Received: 14 February 2022 | Revised: 26 April 2022 | Accepted: 28 June 2022

DOI: 10.1111/jeb.14064

JOURNAL OF Evolutionary Biology eseb

RESEARCH ARTICLE

Evolution and dosage compensation of nucleolar organizing regions (NORs) mediated by mobile elements in turtles with female (ZZ/ZW) but not with male (XX/XY) heterogamety

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Funding information
National Science Foundation of the United States, Grant/Award Number: IOS 1555999, MCB 0815354 and MCB 1244355

Abstract

Understanding the evolution and regulation of nucleolar organizing regions (NORs) is important to elucidate genome structure and function. This is because ribosomal gene (rDNA) copy number and activity mediate protein biosynthesis, stress response, ageing, disease, dosage compensation and genome stability. Here, we found contrasting dosage compensation of sex-linked NORs in turtles with male and female heterogamety. Most taxa examined exhibit homomorphic rRNA gene clusters in a single autosome pair (determined by 28S rDNA fluorescence in situ hybridization), whereas NORs are sex-linked in *Apalone spinifera*, *Pelodiscus sinensis* and *Staurotypus triporcatus*. Full-dosage compensation upregulates the male X-NOR (determined via silver staining—AgNOR) in *Staurotypus* (who lacks Y-NOR) compared with female X-AgNORs. In softshell *Apalone* and *Pelodiscus*, who share homologous ZZ/ZW microchromosomes, their enlarged W-NOR is partially active (due to 28S rDNA invasion by R2 retroelements), whereas their smaller Z-NOR is silent in females but active in both male-Zs (presumably because the W-NOR meets cellular demands and excessive NOR activity is costly). We hypothesize that R2 disruption favoured W enlargement to add intact 28S-units, perhaps facilitated by reduced recombination during sex chromosome evolution. The molecular basis of the potentially adaptive female Z-silencing is likely intricate and perhaps epigenetic, as non-ribosomal Z genes are active in *Apalone* females. Yet, *Emydura maquarii* exhibit identical heteromorphism in their autosomal NOR (R2 invaded 28S-units and the small-autosome NOR is silent), suggesting that the softshell turtle pattern can evolve independent of sex chromosome evolution. Our study illuminates the complex sex chromosome evolution and dosage compensation of non-model systems that challenges classic paradigms.

KEYWORDS
18S/28S repetitive DNA, adaptive evolution of sex chromosome dosage compensation, gene regulation silencing, nucleolar organizing region, R2 retrotransposon retroelement transposable element, ribosomal RNA genes (rRNA, rDNA), silver-staining AgNOR, temperature-dependent (TSD) and ZZ/ZW XX/XY genotypic sex determination (GSD), turtle reptile vertebrates

1 | INTRODUCTION

The nucleolus is a nuclear organelle unbound by membranes where ribosomal RNA is produced and ribosomes are assembled, helping set cellular metabolism. Furthermore, the association between nucleolar function and stress response, development and ageing is increasingly recognized (Tiku & Antebi, 2018). Expression of 18S, 5.8S and 28S ribosomal subunit genes located in the nucleolar organizing region (NOR) of the genome (Shaw & McKeown, 2011) initiates ribosomal biogenesis needed for protein synthesis, and the metabolic and cell growth demands are met by both rDNA copy number and activity (Lawrence & Pikaard, 2004; Shaw & Doonan, 2005). Therefore, it is important to understand the evolution and functional consequences of rRNA gene copy number variation within NORs observed among individuals, chromosomes, and species (Britton-Davidian et al., 2012; Literman et al., 2014). Indeed, some genomes contain a single NOR locus, as is common in reptiles, amphibians and fish (Gornung, 2013; King et al., 1990; Montiel et al., 2016; Olmo & Signorino, 2005; Schmid & Steinlein, 2015), whereas other taxa possess multiple NOR loci, including mammals and birds (Barbosa et al., 2013; Britton-Davidian et al., 2012; Farley et al., 2015; Rocha & Delucca, 1988; Supanuam et al., 2012).

Because not all of the NOR loci may be transcriptionally active (Farley et al., 2015) detection methods are noteworthy (Bickham & Rogers, 1985). NORs have been identified by (1) secondary constrictions of chromosomes (an unreliable technique because not all secondary constrictions harbour NORs) (Bickham & Rogers, 1985; King et al., 1990; Montiel et al., 2016; Prieto & McStay, 2008); (2) silver staining (AgNOR) of transcriptionally active NORs (Goodpasture & Bloom, 1975) (since Ag reacts with proteins associated with rDNA activity; Howell, 1977); and by (3) rDNA fluorescence in situ hybridization (rDNA FISH, e.g. using 18S or 28S rDNA probes) which identifies transcriptionally active and inactive NORs (Leitch et al., 1992; Weisenberger & Scheer, 1995). In vertebrates, NORs change chromosomal location across lineages during evolution (Odierna et al., 1987), and may be present in autosomes (e.g. Odierna et al., 1987; Rocha & Delucca, 1988) or sex chromosomes, as occurs in some turtles (e.g. Badenhorst et al., 2013; Bickham & Rogers, 1985; Kawai et al., 2007; Montiel et al., 2016; Sites et al., 1979; Supanuam et al., 2012).

Consequently, sex-linked NORs may be affected differently by the idiosyncratic molecular evolution characteristic of sex chromosomes compared to autosomal NORs, such as mutation accumulation and dosage compensation that influence other sex-linked loci (Bachtrog et al., 2011). Indeed, reduced recombination during sex chromosome evolution (XX/XY or ZZ/ZW) can cause loss of Y or W genes (Charlesworth, 1996), leading XX females to carry twice as many copies of X genes as XY males (similarly for ZZ males compared with ZW females). While less common, the differentiation of sex chromosomes may lead to the accumulation of repeat DNA or gene duplications in the heterogametic sex chromosome, causing its expansion instead of its attrition (Kratochvíl et al., 2021). Dosage compensation mechanisms (DCMs) evolved that alter gene regulation and equilibrate protein levels affected by gene dosage imbalance between sex chromosomes and autosomes (Chandler, 2017; Gu & Walters, 2017; Lucchesi, 1978; Ohno, 1967). This is called sex-chromosome dosage compensation (SCDC). But dosage compensation per se is not a process unique to sex chromosomes. Indeed, dosage-sensitive genes in autosomes that suffer altered copy number due to duplication or deletion of small regions or whole chromosomes (e.g. aneuploidy, trisomy) may also undergo compensatory adjustments (autosomal dosage compensation—ADC) to ameliorate suboptimal phenotypes (Disteche, 2016).

Here, we examine rDNA cluster activity in several turtle genomes, emphasizing taxa whose NORs are sex-linked and suffer gene dose imbalance between the sexes. Namely, the Mexican giant musk turtle *Staurotypus triporcatus* displays male heterogamety (XX/XY) with X-linked NORs but lacks Y-linked NORs (Montiel et al., 2016; Sites et al., 1979). Thus, females carry a double dose of rRNA genes compared with males, a condition that should be typically compensated to equalize gene activity. Additionally, the softshell turtle *Apalone spinifera*, exhibits female heterogamety (ZZ/ZW) with greatly expanded W-NOR clusters compared with the Z-NORs (Badenhorst et al., 2013), likely due to reduced recombination, such that ZW females carry on average four times as many copies of rRNA genes than ZZ males (Literman et al., 2014).

Enlargement of the heterogametic sex chromosome has been observed in other vertebrates and can result from the expansion of rDNA sequences or other repetitive elements (Cioffi et al., 2010; Martinez et al., 2008; Matsubara et al., 2014; Nanda et al., 2000). Indeed, NORs host R2 retroelements, the oldest known mobile element, which arose during the origin of metazoans and is widely distributed among animals (Kojima et al., 2016). R2 inserts itself at a specific site of the 28S rRNA gene, invading R2-free units via an RNA intermediary (Eickbush & Eickbush, 2015). Although R2 function is unclear, its density in the rRNA locus is linked to NOR inactivation and pseudogenization of the invaded units, suggesting that R2 influences NOR dynamics (Eickbush et al., 2008; Zhou et al., 2012, 2013). Importantly, because increased copy number of functional genes can also alter the stoichiometry between sex-linked and autosomal genes, it is expected that expanded sex chromosomes should select for dosage compensation. Such balance is expected between ribosomal genes and genes functionally associated with the nucleolus (Gibbons et al., 2014). Additionally, a balance between the NOR locus (which encodes the 18S, 5.8S and 28S ribosomal subunits) and the 5S rRNA locus located outside the NOR is also expected, because all four rRNAs are essential for the structural and catalytic integrity of the ribosomes (Gibbons et al., 2015), which has led to compensatory adjustments or concerted evolution in various eukaryotes (Disteche, 2016; Gibbons et al., 2015).

Here, we present AgNOR data for 13 turtle species, 7 of which lack AgNOR information altogether, and contrast these data with previous reports using 18S rDNA FISH (Montiel et al., 2016). AgNOR is a well-established proxy of rDNA transcription (Krzyzanowska et al., 2015). We then use AgNOR data to test for chromosome-specific and sex-specific activity of sex-linked NORs in turtles with contrasting heterogamety (*A. spinifera*—ZZ/ZW, *Pelodiscus sinensis*—ZZ/ZW, and

S. triporcatus—XX/XY) to test whether ribosomal DNA is subject to gene dosage compensation in turtles. We also examine the co-distribution of R2 retrotrasposons in these taxa to test whether rDNA silencing by R2 retrotransposons may mediate the rDNA activity measured by silver staining. Ours is the first chromosomal mapping of R2 retroelements in chelonians. Our combined results reveal the existence of rDNA dosage compensation by male-X upregulation in XX/XY turtles, and by female-Z-rDNA silencing in ZZ/ZW turtles. Additionally, the dynamics of NOR evolution and NOR expression in ZZ/ZW turtles correlate with of R2-mediated rDNA silencing. Our data support the hypothesis that alternative dosage compensation mechanisms for rDNA are utilized in chelonians with contrasting heterogamety. Our results also reveal some unanticipated commonality in the mechanisms of rDNA regulation in turtles with Z/W-NORs and autosomal NORs, illuminating their potential evolutionary origin. Our study expands the breath of the known diversity and evolution of dosage compensation in eukaryotes.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Primary fibroblast cell cultures were established from embryonic and hatchling muscle tissue following standard procedures (Badenhorst et al., 2013; Lee et al., 2019; Montiel et al., 2016) for 13 target turtle species (*A. spinifera*—ASP, *Glyptemys insculpta*—GIN, *Sternotherus odoratus*—SOD, *Carettochelys insculpta*—CIN, *Emydura macquarii*—EMA, *Chelodina oblonga*—COB, *Pelomedusa subrufa*—PSU, *P. sinensis*—PSI, *S. triporcatus*—STR, *Chrysemys picta*—PI, *Trachemys scripta*—TSC, *Chelydra serpentina*—CSE, *Podocnemis unifilis*—PUN) and correspond to specimens studied previously (Montiel et al., 2016). Hereafter, species will be referred to by their genus name or 3-letter acronym. For species with sex-linked NORs (*Apalone*, *Pelodiscus*, and *Staurotypus*), at least one male and one female were examined. Four hours prior to harvesting, 10 μg/ml colcemid (Karyo-MAX®; Invitrogen) was added to the cultures, and cells were harvested after hypotonic exposure (20 min at 29°C in 10 ml of a solution of 0.034 M KCl and 0.007 M sodium citrate) and then fixed in 3:1 methanol:acetic acid following standard procedures (Badenhorst et al., 2013; Ezaz et al., 2006; Martinez et al., 2008). To obtain interphase nuclei and metaphase chromosome preparations, cell suspensions were dropped onto glass slides and air-dried. The sex of hatchlings was diagnosed by gross gonadal morphology, whereas the sex of *Apalone* embryos was diagnosed via PCR amplification of sex-linked markers (Literman et al., 2017), a simpler method than by qPCR of rDNA repeats (Literman et al., 2014).

2.2 | Silver staining

Silver staining was used to identify transcriptionally active NORs (Howell & Black, 1980). Briefly, 50% $AgNO_3$ solution and 2% gelatin developer (1:1 proportion) were dropped onto the chromosome slides and covered with cover slips. Slides were incubated for 8 min at 53°C, then washed with water and dried. A minimum of 25 interphase nuclei were analysed per sex and species.

2.3 | Interphase nucleoli quantification

For species with sex-linked NORs (*Apalone*, *Pelodiscus*, and *Staurotypus*), the area of the nucleus and nucleolus was measured in arbitrary units using ImageJ (version 1.48) and data (Table S1) were analysed following (Montiel et al., 2012). Briefly, four variables were measured for each cell: (a) nucleus area, (b) nucleolus area, (c) the ratio of nucleolus area to nucleus area and (d) number of nucleoli, parameters that are well-known surrogates of rDNA transcription (Krzyzanowska et al., 2015 and references therein). Each variable was tested for differences between sexes within species using Welch's *t*-test for continuous variables (nucleus area, nucleolus area and their ratio) and Chi-square test for discrete variable (number of nucleoli), in R (v3.3.2) (R Core Development Team, 2016). Because additional *Apalone* specimens were available, four adult females, two adult males, plus four male and four female embryos incubated at 26°C were added to this analysis.

2.4 | Novel FISH of 28S rDNA, 18S rDNA and R2-transposon DNA

New FISH data were collected to confirm the presence of a single rDNA cluster in the genomes of species with sexually dimorphic or heteromorphic NORs (*Apalone*, *Pelodiscus*, *Staurotypus* and *Emydura*) following Montiel et al. (2016). A previously designed turtle-specific probe for the 18S rRNA gene (Montiel et al., 2016), and a newly designed turtle-specific probe for the 28S rRNA gene were amplified in each species using primers: 18SF: 5′ GACCCTGTAATTGGAATGAGTAC, 18SR: 5′ GTTCATTATCGGAATTAACCAGAC, 28SF: 5′ ATGAATGG ATGAACGAGATTC, 28SR: 5′ CAACGCTTGGTGAATTCTG. The distribution of R2 element population in these genomes was examined with FISH using an R2 probe obtained by PCR amplification with primers designed in conserved regions of the *Chrysemys picta* R2 element: R2_CPB_F: 5′ GGCTCCGGGTCCTGAC, R2_CPB_R: 5′ CGAGTCACTGAGCATGACCA.

All probes were amplified from genomic DNA (gDNA) by PCR in a 25-μl reaction containing 20–100 ng gDNA, 2.5 μl Taq buffer (10×), 0.75 μl $MgCl_2$ (50 mM), 2 μl dNTPS (2.5 mM), 1 μl of each forward and reverse primer (10 μM) and 0.5 μl Taq polymerase (5 U/μl), and dH_2O. Cycling parameters included initial denaturation at 94°C for 3 min followed by 25 cycles of: 94°C for 45 s, 54°C for 45 s and 72°C for 50 s and a final extension of 72°C for 10 min. PCR products were labelled by PCR with biotin or digoxingenin dUTPs (Roche) in a 25 μl reaction as described above, where the dNTPs mix consisted of 1dATP:1dCTP:1dGTP:1/2 dTTP. FISH was performed following Badenhorst et al. (2013). Images were taken

with a Photometrics CoolSnap ES2 Digital Monochrome camera attached to an Olympus BX41 fluorescence microscope and analysed using CytoVision® cytogenetic analysis system (Applied Imaging/Genetix).

3 | RESULTS

3.1 | Turtles possess a single NOR locus that is heteromorphic mostly when sex-linked

Our new 28S rDNA FISH data (Figure 1, Figure S1) confirmed observations using 18S FISH (Montiel et al., 2016) that most taxa examined carry a unique cluster of rDNA in a single pair of autosomes. Exceptions are *Staurotypus* (who has an X-linked NOR but lacks a Y-linked NOR), the softshell turtles *Apalone* and *Pelodiscus* (whose W-NOR is much larger than the Z-NOR) (Figure 1), and *Emydura* (Figure 2) whose rDNA clusters display a strikingly similar heteromorphism in size between the two homologues of a pair of micro-autosomes, as that observed in softshell turtles between the Z and W (Figure 1).

3.2 | NORs are equally active when they are homomorphic but not when they are heteromorphic

AgNOR staining revealed similar NOR activity in both autosomal homologues in all taxa with homomorphic NORs (Figure S2, except S2j). However, heteromorphic rDNA clusters display differential activity that varied by species. Specifically, AgNORs were detected in both Zs in *Apalone* and *Pelodiscus* males, but only the W- (and not the Z-NOR, which is detectable by rDNA FISH) is silver-stained in females (Figure 3), indicating full Z-NOR silencing in females. Moreover, silver staining in softshell females covers only a portion of the W-rDNA cluster identified by rDNA FISH (Figure 2), revealing further regulation of rDNA activity by partial W-NOR silencing. Intriguingly, *Emydura* displayed silver

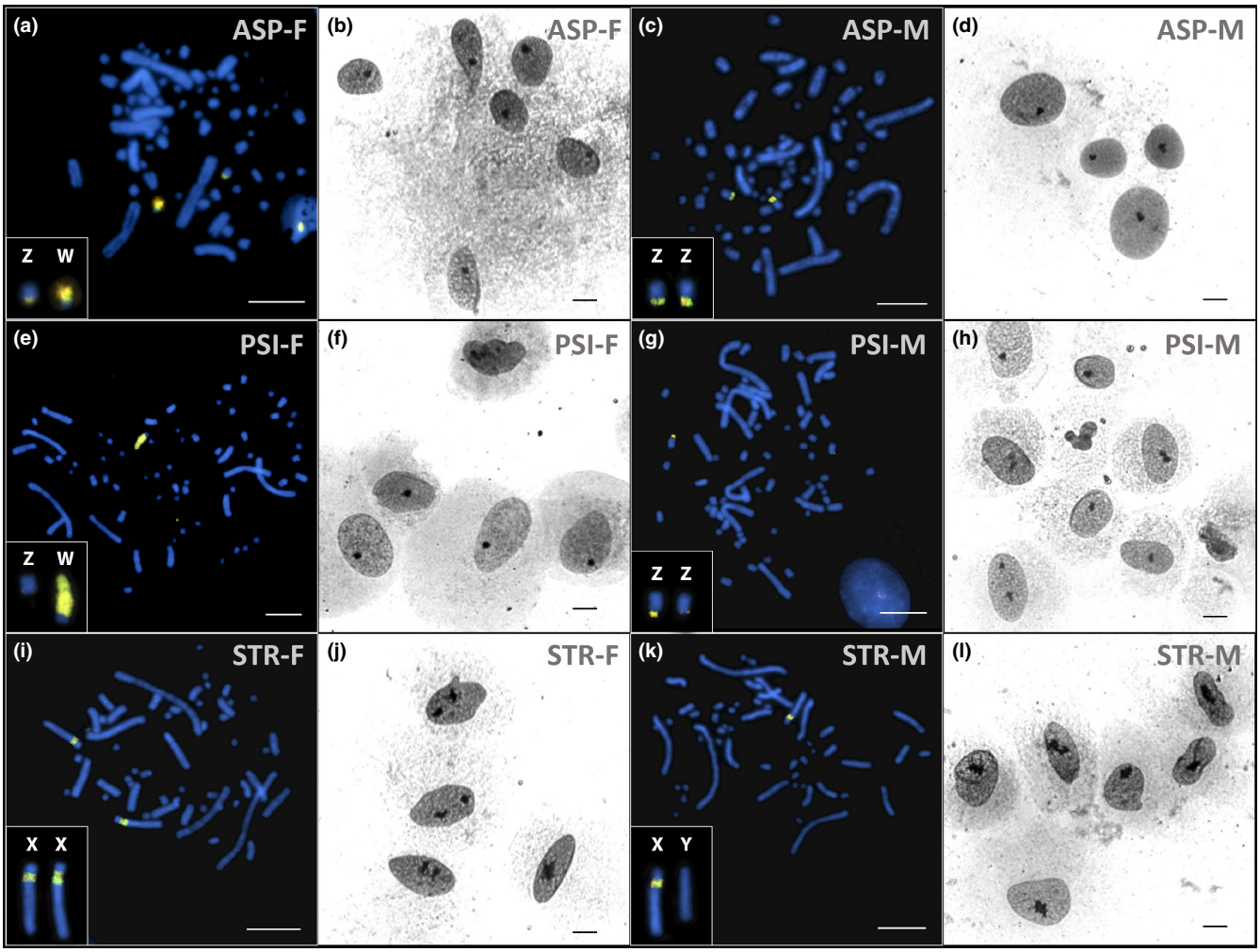

FIGURE 1 rDNA cluster mapping using 18S RNA gene probe (green) and 28S RNA gene (red) in *Apalone* female (a) and male (c), *Pelodiscus* female (e) and male (g) and *Staurotypus* female (i) and male (k). Pseudo-yellow indicates colocalization of green (18S) and red (28S) probes. Additionally, silver-stained interphase nuclei are included from *Apalone* (b, d), *Pelodiscus* (f, h) and *Staurotypus* (j, l). Boxes contain the enlarged sex chromosomes from each spread. F = female, M = male. Scale bar = 10 μm

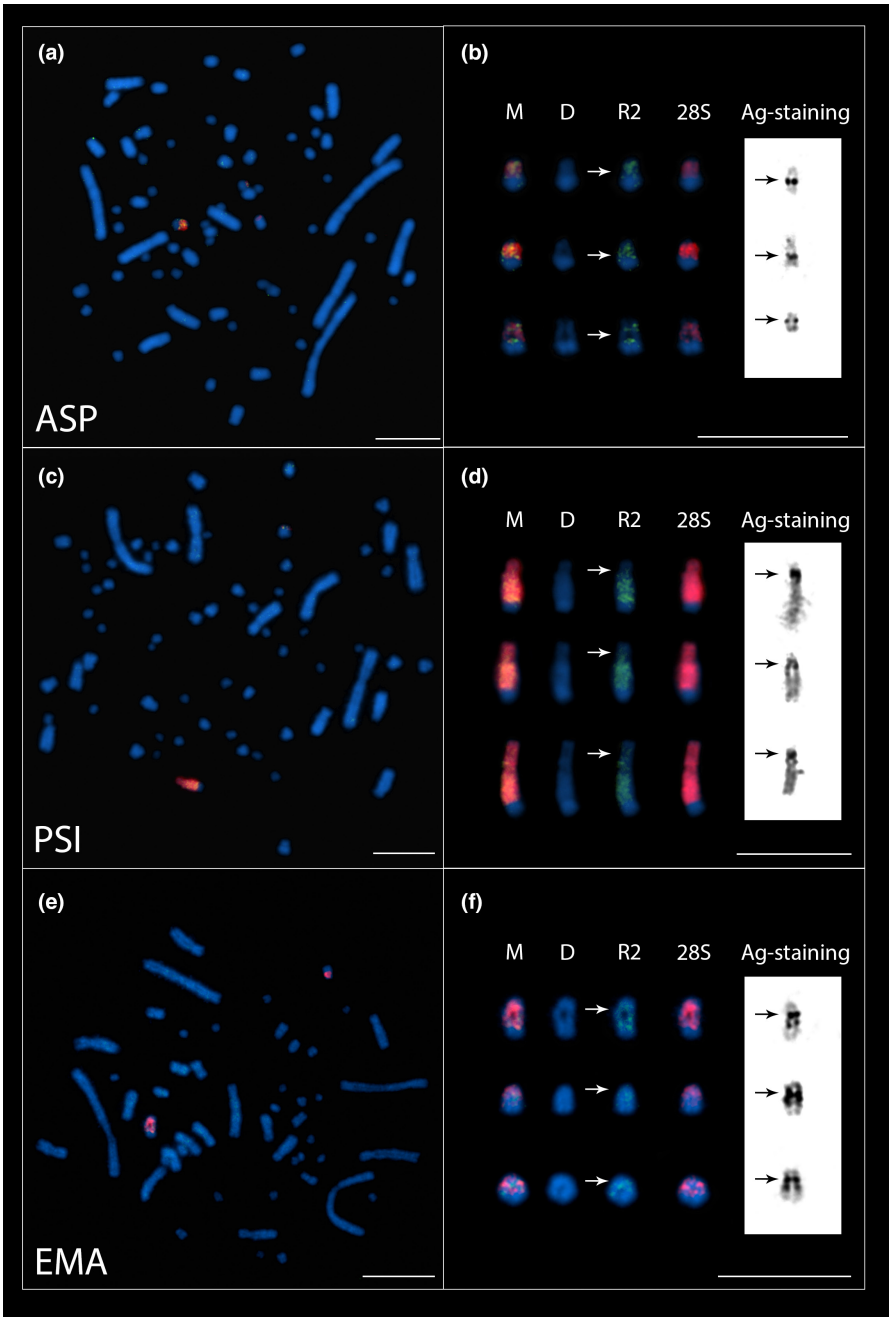

FIGURE 2 FISH using R2 element probe (green) and 28S RNA gene (red) in *Apalone spinifera* female (a), *Pelodiscus sinensis* female (c) and *Emydura macquarii* (e). Enlarged image of W chromosome of *Apalone* (b) and *Pelodiscus* (d), and the autosome carrying the larger NOR of *Emydura* (f) showing DAPI staining, hybridization signal of R2 element, 28S RNA gene and both probe signals merged. White boxes contain silver-stained W chromosomes and larger-NOR autosome for comparison between active NOR position and R2 population. White arrows denote low R2 hybridization signal in rDNA cluster while black arrows denote the active fraction of the rDNA cluster. Scale bar = 10 μm

staining only in part of the rDNA cluster on the larger NOR-carrying micro-autosome (Figure S2j), revealing identical heteromorphic silencing pattern to female softshell turtles. Additionally, equal-sized AgNORs were present in both X chromosomes in *Staurotypus* females (Figure 3e), whereas males, who lack Y-linked NORs (Montiel et al., 2016) (Figure 1), displayed an X-AgNOR twice as large compared female X-NORs, revealing the likely upregulation of male X-NOR (Figure 3f).

3.3 | Quantitative differences exist between homologous chromosomes in sex-linked NOR activity

Staining interphase nucleoli permitted quantifying differences in NOR activity between homologous chromosomes in species with sex-linked NORs. First, nucleoli provided quantitative evidence of differential X-rDNA activity between *Staurotypus* male and female cells. Specifically, because Y-NORs are absent, *Staurotypus* females carry double the rRNA gene copies than males as evidenced by rDNA FISH (Figure 1), which correlated with a greater number of nucleoli by cell ($p < 0.0001$), larger nucleus area ($p = 0.0037$) (Table 1) and larger nucleolus area ($p = 0.0272$) than male cells. However, the ratio of the nucleolus area to nucleus area was similar between the sexes ($p = 0.3191$) (Table 1). This result indicates similar expression of rRNA genes between the sexes relative to nucleus area, revealing dosage compensation of rDNA cluster genes via X-rDNA upregulation in *Staurotypus* males.

Second, in *Apalone* the W-rDNA cluster is significantly larger than the Z-rDNA cluster (Figure 1e–h) such that ZW females carry

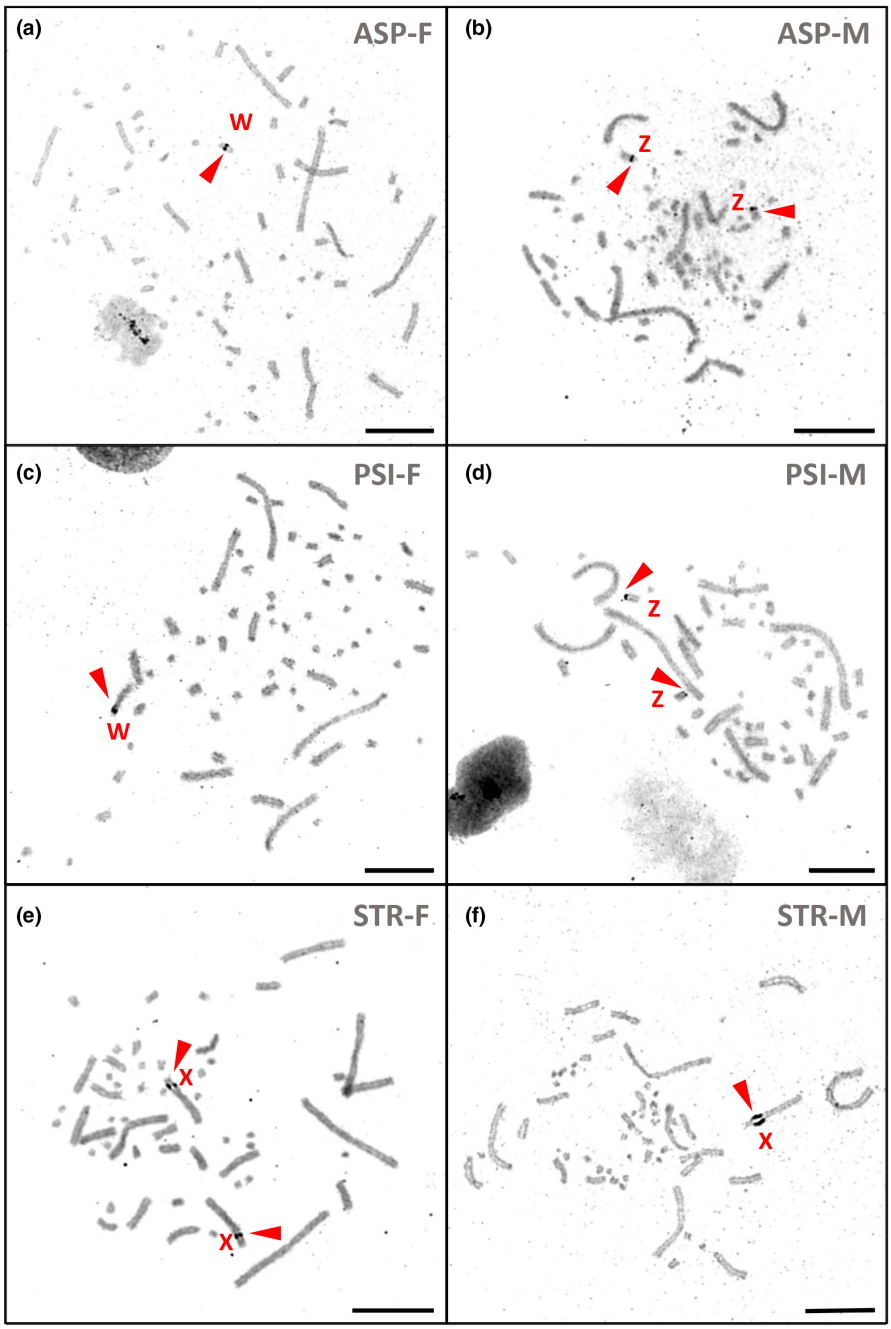

FIGURE 3 Activity of sex-linked NORs in turtles detected via silver staining (AgNOR). Metaphase chromosomes from *Apalone spinifera* female (a) and male (b), *Pelodiscus sinensis* female (c) and male (d), and *Staurotypus triporcatus* female (e) and male (f). Red arrow heads point to Ag-stained NORs. Scale bar = 10 μm

more copies of rRNA genes on average than ZZ males. Consistently, *Apalone* female cells showed larger nucleoli area ($p = 0.0426$), although nuclear area was smaller in females than males ($p = 0.0340$) (Table 1). Indeed, the ratio of nucleolus-to-nucleus area was similar between the sexes in *Apalone* ($p = 0.1924$) revealing female dosage compensation of rRNA genes even without female Z-NOR activity (Z-AgNOR is absent in females—Figure 3). Among-individual variation in rDNA activity was detected in *Apalone* within sexes, and embryonic rDNA activity was higher on average than in adults, consistent with higher metabolic demands (Figure 4).

Third, *Pelodiscus* heteromorphic micro sex chromosomes are homologous and similar in shape to *Apalone*'s (Figure 2; Badenhorst et al., 2013), and their W-rDNA cluster is much larger than the Z-linked cluster (Figure 1i–l), and correlates with larger nuclear area in female than male cells ($p < 0.0001$). Yet, the ratio of the nucleolus area to nucleus area was smaller in the available *Pelodiscus* female than in the male ($p < 0.0001$), and no difference was detected in the number of nucleoli per cell nor in the nucleolus area (Table 1). These results imply substantial silencing of W-rDNA genes in this female beyond Z-rDNA silencing (AgNOR is also absent in *Pelodiscus* female-Z; Figure 3).

3.4 | R2 distribution correlates with rDNA silencing

To investigate possible causes of the limited W-rDNA expression in *Apalone* and *Pelodiscus* females, we examined the distribution of R2

TABLE 1 Differences between sexes of number of nucleoli, nucleus area and nucleolus area in *Staurotypus triporcatus*, *Apalone spinifera* and *Pelodiscus sinensis*.

	Males	Females				
	Mean ± SD	Mean ± SD	χ^2/t	df	*p*	F/M
S. triporcatus						
No. of nucleoli per cell	1.00 ± 0.00	1.80 ± 0.40	32.8217	1.00	**<0.0001**	1.81
Nucleus area (AU)	1.13 ± 0.33	1.40 ± 0.30	3.0413	50.92	**0.0037**	1.23
Nucleolus area (AU)	0.09 ± 0.02	0.11 ± 0.03	2.2766	49.35	**0.0272**	1.17
Nucleolus/Nucleus area	0.08 ± 0.02	0.08 ± 0.02	−1.0062	50.97	0.3191	0.93
A. spinifera						
No. of nucleoli per cell	1.23 ± 0.43	1.16 ± 0.37	0.080	1.00	0.7767	0.94
Nucleus area (AU)	1.49 ± 0.47	1.21 ± 0.24	−2.749	37.69	**0.0091**	0.83
Nucleolus area (AU)	0.03 ± 0.01	0.04 ± 0.01	−3.214	39.08	**0.0026**	0.74
Nucleolus/Nucleus area	0.02 ± 0.01	0.02 ± 0.01	−1.326	39.24	0.1924	0.90
P. sinensis						
No. of nucleoli per cell	1.39 ± 0.50	1.19 ± 0.40	1.4991	1.00	0.2208	0.86
Nucleus area (AU)	1.01 ± 0.34	1.57 ± 0.38	4.7531	49.37	**<0.0001**	1.43
Nucleolus area (AU)	0.04 ± 0.01	0.03 ± 0.01	−1.2111	39.78	0.2330	0.90
Nucleolus/Nucleus area	0.03 ± 0.01	0.02 ± 0.01	−5.9502	34.82	**<0.0001**	0.63

Note: Bold font indicates significant sex differences.

Abbreviations: AU, arbitrary units; F/M, female/male ratio.

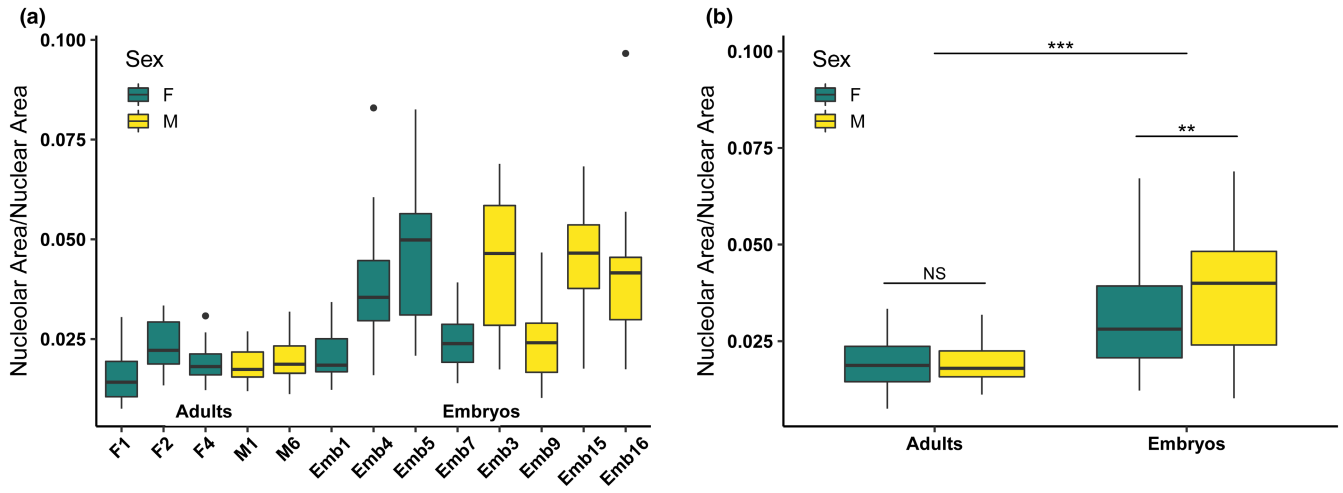

FIGURE 4 Ratio of nucleolus area to nucleus area in *Apalone spinifera* adults and embryos (a) and differences between stages and sexes (b). NS, not significant; $^{**}p < 0.01$; $^{***}p < 0.001$

transposable elements since R2 modulates rDNA expression in other taxa (Eickbush et al., 2008; Zhou et al., 2012, 2013). Because R2 elements target the 28S rRNA units specifically, R2 and 28S colocalization was tested by double FISH using species-specific R2 and 28S DNA probes obtained by PCR (Figure 2). Both probes hybridized on the sex chromosomes of *Apalone* and *Pelodiscus*, but R2 signals cover only part of the W-NOR while the 28S rRNA gene region was more expanded. Furthermore, the W-NOR portion densely populated by R2 coincided with the rDNA portions that fail to silver-stain, while the W-NOR regions depauperate of R2 (Figure 2b,d) are those transcriptionally active as determined by silver staining (AgNOR), thus revealing the potential R2-mediated silencing of W-rDNA in trionychid turtles.

We used the same approach in *Emydura* to test if R2 was also associated with the expanded 28S cluster observed in the NOR-carrying micro-autosome pair and found that the R2 probe hybridized mostly in the larger NOR, although some signal was detected in the smaller NOR (Figure 2e). Like in *Apalone* and *Pelodiscus*, low-R2 regions in the larger NOR-carrying autosome co-localized with the transcriptional active NOR identified by silver-staining AgNOR (Figure 2f).

4 | DISCUSSION

Understanding the evolution and regulation of the NOR is fundamental to elucidate how nucleoli evolve to supply the metabolic demands of the cell (Kurata et al., 1978), and how nucleolar function affects stress response, development and ageing (Tiku & Antebi, 2018). Additionally, growing recognition exists about the diversity of sex chromosome dosage compensation in eukaryotes and its evolution in species with male and female heterogamety. Where XY males or ZW females solve the challenge of X(or Z)-to-autosomes gene dose imbalance via dosage compensation mechanisms that equalize the activity of X- or Z-linked and autosomal genes, leading often to a balanced expression between the sexes (Gu & Walters, 2017). Expression of rRNA genes is associated with dosage compensation in some animals such as *Xenopus laevis* and *Drosophila melanogaster* (Barr & Esper, 1963; Miller & Gurdon, 1970; Semionov & Kirov, 1986; Tartof, 1971).

Here, we uncovered sexually dimorphic size and activity of turtle sex-linked NORs, which undergo contrasting dosage compensation in XX/XY versus ZZ/ZW systems as discussed below, mediated by retroelements in one but not the other, and adding to the complexity of sex chromosome dosage compensation reported in turtles (Bista et al., 2021).

4.1 | NOR activity is evolutionarily labile in turtles

All turtle species examined possess a single NOR locus as determined by rDNA FISH, located on both homeologs of a pair of autosomes in all but 3 of the 13 target taxa (Figure 2, Figure S1; Montiel et al., 2016): *Glyptemys*, *Sternotherus*, *Carettochelys*, *Chelodina*, *Pelomedusa*, *Chrysemys*, *Trachemys*, *Chelydra*, *Podocnemis* and *Emydura*. In contrast, the NOR is sex-linked in *Apalone*, *Pelodiscus* and *Staurotypus* (Figure 1; Badenhorst et al., 2013; Montiel et al., 2016). Sex linkage of NORs is not unique to turtles, but observed in several other reptiles, mammals, amphibians and fish (Abramyan et al., 2009; Born & Bertollo, 2000; Goodpasture & Bloom, 1975; Hsu et al., 1975; Porter et al., 1994; Schmid et al., 1983, 1993; Takehana et al., 2012; Wiley, 2003), and in some invertebrates (e.g. Monti et al., 2011; Montiel et al., 2012).

Sex-linked NORs in turtles are dimorphic in size or presence/absence (Figure 1), but heteromorphism is not an exclusive trait of sex-linked NORs. Indeed, size heteromorphism of rDNA clusters was also observed by rDNA FISH in *Emydura* and *Chelodina*, whose NORs are harboured by micro-autosomes (Figure 2, Figure S2; Montiel et al., 2016). In contrast, autosomal rDNA cluster in the other turtles examined were homomorphic. NOR activity as determined by silver staining (AgNORs) here varied among species in association with their level of heteromorphism in gene copy number, such that both homologues of autosomal NORs that are homomorphic in size exhibited equal activity within cells, as is commonly found across several turtle families (Bickham & Rogers, 1985), whereas taxa with heteromorphic rDNA clusters exhibited heteromorphic activity, but this heteromorphism varied (Figure 3 and Figure S2). AgNOR heteromorphism was reported also in *Cuora ambionensis* whose NOR is located in a small macro-autosome (Bickham & Rogers, 1985), but no FISH data exist to determine whether this corresponds with differences in ribosomal gene copy number.

4.2 | Sex-chromosome dosage compensation of rDNA in X-linked NORs of *Staurotypus* via X upregulation in males

The simpler case was observed in *Staurotypus* whose X-NOR is hemizygous in males as they lack a Y-NOR (Figure 1k; Montiel et al., 2016), and the X-NOR in males is twice as active in males than in females. This was evidenced by similar nucleolus-to-nucleus ratio in both sexes, and by the larger AgNOR in the male X compared with females (Figure 3e,f), which was detected also in the congener *S. salvinii* (Bickham & Rogers, 1985; Sites et al., 1979). The upregulation of X-rDNA cluster genes in *Staurotypus* males compared with females suggest a dosage compensation mechanism by X-NOR upregulation in males similar to that observed in *Drosophila* (Mukherjee & Beermann, 1965) and other animals with X-limited NORs such as wallabies and gnats (Dacunha et al., 1994; Dhaliwal et al., 1988), but not in others such as some frogs (Schmid et al., 1986; Schmid & Steinlein, 2003). Further research is needed to test whether the *Staurotypus* X-rRNA gene upregulation we observed in males is local and affects only some X-linked genes and not others as occurs in *Apalone* (Bista et al., 2021), or whether it is a global mechanism of dosage compensation that impacts all X-linked genes in *Staurotypus* as it does in other XX/XY animals beyond *Drosophila*, such as *Caenorhabditis elegans* and mammals (Gu & Walters, 2017). Our observations underscore the notion that rRNA genes are dosage-sensitive, and that cellular demands must be met by either sufficient copies of rRNA genes, or by their increased activity when fewer than a threshold number of copies are available.

Loss of a NOR by a deletion mutation in one homologue of the chromosomal pair harbouring them may explain the evolutionary origin of the absence of Y-NORs observed in *Staurotypus*, as occurs in other turtles. Indeed, a single NOR was detected in one individual examined of the TSD turtle *Kinosternon subrubrum*, and in one out of four individuals studied of the XX/XY turtle *Siebenrockiella crassicollis* whose NORs are autosomal (Bickham & Rogers, 1985). Alternatively, the lack of Y-NOR in *Staurotypus* may be the result of Y degeneration that characterizes the evolutionary dynamics of sex chromosomes often (Bachtrog et al., 2011), but not always (Kratochvíl et al., 2021). However, the absence of Y-NOR is consistent with the hypothesis that the acrocentric Y, which is homologous to the third group-B pair of several other turtles, represents the ancestral condition, and that the X is the derived sex chromosome (Sites et al., 1979). This derived X would have arisen by the translocation of the NOR to the X (and not the Y) as occurred in *Hyla femoralis* (Wiley, 2003), plus additional heterochromatin, which formed the short X arm in the genus *Staurotypus* (Sites et al., 1979). Further research is needed to fully test these alternatives.

4.3 | Complex dosage compensation of rDNA in *Apalone* and *Pelodiscus* by full Z-NOR shut-off and partial W-NOR silencing in females

In contrast to *Staurotypus*, the pattern observed in softshell turtles is much more intricate. In *Apalone* the W-rDNA cluster is significantly larger than the Z-rDNA cluster (Figure 1a,c; Badenhorst et al., 2013; Kawai et al., 2007) such that on average, ZW females possess four times more copies of rRNA genes than ZZ males, as determined by qPCR (Literman et al., 2014). Likewise, the size of *Pelodiscus* W-rDNA cluster far exceeds the Z-NOR size in females (Figure 1e,g). Since rRNA genes require an expression balance with regulatory genes that associate with the nucleolus for the proper structure and function of the ribosomes (Gibbons et al., 2014), sex chromosome differentiation via W expansion instead of attrition of the NOR clusters poses a challenge to that balance that requires a compensatory adjustment. Thus, it is not surprising that the female Z-NOR in both species is completely silenced, that is, females lack AgNOR in the Z (Figure 3a,c; Kawai et al., 2007). However, it is unclear how the female Z-NOR becomes inactive, although epigenetic mechanisms of rRNA regulation may play a role as demonstrated in other eukaryotes (Gibbons et al., 2014; Lawrence & Pikaard, 2004; Parks et al., 2018). Because rRNA gene copy number mediates rRNA activity (Grummt, 2003; Montiel et al., 2012), the observations in softshell turtles support the hypothesis that the W, but not the single Z, carries sufficient rRNA gene copies to meet the cellular demands (which is evidenced also by the similar nucleolus-to-nucleus ratio in both sexes detected here in adults), and that activating both the Z-NOR and W-NOR might be excessive and costly, suggesting that silencing the female Z-NOR could be adaptive. This is particularly intriguing, because non-ribosomal Z-linked genes that are hemizygous in *Apalone* females (i.e. lack W-gametologs) are trascribed (Bista et al., 2021), such that Z-rDNA silencing reveals the existence of a more nuanced gene-specific and sex-limited dosage compensating mechanism in softshell turtles than previously anticipated. Whether the potentially adaptive Z-NOR silencing might be driven by an autosomal female trans-regulator as proposed by a novel model of sex chromosome evolution (Lenormand & Roze, 2022) remains to be tested.

But this does not explain the expansion of the W-NOR, which instead, appears driven by the invasion of the W-NOR by the R2 retroelement (Figure 2b,d). The similarity of the pattern of R2 distribution and expansion of the W in both softshell turtles indicates that it is ancestral to trionychids. In both taxa, R2 appears to disrupt the host 28S units it invades, silencing them (Eickbush & Eickbush, 2015), such that the W-NOR activity (AgNOR) was restricted to the 28S units uninterrupted by R2 (Figure 2b,d). Thus, we hypothesize that as copies of the 28S became non-functional by R2 disruption, selection may have favoured the addition of new intact 28S copies that extended the size of the W, perhaps aided by the reduced recombination characteristic of sex chromosomes.

The accumulation of transposable elements in sex chromosomes is well documented across the animal, plant and fungi kingdoms (Sliwinska et al., 2016), leading sometimes to the expansion of the heterogametic chromosome (Y or W), including in frogs, birds, lizards and turtles (Gunski et al., 2019; Matsubara et al., 2013, 2015; Schmid et al., 2002). Further, retroelements are known to be involved in gene dosage compensation in mammals and *Drosophila* (Chow et al., 2010; Matyunina et al., 2008) as they silence adjacent genes in sex chromosomes (Gdula et al., 1996; Steinemann & Steinemann, 2005). Our R2 data from *Apalone* and *Pelodiscus* represent the first evidence suggesting that the same may be true for rDNA genes in turtles, a clade whose sex chromosome evolution is receiving growing attention (Bista & Valenzuela, 2020).

Consistently with observations in turtles, a large body of work has uncovered variability in the number of the rRNA gene copies that compose the NOR clusters among individuals and species across eukaryotes (Long & Dawid, 1980; Prokopowich et al., 2003). And understanding the evolution of this variability in NOR size is critical to decipher genome evolution because rRNA genes act as heterochromatic factors, and their copy number affects genome-wide gene expression (driving certain sex-biased diseases (Silkaitis & Lemos, 2014)), and this influence is exerted by both active and inactive rDNA copies that were considered superfluous until recently (Paredes et al., 2011). Thus, stabilizing selection may prevent the excessive expansion or contraction of the NOR so as to maintain genome stability (Paredes et al., 2011).

4.4 | Autosome dosage compensation of rDNA in *Emydura* by full inactivation of the small-NOR homologue and partial silencing of the large-NOR

The case of *Emydura* is particularly intriguing because the micro-autosome pair carrying the NOR in the individual examined is remarkably similar to the sexually dimorphic Z/W-NOR system in *Apalone* and *Pelodiscus*, with one of the micro-chromosomes containing an enlarged NOR and the other autosome in this pair containing a much smaller NOR. This heteromorphism was undetected in previous cytogenetic examinations of this species (Martinez et al., 2008; Matsubara et al., 2015). And just like in softshell turtles, only the autosome carrying the larger NOR is active (AgNOR) while the homologue carrying the smaller NOR is completely silent (lacks AgNOR). Since autosomes also undergo dosage compensation (Disteche, 2016), this finding in *Emydura* strengthens the notion that maintaining a coherent expression between ribosomal genes and other genes functionally associated with the nucleolus is essential for ribosome biogenesis (Gibbons et al., 2014) and thus likely exerts strong selection for compensatory adjustments irrespective of whether NORs are sex-linked or not. Furthermore, not all rRNA gene copies in *Emydura* are active but only those where the R2 retroelement does not colocalize (Figure 2f). Thus, the mechanism we hypothesize as mediating the pattern of activity of the NOR in softshell turtles is not exclusive to sex-linked NORs and may evolve independent of the evolution of sex chromosomes. But whether such a heteromorphism may have originated in a pair of autosomes in

softshell turtles as in *Emydura* and might have facilitated the evolution of the ZZ/ZW sex chromosome system in trionychids remains a mystery for future testing.

5 | CONCLUSION

Here, we uncover evidence for the first time that dosage compensation for sex-linked rDNA genes exists in turtles, a lineage that holds a critical key to illuminating the evolution of vertebrate sex determination and the consequences of sex chromosome evolution. Turtles they possess XX/XY and ZZ/ZW systems of genotypic sex determination (GSD) that evolved independently and repeatedly from an ancestral temperature-dependent sex determination (Sabath et al., 2016; Valenzuela & Adams, 2011). The mechanism of dosage compensation for rDNA varied among turtle taxa with contrasting sex chromosome systems and included rDNA upregulation in XX/XY *Staurotypus*, and rDNA silencing in ZZ/ZW *Apalone* and *Pelodiscus*. We also uncovered partial rDNA cluster silencing in W chromosomes potentially mediated by R2 retrotransposons, the expansion of which appears associated with the evolution of heteromorphic sex chromosomes in softshell turtles, but whose dimorphism could have arisen in a pair of autosomes that was then co-opted as sex chromosomes in trionychids, as observed in the autosomes of a chelid turtle. Our findings show that sex chromosome differentiation in the form of expansion and not just attrition can generate an imbalance for dose-sensitive genes such as rRNA genes, and can select for compensatory adjustments via Z-NOR silencing and additional mechanisms that are yet unknown in turtles. Our study revealed a rich diversity of ribosomal gene organization in turtles, the evolution of which warrants further research and promises to illuminate the evolution of eukaryote genomes. Results expand the breath of the known diversity and evolution of dosage compensation in eukaryotes and add to the complexity of sex chromosome evolution that challenges the classic paradigm (Kratochvíl et al., 2021; Lenormand & Roze, 2022).

AUTHOR CONTRIBUTIONS

E.E.M. and N.V. conceptualized the project, interpreted the results and wrote the manuscript. E.E.M., D.B. and L.S.L. collected the data. E.E.M. analysed the data. All authors edited the manuscript

ACKNOWLEDGEMENTS

This work was funded in part by the NSF grant MCB 0815354, MCB 1244355 and IOS 1555999 to N.V. Open access funding provided by the Iowa State University Library.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All quantitative data used in this study is provided in the Supplementary Materials.

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REFERENCES

Abramyan, J., Ezaz, T., Graves, J. A. M., & Koopman, P. (2009). Z and W sex chromosomes in the cane toad (*Bufo marinus*). *Chromosome Research*, *17*, 1015–1024.

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*, 350–357.

Badenhorst, D., Stanyon, R., Engstrom, T., & Valenzuela, N. (2013). A ZZ/ZW microchromosome system in the spiny softshell turtle, *Apalone spinifera*, reveals an intriguing sex chromosome conservation in Trionychidae. *Chromosome Research*, *21*, 137–147.

Barbosa, M. O., da Silva, R. R., de Sena Correia, V. C., dos Santos, L. P., Garnero, A. D. V., & Gunski, R. J. (2013). Nucleolar organizer regions in *Sittasomus griseicapillus* and *Lepidocolaptes angustirostris* (Aves, Dendrocolaptidae): Evidence of a chromosome inversion. *Genetics and Molecular Biology*, *36*, 70–73.

Barr, H. J., & Esper, H. (1963). Nucleolar size in cells of *Xenopus laevis* in relation to nucleolar competition. *Experimental Cell Research*, *31*, 211–214.

Bickham, J. W., & Rogers, D. S. (1985). Structure and variation of the nucleolus organizer region in turtles. *Genetica*, *67*, 171–184.

Bista, B., & Valenzuela, N. (2020). Turtle insights into the evolution of the reptilian karyotype and the genomic architecture of sex determination. *Genes*, *11*, 416.

Bista, B., Wu, Z., Literman, R., & Valenzuela, N. (2021). Thermosensitive sex chromosome dosage compensation in ZZ/ZW softshell turtles, *Apalone spinifera*. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *376*, 20200101.

Born, G. G., & Bertollo, L. A. C. (2000). An XX/XY sex chromosome system in a fish species, *Hoplias malabaricus*, with a polymorphic NOR-bearing X chromosome. *Chromosome Research*, *8*, 111–118.

Britton-Davidian, J., Cazaux, B., & Catalan, J. (2012). Chromosomal dynamics of nucleolar organizer regions (NORs) in the house mouse: Micro-evolutionary insights. *Heredity*, *108*, 68–74.

Chandler, C. H. (2017). When and why does sex chromosome dosage compensation evolve? *Annals of the New York Academy of Sciences*, *1389*, 37–51.

Charlesworth, B. (1996). The evolution of chromosomal sex determination and dosage compensation. *Current Biology*, *6*, 149–162.

Chow, J. C., Ciaudo, C., Fazzari, M. J., Mise, N., Servant, N., Glass, J. L., Attreed, M., Avner, P., Wutz, A., Barillot, E., Greally, J. M., Voinnet, O., & Heard, E. (2010). LINE-1 activity in facultative heterochromatin formation during X chromosome inactivation. *Cell*, *141*, 956–969.

Cioffi, M. B., Martins, C., & Bertollo, L. A. C. (2010). Chromosome spreading of associated transposable elements and ribosomal DNA in the fish *Erythrinus erythrinus*. Implications for genome change and karyoevolution in fish. *BMC Evolutionary Biology*, *10*, 271.

Dacunha, P. R., Granadino, B., Perondini, A. L. P., & Sanchez, L. (1994). Dosage compensation in *Sciarids* is achieved by hypertranscription of the single X-chromosome in males. *Genetics*, *138*, 787–790.

Dhaliwal, M. K., Pathak, S., Shirley, L. R., & Flanagan, J. P. (1988). Ag-NOR staining in the Bennett wallaby, *Macropus rufogriseus* – Evidence for dosage compensation. *Cytobios*, *56*, 29–38.

Disteche, C. M. (2016). Dosage compensation of the sex chromosomes and autosomes. *Seminars in Cell & Developmental Biology*, *56*, 9–18.

Eickbush, D. G., Ye, J. Q., Zhang, X., Burke, W. D., & Eickbush, T. H. (2008). Epigenetic regulation of retrotransposons within the

nucleolus of *Drosophila*. *Molecular and Cellular Biology*, *28*, 6452–6461.

Eickbush, T. H., & Eickbush, D. G. (2015). Integration, regulation, and long-term stability of R2 retrotransposons. *Microbiology Spectrum*, *3*, MDNA3-0011-2014.

Ezaz, T., Valenzuela, N., Grutzner, F., Miura, I., Georges, A., Burke, R. L., & Graves, J. A. M. (2006). An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination. *Chromosome Research*, *14*, 139–150.

Farley, K. I., Surovtseva, Y., Merkel, J., & Baserga, S. J. (2015). Determinants of mammalian nucleolar architecture. *Chromosoma*, *124*, 323–331.

Gdula, D. A., Gerasimova, T. I., & Corces, V. G. (1996). Genetic and molecular analysis of the gypsy chromatin insulator of Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 9378–9383.

Gibbons, J. G., Branco, A. T., Godinho, S. A., Yu, S. K., & Lemos, B. (2015). Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 2485–2490.

Gibbons, J. G., Branco, A. T., Yu, S. K., & Lemos, B. (2014). Ribosomal DNA copy number is coupled with gene expression variation and mitochondrial abundance in humans. *Nature Communications*, *5*, 4850.

Goodpasture, C., & Bloom, S. E. (1975). Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma*, *53*, 37–50.

Gornung, E. (2013). Twenty years of physical mapping of major ribosomal RNA genes across the teleosts: A review of research. *Cytogenetic and Genome Research*, *141*, 90–102.

Grummt, I. (2003). Life on a planet of its own: Regulation of RNA polymerase I transcription in the nucleolus. *Genes & Development*, *17*, 1691–1702.

Gu, L. Q., & Walters, J. R. (2017). Evolution of sex chromosome dosage compensation in animals: A beautiful theory, undermined by facts and bedeviled by details. *Genome Biology and Evolution*, *9*, 2461–2476.

Gunski, R. J., Kretschmer, R., de Souza, M. S., Furo, I. D., Barcellos, S. A., Costa, A. L., Cioffi, M. B., de Oliveira, E. H. C., & Garnero, A. D. (2019). Evolution of bird sex chromosomes narrated by repetitive sequences: Unusual W chromosome enlargement in *Gallinula melanops* (Aves: Gruiformes: Rallidae). *Cytogenetic and Genome Research*, *158*, 152–159.

Howell, W. M. (1977). Visualization of ribosomal gene activity – Silver stains proteins associated with ribosomal-RNA transcribed from oocyte chromosomes. *Chromosoma*, *62*, 361–367.

Howell, W. M., & Black, D. A. (1980). Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer – A 1-step method. *Experientia*, *36*, 1014–1015.

Hsu, T. C., Spirito, S. E., & Pardue, M. L. (1975). Distribution of 18+28S ribosomal genes in mammalian genomes. *Chromosoma*, *53*, 25–36.

Kawai, A., Nishida-Umehara, C., Ishijima, J., Tsuda, Y., Ota, H., & Matsuda, Y. (2007). Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenetic and Genome Research*, *117*, 92–102.

King, M., Contreras, N., & Honeycutt, R. L. (1990). Variation within and between nucleolar organizer regions in Australian hylid frogs (Anura) shown by 18s+28s in situ hybridization. *Genetica*, *80*, 17–29.

Kojima, K. K., Seto, Y., & Fujiwara, H. (2016). The wide distribution and change of target specificity of r2 non-LTR retrotransposons in animals. *PLoS One*, *11*, e0163496.

Kratochvíl, L., Stöck, M., Rovatsos, M., Bullejos, M., Herpin, A., Jeffries, D. L., Peichel, C. L., Perrin, N., Valenzuela, N., & Pokorná, M. J. (2021). Expanding the classical paradigm: What we have learnt from vertebrates about sex chromosome evolution. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *376*, 20200097.

Krzyzanowska, M., Steiner, J., Brisch, R., Mawrin, C., Busse, S., Braun, K., Jankowski, Z., Bernstein, H.-G., Bogerts, B., & Gos, T. (2015). Ribosomal DNA transcription in the dorsal raphe nucleus is increased in residual but not in paranoid schizophrenia. *European Archives of Psychiatry and Clinical Neuroscience*, *265*, 117–126.

Kurata, S. I., Koga, K., & Sakaguchi, B. (1978). Nucleolar size in parallel with ribosomal-RNA synthesis at diapause termination in eggs of *Bombyx mori*. *Chromosoma*, *68*, 313–317.

Lawrence, R. J., & Pikaard, C. S. (2004). Chromatin turn ons and turn offs of ribosomal RNA genes. *Cell Cycle*, *3*, 880–883.

Lee, L. S., Montiel Jiménez, E. E., Navarro-Domínguez, B. M., & Valenzuela, N. (2019). Chromosomal rearrangements during turtle evolution altered the synteny of genes involved in vertebrate sex determination. *Cytogenetic and Genome Research*, *157*, 77–88.

Leitch, A. R., Mosgoller, W., Shi, M., & Heslopharrison, J. S. (1992). Different patterns of rDNA organization at interphase in nuclei of wheat and rye. *Journal of Cell Science*, *101*, 751–757.

Lenormand, T., & Roze, D. (2022). Y recombination arrest and degeneration in the absence of sexual dimorphism. *Science*, *375*, 663–666.

Literman, R., Badenhorst, D., & Valenzuela, N. (2014). qPCR-based molecular sexing by copy number variation in rRNA genes and its utility for sex identification in soft-shell turtles. *Methods in Ecology and Evolution*, *5*, 872–880.

Literman, R., Radhakrishnan, S., Tamplin, J., Burke, R., Dresser, C., & Valenzuela, N. (2017). Development of sexing primers in *Glyptemys insculpta* and *Apalone spinifera* turtles uncovers an XX/XY sex-determining system in the critically-endangered bog turtle *Glyptemys muhlenbergii*. *Conservation Genetics Resources*, *9*, 651–658.

Long, E. O., & Dawid, I. B. (1980). Repeated genes in eukaryotes. *Annual Review of Biochemistry*, *49*, 727–764.

Lucchesi, J. C. (1978). Gene dosage compensation and the evolution of sex chromosomes. *Science*, *202*, 711–716.

Martinez, P., Valenzuela, N., Georges, A., & Graves, J. A. M. (2008). An XX/XY heteromorphic sex chromosome system in the Australian chelid turtle *Emydura macquarii*, a new piece in the puzzle of sex chromosome evolution in turtles. *Chromosome Research*, *16*, 815–825.

Matsubara, K., Knopp, T., Sarre, S. D., Georges, A., & Ezaz, T. (2013). Karyotypic analysis and FISH mapping of microsatellite motifs reveal highly differentiated XX/XY sex chromosomes in the pink-tailed worm-lizard (*Aprasia parapulchella*, Pygopodidae, Squamata). *Molecular Cytogenetics*, *6*, 60.

Matsubara, K., O'Meally, D., Azad, B., Georges, A., Sarre, S., Graves, J. M., Matsuda, Y., & Ezaz, T. (2015). Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma*, *125*, 111–123.

Matsubara, K., Sarre, S. D., Georges, A., Matsuda, Y., Graves, J. A. M., & Ezaz, T. (2014). Highly differentiated ZW sex microchromosomes in the australian *Varanus* species evolved through rapid amplification of repetitive sequences. *PLoS One*, *9*, e95226.

Matyunina, L. V., Bowen, N. J., & McDonald, J. F. (2008). LTR retrotransposons and the evolution of dosage compensation in Drosophila. *BMC Molecular Biology*, *9*, 55.

Miller, L. E. O., & Gurdon, J. B. (1970). Mutations affecting the size of the nucleolus in *Xenopus laevis*. *Nature*, *227*, 1108–1110.

Monti, V., Manicardi, G. C., & Mandrioli, M. (2011). Cytogenetic and molecular analysis of the holocentric chromosomes of the potato aphid *Macrosiphum euphorbiae* (Thomas, 1878). *Comparative Cytogenetics*, *5*, 163–172.

Montiel, E. E., Badenhorst, D., Lee, L. S., Literman, R., Trifonov, V., & Valenzuela, N. (2016). Cytogenetic insights into the evolution of chromosomes and sex determination reveal striking homology of

turtle sex chromosomes to amphibian autosomes. *Cytogenetic and Genome Research*, *148*, 292–304.
Montiel, E. E., Manrique-Poyato, M. I., Rocha-Sanchez, S. M., Lopez-Leon, M. D., Cabrero, J., Perfectti, F., & Camacho, J. P. M. (2012). Nucleolus size varies with sex, ploidy and gene dosage in insects. *Physiological Entomology*, *37*, 145–152.
Mukherjee, A. S., & Beermann, W. (1965). Synthesis of ribonucleic acid by X-chromosomes of *Drosophila melanogaster* and problem of dosage compensation. *Nature*, *207*, 785–786.
Nanda, I., Volff, J. N., Weis, S., Korting, C., Froschauer, A., Schmid, M., & Schartl, M. (2000). Amplification of a long terminal repeat-like element on the Y chromosome of the platyfish, *Xiphophorus maculatus*. *Chromosoma*, *109*, 173–180.
Odierna, G., Olmo, E., & Cobror, O. (1987). Taxonomic implications of nor-localization in lacertid lizards. *Amphibia-Reptilia*, *8*, 373–382.
Ohno, S. (1967). *Sex chromosomes and sex-linked genes*. Springer-Verlag.
Olmo, E., & Signorino, G. G. (2005). *ChromoRep: A reptiles chromosomes database*. http://193.206.118.100/professori/chromorep.pdf
Paredes, S., Branco, A. T., Hartl, D. L., Maggert, K. A., & Lemos, B. (2011). Ribosomal DNA deletions modulate genome-wide gene expression: "rDNA-sensitive" genes and natural variation. *PLoS Genetics*, *7*, e1001376.
Parks, M. M., Kurylo, C. M., Dass, R. A., Bojmar, L., Lyden, D., Vincent, C. T., & Blanchard, S. C. (2018). Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. *Science Advances*, *4*, eaao0665.
Porter, C. A., Haiduk, M. W., & Dequeiroz, K. (1994). Evolution and phylogenetic significance of ribosomal gene location in chromosomes of squamate reptiles. *Copeia*, *1994*, 302–313.
Prieto, J.-L., & McStay, B. (2008). Pseudo-NORs: A novel model for studying nucleoli. *Biochimica et Biophysica Acta – Molecular Cell Research*, *1783*, 2116–2123.
Prokopowich, C. D., Gregory, T. R., & Crease, T. J. (2003). The correlation between rDNA copy number and genome size in eukaryotes. *Genome*, *46*, 48–50.
R Core Development Team. (2016). *R: a language and environment for statistical computing*. R Foundation for Statistical Computing. http://cran.R-project.org
Rocha, G. T., & Delucca, E. J. (1988). Nucleolar organizer regions in somatic chromosomes of some species of birds. *Caryologia*, *41*, 299–308.
Sabath, N., Itescu, Y., Feldman, A., Meiri, S., Mayrose, I., & Valenzuela, N. (2016). Sex determination and the birth and death of species. *Ecology and Evolution*, *6*, 5207–5220.
Schmid, M., Feichtinger, W., Steinlein, C., Rupprecht, A., Haaf, T., & Kaiser, H. (2002). Chromosome banding in Amphibia – XXIII. Giant W sex chromosomes and extremely small genomes in *Eleutherodactylus euphronides* and *Eleutherodactylus shrevei* (Anura, Leptodactylidae). *Cytogenetic and Genome Research*, *97*, 81–94.
Schmid, M., Haaf, T., Geile, B., & Sims, S. (1983). Chromosome-banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). *Chromosoma*, *88*, 69–82.
Schmid, M., Ohta, S., Steinlein, C., & Guttenbach, M. (1993). Chromosome-banding in Amphibia. 19. Primitive ZW/ZZ sex-chromosomes in *Buergeria buergeri* (Anura, Rhacophoridae). *Cytogenetics and Cell Genetics*, *62*, 238–246.
Schmid, M., Sims, S. H., Haaf, T., & Macgregor, H. C. (1986). Chromosome-banding in Amphibia. 10. 18S and 28S ribosomal-RNA genes, nucleolus organizers and nucleoli in *Gastrotheca riobambae*. *Chromosoma*, *94*, 139–145.
Schmid, M., & Steinlein, C. (2003). Chromosome banding in Amphibia. XXIX. The primitive XY/XX sex chromosomes of *Hyla femoralis* (Anura, Hylidae). *Cytogenetic and Genome Research*, *101*, 74–79.
Schmid, M., & Steinlein, C. (2015). Chromosome banding in amphibia. XXXII. The genus *Xenopus* (Anura, Pipidae). *Cytogenetic and Genome Research*, *145*, 201–217.
Semionov, E. P., & Kirov, N. K. (1986). Increased number of nucleoli in the salivary-gland cells of *Drosophila-melanogaster* under conditions of rDNA dose compensation. *Chromosoma*, *93*, 477–482.
Shaw, P., & Doonan, J. (2005). The nucleolus – Playing by different rules? *Cell Cycle*, *4*, 102–105.
Shaw, P. J., & McKeown, P. C. (2011). The structure of rDNA chromatin. In M. O. J. Olson (Ed.), *The nucleolus. Protein reviews* (Vol. 15, pp. 43–55). Springer.
Silkaitis, K., & Lemos, B. (2014). Sex-biased chromatin and regulatory cross-talk between sex chromosomes, autosomes, and mitochondria. *Biology of Sex Differences*, *5*, 2.
Sites, J. W., Bickham, J. W., & Haiduk, M. W. (1979). Derived X-chromosome in the turtle genus *Staurotypus*. *Science*, *206*, 1410–1412.
Sliwinska, E. B., Martyka, R., & Tryjanowski, P. (2016). Evolutionary interaction between W/Y chromosome and transposable elements. *Genetica*, *144*, 267–278.
Steinemann, S., & Steinemann, M. (2005). Retroelements: Tools for sex chromosome evolution. *Cytogenetic and Genome Research*, *110*, 134–143.
Supanuam, P., Tanomtong, A., Khunsook, S., Sangpakdee, W., Pinthong, K., Sanoamuang, L.-O., & Keawsri, S. (2012). Localization of nucleolar organizer regions (NORs) of 4 Gibbon species in Thailand by Ag-NOR banding technique. *Cytologia*, *77*, 141–148.
Takehana, Y., Naruse, K., Asada, Y., Matsuda, Y., Shin-I, T., Kohara, Y., Fujiyama, A., Hamaguchi, S., & Sakaizumi, M. (2012). Molecular cloning and characterization of the repetitive DNA sequences that comprise the constitutive heterochromatin of the W chromosomes of medaka fishes. *Chromosome Research*, *20*, 71–81.
Tartof, K. D. (1971). Increasing the multiplicity of ribosomal RNA genes in *Drosophila melanogaster*. *Science*, *171*, 294–297.
Tiku, V., & Antebi, A. (2018). Nucleolar function in lifespan regulation. *Trends in Cell Biology*, *28*, 662–672.
Valenzuela, N., & Adams, D. C. (2011). Chromosome number and sex determination co-evolve in turtles. *Evolution*, *65*, 1808–1813.
Weisenberger, D., & Scheer, U. (1995). Possible mechanism for the inhibition of ribosomal-RNA gene-transcription during mitosis. *Journal of Cell Biology*, *129*, 561–575.
Wiley, J. E. (2003). Replication banding and FISH analysis reveal the origin of the *Hyla femoralis* karyotype and XY/XX sex chromosomes. *Cytogenetic and Genome Research*, *101*, 80–83.
Zhou, J., Eickbush, M. T., & Eickbush, T. H. (2013). A population genetic model for the maintenance of R2 retrotransposons in rRNA gene loci. *PLoS Genetics*, *9*, e1003179.
Zhou, J., Sackton, T. B., Martinsen, L., Lemos, B., Eickbush, T. H., & Hartl, D. L. (2012). Y chromosome mediates ribosomal DNA silencing and modulates the chromatin state in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 9941–9946.

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How to cite this article: Montiel, E. E., Badenhorst, D., Lee, L., & Valenzuela, N. (2022). Evolution and dosage compensation of nucleolar organizing regions (NORs) mediated by mobile elements in turtles with female (ZZ/ZW) but not with male (XX/XY) heterogamety. *Journal of Evolutionary Biology*, *35*, 1709–1720. https://doi.org/10.1111/jeb.14064