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Cellular calcium and redox regulation: the mediator of vertebrate environmental sex determination?

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ABSTRACT

Many reptiles and some fish determine offspring sex by environmental cues such as incubation temperature. The mechanism by which environmental signals are captured and transduced into specific sexual phenotypes has remained unexplained for over 50 years. Indeed, environmental sex determination (ESD) has been viewed as an intractable problem because sex determination is influenced by a myriad of genes that may be subject to environmental influence. Recent demonstrations of ancient, conserved epigenetic processes in the regulatory response to environmental cues suggest that the mechanisms of ESD have a previously unsuspected level of commonality, but the proximal sensor of temperature that ultimately gives rise to one sexual phenotype or the other remains unidentified. Here, we propose that in ESD species, environmental cues are sensed by the cell through highly conserved ancestral elements of calcium and redox (CaRe) status, then transduced to activate ubiquitous signal transduction pathways, or influence epigenetic processes, ultimately to drive the differential expression of sex genes. The early evolutionary origins of CaRe regulation, and its essential role in eukaryotic cell function, gives CaRe a propensity to be independently recruited for diverse roles as a 'cellular sensor' of environmental conditions. Our synthesis provides the first cohesive mechanistic model connecting environmental signals and sex determination pathways in vertebrates, providing direction and a framework for developing targeted experimentation.

Key words: oxidative stress, reactive oxygen species, calcium signalling, temperature dependent sex determination, epigenetics

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I. INTRODUCTION

The mechanisms by which sex is determined and the processes by which sexual phenotypes subsequently differentiate (sexual differentiation) have been a focus of enquiry for many centuries (Mittwoch, 2000, 2013). The structures of the testes and ovaries are highly conserved across vertebrates (Morrish & Sinclair, 2002; Schroeder *et al.*, 2016), so it is not surprising that the genes and regulatory processes governing gonad formation and differentiation share a high degree of commonality (Sarre, Georges & Quinn, 2004; Cutting, Chue & Smith, 2013; Capel, 2017). Despite the conservation of gonadal morphology, sex in vertebrates is influenced by a wide variety of mechanisms, broadly divided into genetic sex determination (GSD) and environmental sex determination (ESD), as well as mixed systems in which genes and environment interact to determine sex (Bachtrog *et al.*, 2014). ESD systems occur in species from 15% of vertebrate orders. They use several different environmental cues including light regime, social stress, pH and temperature (Bachtrog *et al.*, 2014).

Decades of research on model and non-model organisms have documented the extraordinary variety of sex-determining environmental signals, and characterized different downstream elements of sex differentiation pathways in ESD systems. However, recent work implicating ancient, conserved epigenetic mechanisms in the regulatory response to environmental cues suggests that the mechanisms of ESD have a previously unsuspected level of commonality (Rhen & Schroeder, 2010; Deveson *et al.*, 2017; Ge *et al.*, 2018). This poses the fundamental question: what is the mechanism by which such a wide variety of environmental cues are transduced to determine sex by a common molecular sensor?

The conservation of epigenetic elements in ESD suggests the action of a biochemical sensor common to all ESD species. Such a sensor must be (*i*) inherently environmentally sensitive; (*ii*) capable of interacting with components of known sex differentiation pathways; and (*iii*) conserved in function yet plastic enough to be recruited to capture and transduce different environmental signals for different phenotypic outcomes.

Here, we propose a general model in which sex determination is mediated by cellular calcium (Ca^{2+}) and redox (reactive oxygen species; ROS) status, which are subject to environmental influence. Elements of this hypothesis have been discussed in six recent papers that explicitly posited the involvement of either ROS production or Ca^{2+} flux in directing the outcomes of ESD (Yatsu *et al.*, 2015, 2016; Czerwinski *et al.*, 2016; Corona-Herrera *et al.*, 2018; Lin *et al.*, 2018; Hayasaka *et al.*, 2019). We suggest that these two interrelated signalling systems (Richter & Kass, 1991) work together to initiate sex determination.

Here, we refer to calcium and redox status collectively as CaRe status, and propose a model for its biological action in ESD. We review evidence that CaRe status (and its subsequent effects on CaRe-sensitive regulatory pathways) is an environmentally sensitive mediator of complex biochemical cascades, and therefore a promising candidate for the capture and transduction of environmental signals into a sexual outcome. We propose that these CaRe-sensitive regulatory pathways have been co-opted independently and repeatedly to determine sex in different vertebrate lineages, acting as the crucial missing link between sex and the environment.

II. CALCIUM AND REDOX REGULATION IN THE CELL

(1) Roles of ROS and Ca^{2+}

ROS and Ca^{2+} constitute some of the most important signalling molecules in the cell, and are both involved in a staggering variety of essential cellular processes (Gordeeva, Zvyagilskaya & Labas, 2003; Camello-Almaraz *et al.*, 2006; Görlach *et al.*, 2015). The subtle ways in which these interactions can be modulated allows cellular responses to be fine-tuned according to the cellular context (Yan *et al.*, 2006; Metcalfe *et al.*, 2010).

ROS are highly reactive by-products of cellular respiration, and can cause cellular damage when production exceeds that of the cell's antioxidant capacities (Martindale & Holbrook, 2002; Temple, Perrone & Dawes, 2005). ROS are produced mainly in the electron transport chain in the mitochondria, but can be generated elsewhere in the cell. They are typically rapidly dismuted through a series of antioxidant reactions (Camello-Almaraz *et al.*, 2006; Yan *et al.*, 2006; Hamanaka & Chandel, 2010). If ROS production outweighs the antioxidant capacity of the cell, the redox environment can be altered to an oxidizing state (Treidel, Carter & Bowden, 2016). However, at physiologically moderate levels (eustress), ROS possess vital cellular signalling roles in growth, homeostasis, reproduction, and programmed apoptosis (Covarrubias *et al.*, 2008; Dowling & Simmons, 2009; Sies, Berndt & Jones, 2017). When acting in

their capacity as signalling molecules, ROS can influence protein conformation and function through the oxidative modification of accessible cysteine residues and reversible changes to disulfide bonds (Hammond, Lee & Ballatori, 2001; Covarrubias *et al.*, 2008; Morgan & Liu, 2011; Cremers & Jakob, 2013). Even subtle subcellular alterations in redox state can drive differential gene expression (Sen & Packer, 1996; Antelmann & Helmann, 2010) through physiological or epigenetic mechanisms (Cyr & Domann, 2011; Timme-Laragy *et al.*, 2018), and ultimately influence cell and tissue-specific environmental responses.

In close concert with redox signals, Ca^{2+} flux co-regulates many cellular signalling and environmental sensing functions (West *et al.*, 1982; Contreras *et al.*, 2010; Görlach *et al.*, 2015; Plattner & Verkhratsky, 2015), and displays considerable evolutionary flexibility in recruitment to these different functions (Hilton *et al.*, 2015). Ca^{2+} concentrations inside the cell are tightly controlled by numerous calcium pumps and channels on the plasma membrane (Ermak & Davies, 2002), and are mediated by Ca^{2+} release from internal stores in the mitochondria and endoplasmic and sarcoplasmic reticula (Røttingen & Iversen, 2000; Berridge, Bootman & Roderick, 2003; Brostrom & Brostrom, 2003). Ca^{2+}-mediated signalling is crucial for orchestrating cell signalling cascades, which are highly sensitive to and modulated by the amplitude, duration, and subcellular localisation of Ca^{2+} (Røttingen & Iversen, 2000; Dupont & Sneyd, 2017). Such finely tuned signal transduction cascades, which primarily involve protein phosphorylation or dephosphorylation, allow Ca^{2+} to control a wide variety of highly specific responses to environmental variables (Brostrom & Brostrom, 2003; Sharma, Nguyen & Geng, 2014).

(2) Environmental sensitivity of Ca^{2+} and ROS

We propose that CaRe status is the most promising candidate for encoding extrinsic environmental signals in the cell, and provide a framework in which CaRe status determines sex in environmentally sensitive species. On a biochemical level, ROS and Ca^{2+} levels in the cell are affected by many environmental factors, such as temperature (Ahn & Thiele, 2003), ultraviolet (UV) light (Schieven *et al.*, 1993; Gniadecki *et al.*, 2000), and hypoxia (Chandel *et al.*, 2000). CaRe status can therefore indicate the presence and magnitude of an environmental signal and initiate a cellular response.

Ca^{2+} signalling has been implicated in temperature-dependent sex determination (TSD) through the temperature-sensitive regulation of transient receptor potential (TRP) cation channel expression in two TSD alligator species [American alligator, *Alligator mississippiensis* (Yatsu *et al.*, 2015) and Chinese alligator, *Alligator sinensis* (Lin *et al.*, 2018)] and a freshwater turtle *Mauremys reevesii* (Ye *et al.*, 2019). These plasma membrane channels control the flow of Ca^{2+} ions into the cell, and are thermosensitive at least in mammals (Hilton *et al.*, 2015), although TRP channel function is unknown for other vertebrates (Hilton *et al.*, 2015; Yatsu *et al.*, 2015). Within the TRP family, *TRPV4* exhibits temperature-specific differential expression in *A. mississippiensis* (Yatsu *et al.*, 2015), and three other TRP family genes (*TRPV2*, *TRPC6*, and *TRPM6*) display temperature- and sex-biased expression in *A. sinensis* (Lin *et al.*, 2018). It was suggested that these channels act as the initial temperature sensor mechanism in alligators that regulates the expression of downstream sexual development genes through Ca^{2+} signalling (Lin *et al.*, 2018). The application of TRPV4 antagonist drugs in *A. mississippiensis* partially interfered with male development, producing testes-like gonads with incomplete Mullerian ducts (Yatsu *et al.*, 2015). This suggests that TRPV4 operates alongside other, as yet unidentified, thermosensitive mechanisms acting in concert with Ca^{2+}, such as those involving ROS. In the turtle *M. reevesii*, the application of a TRPV1 and TRPM8 inhibitor altered sex ratios under certain incubation conditions, and although the authors accredited this to inhibited thermoregulatory behavior rather than altered sex gene expression, the result could be due to interference with Ca^{2+} signalling (Ye *et al.*, 2019).

TRP channels also respond to different wavelengths of visible light (Wang et al., 2016), and other research has proposed the effect of light on intracellular calcium concentrations to be mediated by ROS production (Lavi *et al.*, 2003). Additionally, the oxidation of cysteine residues can sensitize and activate TRPA1 (Materazzi *et al.*, 2012) and TRPV1 (Kozai, Ogawa & Mori, 2013; Ogawa *et al.*, 2016), further substantiating the link between the two messenger systems in response to various stimuli. TRP channels are also sensitive to and can be modulated by steroid hormones, particularly in sperm cells (Kumar *et al.*, 2015).

ROS production is directly influenced by the environment, primarily through the metabolism-enhancing effects of temperature (Clarke & Fraser, 2004; Halliwell & Gutteridge, 2015), although pH (Maurer *et al.*, 2005; Wang *et al.*, 2009), UV light (de Jager, Cockrell & Du Plessis, 2017) and photoperiod-influenced circadian rhythms (Hirayama, Cho & Sassone-Corsi, 2007) can also alter oxidative state. Developmental rate in some reptiles accelerates with temperature, as does mitochondrial respiration (Sun *et al.*, 2015), so it is feasible that that ROS could accumulate more quickly at a higher temperature, activating responses to oxidative stress. Further, antioxidant capacity in embryos varies in response to incubation temperature in a TSD turtle (red-eared slider, *Trachemys scripta elegans*), indicating that metabolic rate and ROS accumulation vary with temperature (Treidel *et al.*, 2016). Additionally, yolk deposition of antioxidants is greater in birds with shorter developmental periods (Deeming *et al.*, 2013), suggesting that even in a homeothermic taxon, faster development results in greater oxidative stress. In some fish species, water temperature affects redox status and oxidative damage, although the effects have not been investigated in the context of sex determination (Birnie-Gauvin *et al.*, 2017).

Environmental cues do not necessarily need to be abiotic, as many species of fish display forms of socially cued sex change, commonly through the reorganization of dominance hierarchies (Todd *et al.*, 2016). Oxidative stress has been shown to correlate with social status in species of fish

(Border *et al.*, 2019) and primates (Beaulieu *et al.*, 2014), probably through the increased behavioral costs of defending and maintaining dominance. Signals of differential calcium regulation and responses to oxidative stress were both observed in dominant male bluehead wrasse (*Thalassoma bifasciatum*), further indicating differential regulation of these messenger systems during sex change (Todd *et al.*, 2018).

Combined with evidence on the environmental sensitivity of calcium channels, these studies show that a wide range of environmental conditions, including temperature, during development can alter both redox state and calcium flux. This raises the possibility that CaRe status could have a role as a cellular sensor for a broad range of environmental cues responsible in developmental programming and variation in different species.

III. CONNECTIONS BETWEEN CaRe STATUS AND SEX DETERMINATION

(1) Signal transduction pathways

As discussed above, CaRe status is clearly a strong candidate for the capture of environmental signals by the cell. We propose here that the signal captured by CaRe status is then transduced *via* ubiquitous signalling pathways that influence epigenetic processes to govern sex differentiation.

The interactions between CaRe status and cellular organization and function are complex, and so can interact with a variety of pathways involved in sex determination. Here we discuss CaRe-sensitive candidates likely to transduce an environmental signal; the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), heat shock response and antioxidant response pathways, and explore the potential interactions between CaRe status and another candidate pathway for ESD, the vertebrate stress axis (Table 1, Fig. 1).

(*a*) *The NF-κB pathway*

The NF-κB pathway is involved in a wide variety of cellular processes and can be activated by Ca^{2+} influx, ROS, and ROS-induced glutathione production (Røttingen & Iversen, 2000; Hammond *et al.*, 2001; Antonsson *et al.*, 2003; Morgan & Liu, 2011; Fig. 1).

The NF-κB pathway has well-established associations with numerous sex determination genes in mammalian development. However, its role has been less well studied in ESD taxa (Josso & di Clemente, 2003; Hong *et al.*, 2003; Delfino & Walker, 2014; Table 1). Analysis of the transcriptome during development in two TSD species (the alligator *A. sinensis* and painted turtle, *Chrysemys picta*) showed that differential expression of various genes in the NF-κB pathway is associated with temperature at key developmental stages, but this has not been backed up by functional studies (Radhakrishnan *et al.*, 2017; Lin *et al.*, 2018).

A single study directly demonstrated a role for NF-κB in vertebrate sex determination using the zebrafish (*Danio rerio*) (Pradhan *et al.*, 2012). While the genetics of sex determination in laboratory strains of *D. rerio* lacking a W chromosome (Wilson *et al.*, 2014) are not yet well understood, it appears to have a polygenic basis that is sensitive to environmental factors such as temperature and hypoxia (Ribas *et al.*, 2017; Santos, Luzio & Coimbra, 2017). *Danio rerio* is unusual in that a juvenile ovary initially forms, and either continues to mature as an ovary, or transitions into testes through the promotion of selective apoptosis (Uchida *et al.*, 2004; Chen, Liu & Ge, 2017). Manipulating the induction or inhibition of the NF-κB pathway prior to gonadal commitment led to a female or male bias, respectively, demonstrating its role in suppressing the apoptotic pathways that trigger the transition to testis development (Pradhan *et al.*, 2012). Sex cell-specific apoptosis is a well-established mechanism in sex determination in *D. rerio* (Uchida *et al.*, 2002), as well as in other teleosts (He *et al.*, 2009; Yamamoto *et al.*, 2013; Sarida *et al.*, 2019) and other model organisms such as *Drosophila melanogaster* (DeFalco *et al.*, 2003) and *Caenorhabditis elegans* (Gumienny *et al.*, 1999; Kuwabara & Perry, 2001; Peden *et al.*, 2007). Manipulating the NF-κB pathway thus presents opportunities for exploring the link between CaRe regulation and ESD (Fig. 2).

(*b*) *Heat shock proteins and the heat shock response*

Several authors have proposed a role in TSD for heat shock proteins (HSPs) (Harry, Williams & Briscoe, 1990; Kohno *et al.*, 2010; Bentley *et al.*, 2017; Table 1). These proteins are chaperones and regulators of transcription factor binding, functions which are essential for maintaining cell function at extreme incubation temperatures (Haslbeck & Vierling, 2015; Ikwegbue *et al.*, 2018).

Heat shock causes Ca^{2+} concentration to rise according to time and temperature, and concurrently increases levels of the oxidizing agent hydrogen peroxide (Soncin *et al.*, 2000; Ahn & Thiele, 2003). This change in CaRe status can activate heat shock factor 1 (HSF1), which in turn regulates expression of heat shock protein genes (notably *HSP70*), whose actions are required for protection against heat-induced cell damage (Soncin *et al.*, 2000; Ahn & Thiele, 2003; Tedeschi *et al.*, 2015, 2016; Fig. 1). Incubation temperature affects the expression of many HSPs in reptiles (Table 1), however, no consistent patterns have emerged even between closely related species, suggesting that HSPs exhibit considerable evolutionary flexibility (Harry *et al.*, 1990; Kohno *et al.*, 2010; Haslbeck & Vierling, 2015; Czerwinski *et al.*, 2016; Bentley *et al.*, 2017). Inconsistent patterns of expression of HSPs across species, and their role as molecular chaperones across a wide range of temperatures, might explain the variety of ESD responses to temperature across species (Hilton *et al.*, 2015; Tedeschi *et al.*, 2016).

Particularly interesting is that environmental triggers of HSPs extend beyond temperature. Some members of the HSP family show differential expression during socially induced sex change in the two-banded anemonefish (*Amphiprion bicinctus*) (Casas *et al.*, 2016), and HSP10 is associated

Table 1. Calcium and redox (CaRe)-sensitive elements, their functions relating to epigenetic modulation, cellular localisation and their roles in environmental sex determination (ESD) or temperature sex determination (TSD)

Candidate element	Cellular functions and known roles in environmental sex determination		References
Nuclear to cytoplasmic translocation			
CIRBP Cold-inducible RNA-binding protein	Functions	(1) Translocation to cytoplasm induced by numerous environmental stressors including temperature and oxidative state (2) Typically associates with cytoplasmic stress granules where it acts as a mRNA chaperone	De Leeuw *et al.* (2007); Rhen & Schroeder (2010); Schroeder *et al.* (2016); Radhakrishnan *et al.* (2017); Zhong & Huang (2017)
	ESD roles	(1) Candidate gene for TSD in *Chelydra serpentina* (2) Thermosensitive expression in *Chrysemys picta* and *Apalone spinifera*	
hnRNPs Heterogeneous ribonucleoprotein particle family	Functions	(1) Involved in numerous cellular processes including splicing regulation, pre-mRNA processing, nuclear export of mRNA, chromatin remodeling (2) Interacted with *p38 MAPK* stress-induced signalling pathway, and the EED subunit of the PRC2 complex	Harry *et al.* (1990, 1992); Huelga *et al.* (2012); Kim *et al.* (2017)
	ESD roles	(1) Thermosensitive expression in *Caretta caretta* (2) Posited as candidates for the regulation of TSD	
Cytoplasmic to nuclear translocation			
NRF2 Nuclear factor (erythroid-derived 2)-like 2	Functions	(1) Regulates expression of antioxidant genes under oxidative stress through transactivation of antioxidant response elements	Covarrubias *et al.* (2008); Loboda *et al.* (2016)
HSF1 Heat shock factor 1	Functions	(1) Transcriptional regulator of all heat shock proteins (2) Redox and temperature regulated (3) Induced by *p38 MAPK* phosphorylation	Harry *et al.* (1990); Kohno *et al.* (2010); Tedeschi *et al.* (2015, 2016); Bentley *et al.* (2017); Lin *et al.* (2018); Furukawa *et al.* (2019)
	ESD roles	(1) Role of heat shock response established for majority of TSD species (2) Involved in female sexual development in *Oryzias latipes*	
HSPs Heat shock protein family	Functions	(1) Molecular chaperone for steroids and hormones, participates in cell signalling (2) Roles in maintaining protein stability, folding, and transmembrane transport	Harry *et al.* (1990); Brostrom & Brostrom (2003); He *et al.* (2009); Kohno *et al.* (2010); Tedeschi *et al.* (2015, 2016); Casas *et al.* (2016); Czerwinski *et al.* (2016); Bentley *et al.* (2017); Lin *et al.* (2018); Tao *et al.* (2018); Wang *et al.* (2019)
	ESD roles	(1) Thermosensitive expression in *Alligator mississippiensis* and *Alligator sinensis* (2) Markers of thermal stress, and thermosensitive expression in *Caretta caretta* (3) Downregulation of *HSP10*-associated apoptosis during sex reversal in *Monopterus albus* (4) Various HSPs associated with social sex change in *Amphiprion bicinctus* (5) *HSP90* upregulated in *Oreochromis niloticus* undergoing temperature-induced sex reversal	
Protein kinases Family includes mitogen-activated, cAMP-dependent, calcium/calmodulin-dependent	Functions	(1) Multitude of cellular roles centering on ability to catalyze protein phosphorylation; integral role in numerous signal transduction cascades	Radhakrishnan *et al.* (2017); Lin *et al.* (2018); Tsakogiannis *et al.* (2018)
	ESD roles	(1) Temperature-dependent expression in *Alligator sinensis* and *Chrysemys picta* (2) Male-biased expression in *Pagellus erythrinus* and *Pagrus pagrus*	
JAK-STAT pathway Janus kinase/signal transducers and activators of transcription	Functions	(1) Redox-regulated signalling cascade for stress response	Simon *et al.* (1998); Radhakrishnan *et al.* (2017); Todd *et al.* (2019)

(Continues)

Table 1. *(Cont.)*

Candidate element	Cellular functions and known roles in environmental sex determination		References
	ESD roles	(1) Components of pathway show thermosensitive expression in *Chrysemys picta* (2) Progressive upregulation during sex change in *Thalassoma bifasciatum*	
NF-κB pathway Nuclear factor kappa light-chain-enhancer of activated B cells	Functions	(1) Redox-regulated signalling cascade for environmental stress response (2) Activation has anti-apoptotic effects	Pradhan *et al.* (2012); Ravi *et al.* (2014); Radhakrishnan *et al.* (2017); Lin *et al.* (2018)
	ESD roles	(1) Components of pathway show thermosensitive expression in *Chrysemys picta* and *Alligator sinensis* (2) Crucial for sexual differentiation in *Danio rerio* (3) Male-biased expression in *Lates calcarifer*	
No subcellular translocation known/not applicable			
JARID2 & JMJD3 Jumonji and AT-rich interaction domain-containing 2 (*JARID2*) and lysine demethylase 6B (*JMJD3/KDM6B*)	Functions	(1) Members of the Jumonji chromatin remodeling gene family (2) *JARID2* mediates Polycomb repressive complex (PRC2) deposition of silencing H3K27me3 marks (3) *JMJD3* catalyzes demethylation of H3K27me3	Díaz & Piferrer (2015); Akashi *et al.* (2016); Deveson *et al.* (2017); Radhakrishnan *et al.* (2017); Ge *et al.* (2018); Todd *et al.* (2019)
	ESD roles	(1) Retained intron associated with sex reversal in *Pogona vitticeps*, *Alligator mississippiensis* and *Trachemys scripta elegans* (2) TSD in *Trachemys scripta elegans*, *Chrysemys picta*, and *Apalone spinifera* (3) Thermal adaptation in *Anolis* lizards (*A. allogus, A. homolechis, A. sagrei*) (4) Associated transition to masculine phenotype during sex change in *Thalassoma bifasciatum* (5) Upregulated in response to temperature in *Dicentrarchus labrax*	
AP1 Transcription factor, activator protein-1	Functions	(1) Acts as a point of integration of many signalling pathways involved in responses to environmental signals (e.g. MAPKs, NF-κB, HSPs) (2) Redox-controlled switch determines ability to bind DNA	Yin *et al.* (2017)
TRPs Transient receptor potential cation channels	Functions	(1) Innately thermosensitive channels that allow the passive transfer of Ca^{2+} across the plasma membrane	Yatsu *et al.* (2015); Liu *et al.* (2015); Lin *et al.* (2018); Todd *et al.* (2019)
	ESD roles	(1) Known thermosensitivity, temperature-dependent expression in *Alligator sinensis* and *Alligator mississippiensis* (2) Calcium signalling enrichment during sex change in *Thalassoma bifasciatum*	
TET enzymes Ten-eleven translocation methylcytosine dioxygenases	Functions	(1) Redox-dependent DNA methylation	Todd *et al.* (2019)
	ESD roles	(1) Expression strongly associated with sex change in *Thalassoma bifasciatum*	
DNMTs DNA methyltransferases	Functions	(1) Sensitive to redox state and calcium concentration (2) Action influenced by the redox microenvironment of chromatin	van der Wijst *et al.* (2015); Tsakogiannis *et al.* (2018); Todd *et al.* (2019)
	ESD roles	(1) Associated with sex change in *Thalassoma bifasciatum* (2) Sex-biased expression in *Pagellus erythrinus* and *Pagrus pagrus*	

MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; PRC2, polycomb repressive complex 2.

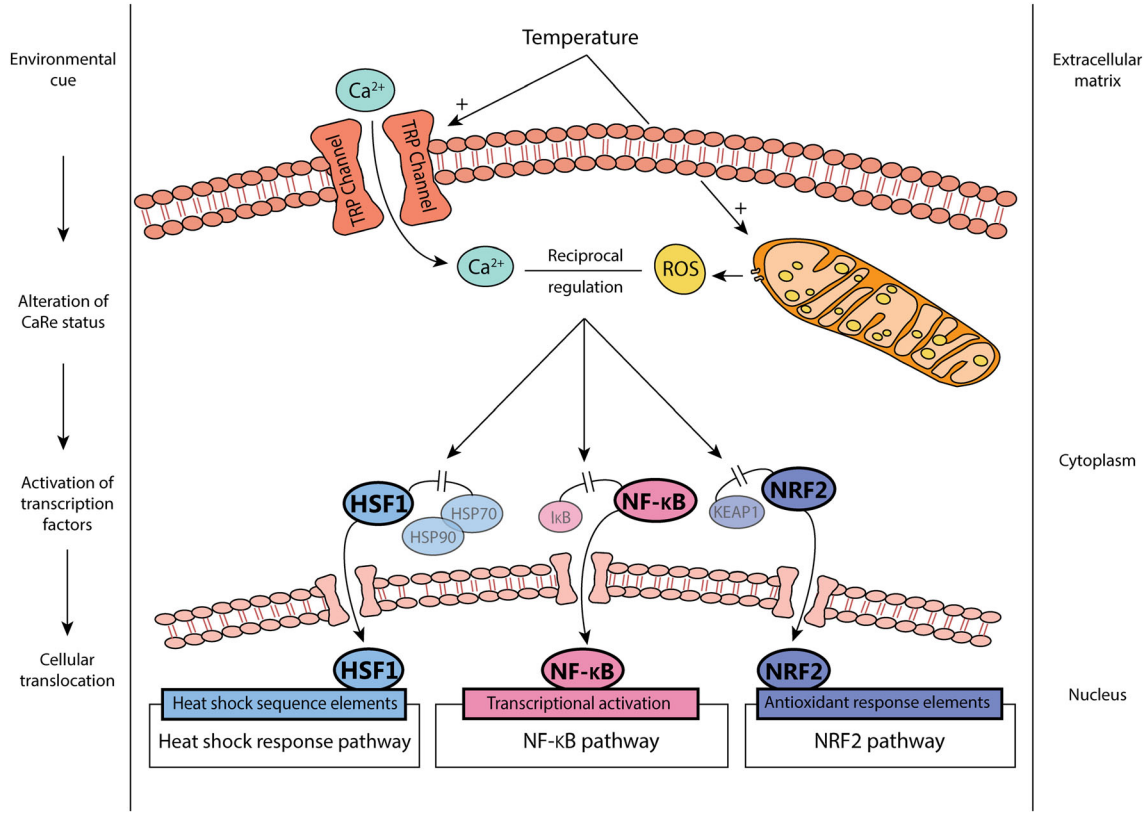

Fig. 1. A subset of environmental response pathways hypothesized to be involved in environmental sex determination, activated by external signals integrated into the cell as calcium and redox (CaRe) status. This simplified model outlines how an environmental cue, in this case temperature, can alter CaRe status by causing an influx of Ca^{2+} ions through innately thermosensitive transient receptor potential (TRP) channels, and an increase in reactive oxygen species (ROS) production by mitochondria through increased metabolic rate. With their reciprocal co-regulation, both Ca^{2+} and ROS can act in concert to activate transcription factors [heat shock factor 1 (HSF1); nuclear factor erythroid-related factor 2 (NRF2)] and pathways [nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)], which then translocate from the cytoplasm to the nucleus to alter the transcription of target genes involved in sex determination. HSP, heat shock protein; KEAP1, Kelch-like ECH-associated protein 1.

with female to male sex reversal (the trigger of sex reversal is not yet known) in the rice field eel (*Monopterus albus*), where it plays a role in inhibiting apoptosis in male germ cells (He *et al.*, 2009). Given HSPs demonstrated roles in sex determination across ESD taxa, and responsiveness to diverse environmental stimuli, they are promising candidates for further study (Fig. 2).

(*c*) *Oxidative stress and the antioxidant response*

Cellular responses to oxidative stress commonly involve induction of the cell's inbuilt antioxidant defense system (Kobayashi *et al.*, 2009). The response is generally initiated by nuclear factor erythroid-related factor 2 (NRF2), whose action is critical for the oxidative stress response and cytoprotection (Brigelius-Flohé & Flohé, 2011; Loboda *et al.*, 2016). Ordinarily NRF2 persists in the cytoplasm at low levels bound in an inactive state with KEAP1 (Kelch-like ECH-associated protein 1). However, in a state of oxidative stress the bond with KEAP1 is broken, allowing NRF2 to translocate to the nucleus where it binds to antioxidant responsive elements. This initiates expression of genes such as thioredoxins, peroxiredoxins, and glutaredoxins that are critical to launching an antioxidant response to oxidative stress (Nguyen, Nioi & Pickett, 2009; Fig. 1).

These antioxidants quench ROS and cross-talk with proteins involved in the NF-κB pathway (Morgan & Liu, 2011). Glutathione is particularly crucial in the oxidative stress response, as the ratio of its oxidized and reduced states (GSH:GSSG ratio) is responsible for sensing the redox status of the cell (Storey, 1996; Hammond *et al.*, 2001; Robert, Brunet-Rossinni & Bronikowski, 2007; Cyr & Domann, 2011). Glutathione directly modifies chromatin structure *via* histone glutathionylation, increasing the binding of transcription factors and upregulating gene expression (Olaso *et al.*, 2013). This has been demonstrated in mammals, in which glutathione enhances decondensation of the paternal genome in a newly fertilized egg (Reyes *et al.*, 1989; Sutovsky & Schatten, 2005; Sánchez-Vázquez *et al.*, 2007).

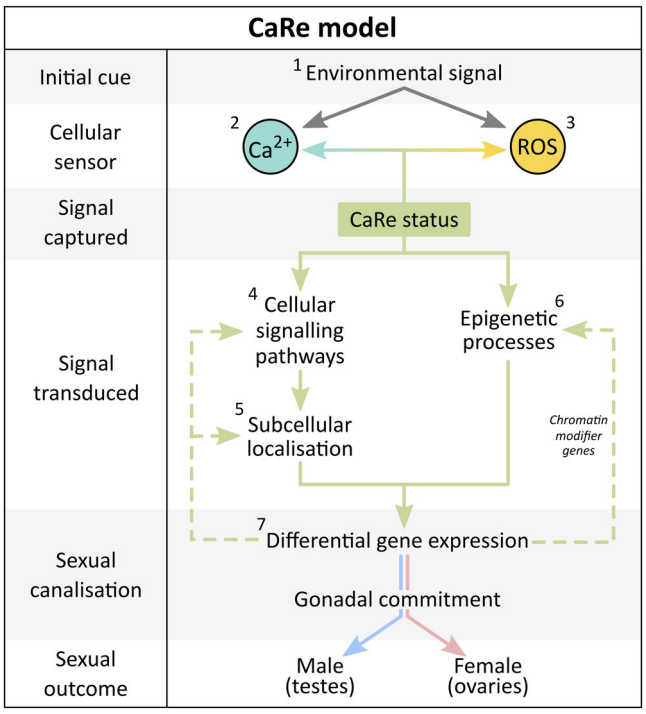

Fig. 2. Generalised model for the influence of environment on sexual fate in vertebrates, identifying target stages for manipulation techniques that facilitate rigorous testing of the model. Solid lines indicate the top-down influence from environmental cue to sexual outcome, while dashed lines indicate areas where there is potential for feedback loops to occur. Incubation/rearing conditions during the environmentally sensitive period can be expanded to include not just the environmental stimulus the species is known to respond to, but other calcium and redox (CaRe)-altering stimuli, such as ultraviolet (UV) light, green light, or pH (1). Ca^{2+} flux can be manipulated either through the addition of calcium (typically accompanied by the calcium transporter ionomycin) or through altering the function of transient receptor potential (TRP) channels, either through RNA interference or the administration of TRP channel agonist and antagonist drugs (2). Reactive oxygen species (ROS) production can be manipulated by the direct addition of oxidants (e.g. H_2O_2) or antioxidants, or by application of ROS-inducing drugs (e.g. doxorubicin) (3). A range of approaches could be taken to interfere with cellular signal transduction pathways, which would vary depending on the pathway of interest (4). Subcellular localisation is similarly pathway specific, but for example small peptides can be used to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation (Gupta *et al.*, 2011) (5). The role of epigenetic regulators can be investigated using agents for histone demethylation (e.g. 5-azacytidine), or through agents that inhibit the epigenetic regulatory machinery, for example polycomb repressive complex 2 (PRC2) inhibitors (Danishuddin *et al.*, 2019) (6). Genes suspected to be involved in the determination of sexual fate can be downregulated through gene knock-down, RNA interference, or the addition of downstream products including hormones or hormone disruptors (e.g. estrogen, testosterone, corticosterone, or fadrozole) (7).

Broadly, the response of antioxidant genes to environmental changes may be able to affect chromatin structure, essentially 'priming' key regions for binding by transcription factors, such as components of the NF-κB pathway (Hammond *et al.*, 2001), and the polycomb repressive complex PRC2, which is likely to be involved in reptile sex reversal (Deveson *et al.*, 2017; Georges & Holleley, 2018). The antioxidant response can therefore induce changes in gene expression and protein function which may contribute to the broader processes taking place during sex determination and differentiation in environmentally sensitive species (Table 1).

(d) *Synergism between hormonal and oxidative stress*

The hypothalamic–pituitary–adrenal (HPA) axis in reptiles, birds and mammals or inter-renal (HPI) axis in fish and amphibians has a role in sex determination in a range of taxa [see reviews in Goikoetxea, Todd & Gemmell (2017) and Geffroy & Douhard (2019)]. Among gonochoristic (single-sex) fish, cortisol-mediated sex determination in response to temperature is well supported by experimental application of cortisol (Hattori *et al.*, 2009; Hayashi *et al.*, 2010; Castañeda Cortés *et al.*, 2019; Miller *et al.*, 2019). Cortisol has not yet been experimentally demonstrated to be a mediator of sex change in sequentially hermaphroditic teleost fish, but transcriptomic evidence suggests cortisol upregulation, supporting a role for the HPI axis in the repression of aromatase and the regulation of downstream epigenetic effectors of gene regulation (Fernandino *et al.*, 2013; Solomon-Lane, Crespi & Grober, 2013; Goikoetxea *et al.*, 2017; Todd *et al.*, 2019).

Even in these fish species in which the stress axis has been co-opted as the environmental sensory mechanism, CaRe pathways may play a synergistic role in initiating, maintaining or mediating sex determination or sex change. Hormonal stress results in oxidative stress *via* an increase in metabolic rate (Spiers *et al.*, 2015), and Ca^{2+} has a very strong association with sexual reproduction in fish (Persson *et al.*, 1998; Johnson & Chang, 2002; Norberg *et al.*, 2004). For example, a social cue such as the removal of a dominant male induces HPI activation and glucocorticoid production in the dominant female of some species (Goikoetxea *et al.*, 2017). Elevated hormonal stress then results in aromatase repression and elevated androgen production through glucocorticoid receptor (GR) nuclear localisation and glucocorticoid receptor element (GRE) occupation in key genomic regions (Adolfi *et al.*, 2019; Todd *et al.*, 2019). Concurrently, hormonal stress leads to oxidative stress through elevated metabolism and energy production (Spiers *et al.*, 2015), and alteration in CaRe status through one or more of the mechanisms described herein. There is extensive cross-talk between the hormonal stress axis and CaRe-sensitive pathways, creating opportunities for the two to synergise. CaRe-sensitive HSPs chaperone GRs, and GRs further interact extensively with the NF-κB pathway in a stimulus-, time-, and cell-specific manner to control responses to stimuli (Bekhbat, Rowson &

Neigh, 2017). Whether CaRe pathways play a causative or synergistic role with stress hormones in species that have co-opted the HPI axis for sex determination (as many teleost fish clearly have) is not yet known, but there is evidence to suggest that these interactions exist.

Among crocodilians, turtles, and squamates there is little, and contradictory, evidence for the involvement of stress hormones in ESD. Temperature sex-reversed adult bearded dragons (*Pogona vitticeps*) display greatly upregulated pro-opiomelanocortin (*POMC*) gene expression in the brain, suggesting stress axis upregulation (Deveson *et al.*, 2017). However, in other reptiles, manipulating incubation temperature and yolk corticosteroids during the embryonic period of sex determination has not demonstrated a causal link between temperature and glucocorticoid production (Uller *et al.*, 2009; Warner, Radder & Shine, 2009; Iungman, Somoza & Piña, 2015; Marcó *et al.*, 2015). Additionally, gonads of TSD reptiles cultured in isolation from the brain were still found to respond to temperature, suggesting that the effect of temperature on the HPA axis is not the temperature-sensitive mechanism in reptiles (Moreno-Mendoza, Harley & Merchant-Larios, 2001; Shoemaker-Daly *et al.*, 2010; Mork, Czerwinski & Capel, 2014). Thus, there is substantial evidence that the stress axis plays a role in ESD in teleost fish, but evidence for stress axis activation as a cause or consequence of sex reversal among reptiles remains equivocal. It is therefore unlikely that the stress axis is central to the temperature-sensitive mechanism in all vertebrates, but a common role for CaRe mechanisms is plausible in both teleost fish and reptiles with ESD.

(2) Subcellular localisation

A commonality among many of the candidate pathways and proteins discussed herein is that their mode of action requires cellular translocation in response to changes in CaRe status (Nelson *et al.*, 2004; Awad *et al.*, 2013) (Fig. 1, Table 1). A change in localisation of transcription factors is necessarily upstream of any changes in nuclear organization and gene expression. For example, in mammals the testis-inducing transcription factor (SOX9) must be translocated from the cytoplasm to the nucleus for normal testes development to occur. Otherwise, the developing gonads retain ovary-like characteristics even when expression levels of *SOX9* are maintained (Chen *et al.*, 2017). This process in mammals is regulated by the CaRe-sensitive catabolite activator protein cyclic AMP (cAMP) and protein kinase A phosphorylation (Malki *et al.*, 2005*a*,*b*), and by Ca^{2+}-calmodulin nuclear entry pathways (Hanover, Love & Prinz, 2009). It is plausible that a similar process, linked more directly to environmental conditions, occurs in vertebrates with ESD. While numerous candidates whose function relies on changes in cellular localisation have been associated with ESD, functional studies in this context are currently lacking, so future experimentation would benefit from considering these processes (Fig. 2).

(3) Alternative splicing and epigenetic remodeling

As well as the signal transduction pathways discussed above, there are other mechanisms that can also modulate gene expression in response to environmentally driven changes in CaRe status (Table 1). While these are as yet poorly understood, evidence is building that post-transcriptional processes including alternative splicing and epigenetic remodeling are involved in ESD.

In the 1990s, differential splicing was proposed to control TSD after differential expression of heterogeneous ribonucleoprotein particles (hnRNPs) was discovered in two TSD turtles (diamondback terrapin, *Malaclemys terrapin* and loggerhead turtle, *Caretta caretta*) (Harry *et al.*, 1990; Harry, Briscoe & Williams, 1992; Jeyasuria & Place, 1998; Table 1). Splicing factors in the hnRNP family were suggested to regulate expression of key genes in a temperature-dependent manner at crucial stages in development, although the mechanism by which thermosensitivity is conferred on hnRNPs was (and remains) unidentified (Harry *et al.*, 1992; Matthew Michael, Choi & Dreyfuss, 1995; van der Houven van Oordt *et al.*, 2000; Huelga *et al.*, 2012).

Subsequently, sex-specific associations with a single nucleotide polymorphism, embryonic expression profiles, and protein localisation in the TSD snapping turtle (*Chelydra serpentina*) suggested that *CIRBP* (cold-inducible RNA-binding protein; *CIRP*, *A18 hNRNP*) was critical for determining sex (Schroeder *et al.*, 2016). This gene has thermosensitive expression in the pond slider turtle (*Trachemys scripta*) (Chojnowski & Braun, 2012) and Chinese alligator (*A. sinensis*) (Lin *et al.*, 2018), so this gene may be involved in TSD more broadly. CaRe status may be involved in the regulation of *CIRBP*, as it can be activated by a variety of environmental stressors that cause changes in CaRe, including osmotic shock, hypoxia, heat, and oxidative stress (Zhong & Huang, 2017). *CIRBP* may also be involved in mediating CaRe-regulated feedback loops, as upon activation it can function as an RNA chaperone or post-transcriptional regulator of many CaRe-sensitive genes (Peng *et al.*, 2006; De Leeuw *et al.*, 2007; Zhang *et al.*, 2016; Zhong & Huang, 2017).

Recent work supports the early evidence for a role of alternative splicing of key chromatin remodeling genes in TSD in reptiles. A sex-associated retained intron event in two members of the Jumonji gene family *JARID2* and *JMJD3* (also called *KDM6B*) occurs in three thermally sensitive reptile species (*Pogona vitticeps*, *Alligator mississippiensis*, and *Trachemys scripta*; Deveson *et al.*, 2017). In *P. vitticeps*, intron retention (IR) occurs only in sex-reversed females produced at high incubation temperatures. There is variation among these species in the pattern of sex-associated IR, perhaps arising from different ancestral genetic sex determination systems (Deveson *et al.*, 2017). In a fish that undergoes socially cued sex change, the bluehead wrasse *Thalassoma bifasciatum*, *JARID2* and other cofactors within the PRC2 (*EZH2*, *SUZ12*, *EED*, *RNF2*) are transiently downregulated during female to male transition (Todd *et al.*, 2019). Both *JARID2*

and *JMJD3* also exhibit thermosensitive expression in the brains of sex-reversed (neomale) Nile tilapia (*Oreochromis niloticus*) (Zhao *et al.*, 2019). The PRC2 complex is also involved in orchestrating the commitment of sexual fate in GSD species, primarily through chromatin remodeling on the sex chromosomes (Garcia-Moreno, Plebanek & Capel, 2018).

JARID2 and JMJD3 regulate the tri-methylation of histone H3, lysine 27 (H3K27), and are involved in orchestrating embryonic development and sexual differentiation (Sanulli *et al.*, 2015; Holoch & Margueron, 2017) (Fig. 3). Knockdown of *JMJD3* in a TSD turtle (*T. scripta elegans*) at male-producing temperatures triggers female development in 80% of embryos that survive (Ge *et al.*, 2018). JMJD3 mediates transcription of the male-determining gene *DMRT1* (Ge *et al.*, 2017) by demethylating the repressive H3K27me3 near its promoter (Ge *et al.*, 2018). Downregulation of *JMJD3* by upstream mechanisms responding to high temperature results in persistent tri-methylation of H3K27, which suppresses *DMRT1* and promotes the female developmental pathway (Fig. 3). Upregulation of *JMJD3* in response to lower temperature results in de-methylation of H3K27me3 near the *DMRT1* promoter, activating *DMRT1* expression and promoting the male developmental pathway (Fig. 3). In alligators, switching embryos from a low female-producing temperature to a high male-producing temperature results in downregulation of *JARID2* and *JMJD3*, further demonstrating the commonality of these chromatin remodeling pathways in reptiles (Yatsu *et al.*, 2016). The interplay between thermo-responsive intron retention and activity of *JMJD3* (Deveson *et al.*, 2017; Ge *et al.*, 2018) is not well understood (Georges & Holleley, 2018). However, these recent findings have dramatically shifted the focus of inquiry from direct thermosensitivity of candidate sex-determining genes to higher-order thermosensitive epigenetic processes that differentially downregulate or upregulate influential sex genes (Georges & Holleley, 2018).

CaRe status may be directly linked to the epigenetic processes discussed above. ROS release from mitochondria (Ying *et al.*, 2018) and hydrogen peroxide exposure (Niu *et al.*, 2015) can alter histone methylation, and the oxidative status of a *JMJD3*-regulating transcription factor (STAT6) directly alters *JMJD3* (He *et al.*, 2016). JARID2 and the associated epigenetic remodeling complex PRC2, and JMJD3, exhibit a wide range of responses to oxidative and other cellular stressors, triggered by environmental signals such as heat shock (Marasca, Bodega & Orlando, 2018). The actions of hnRNPs also change depending on their oxidation status. For example, the activity of hnRNPk (a chaperone and inhibitor of HSF1 binding to heat shock elements) alters depending on the oxidation status of a single redox-sensitive cysteine residue, affecting the activation of heat shock response genes (Kim *et al.*, 2017). Alternatively, epigenetic processes may be mediated by the CaRe-responsive signalling pathways detailed above. The NF-κB pathway is known to control some histone methylation marks, perhaps *via* the transcriptional regulation of *KDM2B*, another lysine demethylase (Nakshatri *et al.*, 2015), and HSF1 has been demonstrated to open chromatin structure to assist the recruitment of other transcription factors (Inouye *et al.*, 2007). These examples point to a promising area of future research, directed at the CaRe-sensitive epigenetic processes driving ESD.

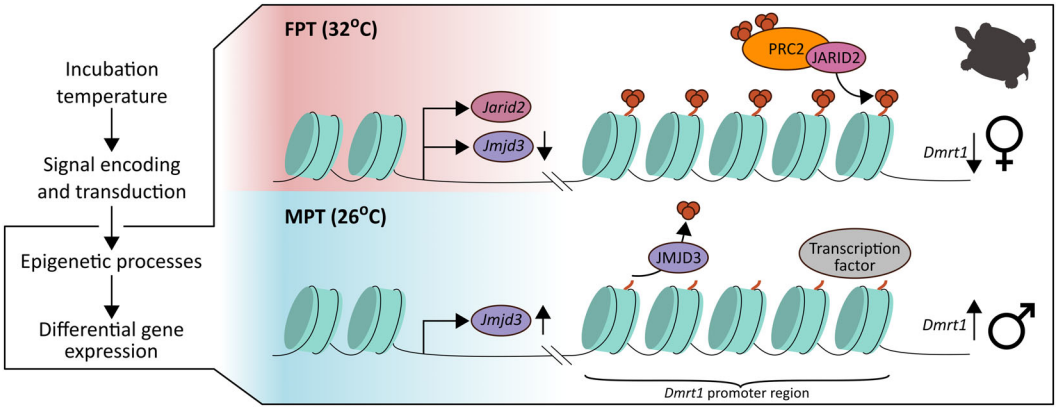

Fig. 3. A schematic diagram showing the action of *Jumonji* family genes in altering the expression of a key sex gene in the red-eared slider turtle (*Trachemys scripta elegans*) based on the work of Ge *et al.* (2017, 2018). At female-producing temperatures (FPT), the chromatin modifier *JMJD3*, a histone demethylase, is downregulated, presumably under the influence of calcium and redox (CaRe)-mediated upstream signal transduction pathways. This allows the polycomb repressive complex 2 (PRC2) complex to deposit heritable methylation marks on histone 3 lysine 27 (H3K27me3), in part due to the action of JARID2. The methylation marks deposited in the *DMRT1* promoter give permanence to the trimethylation and repression through cell division, ultimately leading to ovary development. At male-producing temperatures (MPT), *JMJD3* is upregulated, likely under the influence of upstream CaRe-mediated signal transduction pathways. *JMJD3* removes the H3K27me3 marks deposited by the PRC2 complex on the *DMRT1* promoter, which then opens this region for transcription by as yet unidentified transcription factors, so altering the developmental trajectory toward a male fate. [After Georges & Holleley, 2018]. Image credit (turtle silhouette) Roberto Díaz Sibaja under PhyloPic Creative Commons attribution unported license 3.0.

IV. EVOLUTIONARY SIGNIFICANCE OF CaRe REGULATION

Tightly controlled regulation of intracellular levels of Ca^{2+} and ROS is essential for life, and has been since the emergence of the earliest eukaryotes (Maynard Case *et al.*, 2007). The regulatory mechanisms by which Ca^{2+} and ROS are sensed, and the genetic pathways involved in responding to these signalling molecules, are therefore highly conserved (Aguirre *et al.*, 2005). The evolution of sexual reproduction itself has been proposed as an adaptive response to mitigate the subcellular damage caused by increased production of ROS in an oxygen-rich environment (Nedelcu & Michod, 2003). An alternative view is that ROS production by bacterial endosymbionts may have driven the evolution of sexual reproduction as a mechanism to allow for DNA repair through recombination (Hörandl & Speijer, 2018).

In a facultatively sexual multicellular alga (*Volvox carteri*), temperature-induced ROS production triggered sexual reproduction (Nedelcu, Marcu & Michod, 2004), and treatment with antioxidants completely inhibited temperature-induced sexual reproduction (Nedelcu & Michod, 2003). There is a fundamental association between ROS and the regulation of sexual reproduction in all three eukaryotic domains (Gapper & Dolan, 2006). ROS are known to control sexual/asexual reproductive modes in fungi (Lara-Ortíz, Riveros-Rosas & Aguirre, 2003), affect germination and gametogenesis in plants (Chailakhyan & Khrianin, 1987; Traverso *et al.*, 2013), and influence reproductive phenotypes in multicellular animals (Shibata *et al.*, 2003).

Canalisation of the downstream regulatory pathways of gonad development, indicated by the relative commonality of gonadal structure, releases upstream elements of the regulation from selection. Provided functional ovaries or testes result, diversity in the upstream regulatory processes will be tolerated by selection (Georges *et al.*, 2010; Capel, 2017). The resultant evolutionary flexibility might account for the phylogenetic variability of ESD systems, which has been difficult to explain (Sarre *et al.*, 2004; Bachtrog *et al.*, 2014; Pennell, Mank & Peichel, 2018). In particular, the independent re-emergence of TSD from GSD can be seen as a gain of sensitivity to the environment without the disruption of underlying CaRe mechanisms, which are essential for life (Pokorná & Kratochvil, 2009; Georges *et al.*, 2010; Janes, Organ & Edwards, 2010). Sensitivity to CaRe status can therefore be rapidly regained if there is selective pressure to do so. This may require only small-scale biochemical changes, allowing rapid responses in shorter evolutionary time scales compared with larger scale genetic or physiological changes.

V. APPLYING THE CaRe MODEL IN THEORY AND PRACTICE

(1) Summary of the model

We have provided a simplified and generalized framework that proposes a critical role for CaRe regulation in environmentally sensitive sex determination systems. The CaRe model we present posits that an environmental influence, for example temperature, acts as a cue to stimulate a regulatory cascade that ultimately delivers a sexual outcome (testes or ovaries) (Fig. 2). Such temperature cues act upon thermosensitive ion channels to regulate Ca^{2+} flux, interacting with ROS production driven by metabolic rate, resulting in a CaRe status that captures the environmental signal. CaRe status is decoded and transmitted to the nucleus *via* signal transduction pathways, such as the NF-κB and heat shock response pathways, potentially moderated by antioxidant activity (Fig. 1). Each of these signal transduction pathways is likely to involve changes in subcellar localisation of key transcription factors such as HSF1, which can influence expression of genes responsible for developmental outcomes (Kim *et al.*, 2009; Fig. 1). CaRe status can also be transmitted *via* epigenetic or post-translational modifications, so that a diverse array of CaRe-sensitive cellular pathways can ultimately drive differential gene expression and direct sexual outcomes.

(2) Testing hypotheses derived from the model

While our model is necessarily speculative, it forms a basis for the generation of testable hypotheses and the re-examination of existing data. Models such as this have proven immensely successful in setting priorities and giving direction to research on the genes and gene products responsible for sexual differentiation (Morrish & Sinclair, 2002; Smith & Sinclair, 2004).

Functional analysis will be critical for determining the role of CaRe in ESD systems and elucidating the species-specific pathways involved. Our model identifies target stages at different levels of the pathway for manipulation techniques, which can be applied to a wide range of study species (Fig. 2). Manipulation of such ubiquitous signal transduction pathways is likely to present practical barriers (e.g. lethality), so we suggest that functional manipulation should exploit the wide variety of targeted inhibitor drugs and enhancers in both *in vitro* and *in vivo* experiments. We might borrow approaches from the biomedical and cancer research fields, in which these regulatory pathways are becoming well characterized and techniques for their manipulation are becoming more accessible. Gene editing techniques such as the clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) system (Cong & Zhang, 2015), combined with drug manipulation and transcriptomic approaches, will increase understanding of the role of these ubiquitous signal transduction pathways in both model and non-model species with ESD.

Understanding the mechanisms by which environmental signals are transduced to determine sex will have broader implications beyond the evolution of ESD systems. Practical applications could include manipulation of sex ratios in aquaculture systems, which frequently rear ESD species. Precise control of sex ratios in farmed species could increase efficiency of food production for a growing human population (Budd *et al.*, 2015). More broadly, a better understanding of ESD is increasingly important for assessing the biological

impacts of climate change on environmentally sensitive species (Parmesan & Yohe, 2003; Umina *et al.*, 2005; Etterson *et al.*, 2007; Sinervo, 2010; IPCC, 2013). Already populations of ESD species are experiencing skewed sex ratios caused by rising global temperatures (Mitchell & Janzen, 2010; Refsnider & Janzen, 2016; Bókony *et al.*, 2017; Hays *et al.*, 2017; Honeycutt *et al.*, 2019). By understanding how an environmental signal is transduced to a sexual outcome, novel conservation management strategies could be devised to avoid or mitigate these impacts of climate change.

VI. CONCLUSIONS

(1) A universal cellular sensor in ESD systems must be (*i*) inherently environmentally sensitive; (*ii*) capable of interacting with components of known sex determination pathways; and (*iii*) highly conserved in function yet plastic enough to be recruited for the transduction of different environmental signals for different phenotypic outcomes.

(2) CaRe status meets these requirements for a cellular sensor, and associated CaRe-sensitive pathways are promising candidates for the transduction of the environmental cue to orchestrate sex determination and differentiation in ESD species. Several lines of evidence support our model that CaRe-sensitive pathways have been independently and repeatedly co-opted as the mechanism by which an environmental signal is transduced to a sexual outcome in ESD species.

(3) The CaRe model is so far the only unifying model that has been proposed for ESD in vertebrates. Continued investigation of the role of CaRe regulation in ESD through explicit testing of CaRe mechanisms proposed in this review will not only advance understanding of evolutionary developmental biology and genetics, but may also at last identify the cellular sensing mechanism of ESD.

(4) We posit that what has been viewed as an intractable problem of identifying the environmentally sensitive element(s) among a myriad of possible candidates with putative influences on sexual differentiation, instead involves the more tractable challenge of identifying highly conserved ancestral elements of cellular machinery under the influence of equally highly conserved signalling pathways.

(5) We present this model as a basis for future experimentation that goes beyond simply examining gene expression. Our model incorporates signal reception, capture of the signal by the cell, receipt of the signal by established cellular signal transduction pathways, and the transduction of signals to the epigenome to direct gene expression leading to discrete sexual outcomes.

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VIII. AUTHOR CONTRIBUTIONS

M.A.C. and S.L.W. initially developed the CaRe model and jointly led the formulation of ideas and writing of the manuscript to which A.G. and C.E.H. also contributed substantially.

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