human disease states, the application of omics-based strategies has shown considerable promise as a complimentary assay for characterizing the consequences related to EDC exposure, especially with the increasing body of knowledge that certain EDCs can affect other lesser-studied pathways (e.g., obesogens) (Temkin et al., 2015).

7. Future directions

How do we most effectively build upon the vibrant research program set in motion by Dr. Guillette? First, we should continue to conduct integrative and comparative studies that address both basic and applied questions. As Dr. Guillette often said: We can't understand what is abnormal without a clear understanding of normal. Second, we must explicitly investigate how environmental context influences the endocrine system and its responses (both mean and variance components) to pollutants. Third, we need be innovative and continue to develop novel cutting edge approaches to measure and compare across biological systems. Fourth, we should continue to ask and answer mechanistic questions, but we should also go outside (potentially at night and in the rain) to interact with wildlife, for that is where we learn how actual individuals are exposed to pollutants and respond. Finally, we should look beyond dogma, define novelty, and collaborate to solve large problems unanswerable by single laboratories alone.

8. The take home message

Despite his academic and professional successes, Dr. Guillette was adamant that his true legacy was not his body of work, but the people he worked with. His legacy is one of passion, support, enthusiasm and determination; a true blueprint to follow for all of us in search of research excellence.

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