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Premeiotic endoreplication is the mechanism of obligate parthenogenesis in rock lizards of the genus *Darevskia*

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Abstract

Among vertebrates, obligate parthenogenesis occurs exclusively in squamate reptiles.

Premeiotic endoreplication in a small subset of developing oocytes has been documented as

the mechanism of production of unreduced eggs in minutely explored obligate

parthenogenetic lineages, namely in teiids and geckos. The situation in the lacertid genus

Darevskia has been discussed for decades. Certain observations suggested that ploidy level is

restored during egg formation through a fusion of egg and polar body nuclei in *D. unisexualis*

and *D. armeniaca*. In this study, we re-evaluated the fusion hypothesis by studying diplotene

chromosomes in adult females of sexual species *D. raddei nairensis* and obligate

parthenogens *D. armeniaca, D. dahli* and *D. unisexualis*. We revealed 19 bivalents in the

sexual species and 38 bivalents in the diploid obligate parthenogens, which uncovers

premeiotic endoreplication as the mechanism of the production of non-reduced eggs in

parthenogenetic females. The earlier contradicting reports can be likely attributed to the

difficulty in identifying mispairing of chromosomes in pachytene, and the fact that in

parthenogenetic reptiles relying on premeiotic endoreplication only a small subset of

developing oocytes undergo genome doubling and overcome the pachytene checkpoint. This

study highlights co-option of premeiotic endoreplication for escape from sexual reproduction

in all independent hybrid origins of obligate parthenogenesis in vertebrates studied to date.

Keywords: Asexuality, Lacertidae, Meiosis, Obligatory parthenogenesis, Reptiles,

Vertebrates

Introduction

It has been estimated that approximately 99.9% of vertebrate species reproduce sexually (Avise, 2009). Rare exceptions to this rule can provide us essential information about the cellular, genetic and evolutionary consequences of sexual reproduction and its loss. Despite its rarity, asexual reproduction in vertebrates is surprisingly diverse and relies on different processes. Gynogenesis, also known as sperm-dependent parthenogenesis, and hybridogenesis, the selective transmission of one intact parental genome to the offspring while the other is renewed by mating, occur in teleost fishes and amphibians (Bogart et al., 2009; Lamatsch and Stöck, 2009, Lavanchy and Schwander, 2019, Stöck et al., 2021a). On the other hand, parthenogenesis, the form of asexual reproduction in which embryo growth and development occur without sperm, has only been documented in cartilaginous fishes (sharks and rays) and sauropsids (non-avian reptiles and birds) (Stöck et al., 2021b).

Facultative parthenogenesis, the type of parthenogenesis in which a female individual can reproduce by both sexual and asexual means, is generally thought to be rare in most cartilaginous fishes and sauropsids. The only species up to now where it appears to be very common is a night lizard of the genus *Lepidophyma* (Kratochvíl et al., 2020). Therefore, in most cases it would be more correct to speak of accidental or spontaneous parthenogenesis, but for simplicity, we use the term facultative parthenogenesis here. In all vertebrates studied, facultative parthenogenesis has been associated with partial or even complete loss of heterozygosity in the offspring (Booth et al., 2012, 2014; Booth and Schuett, 2016; Kratochvíl et al., 2020; Ho et al., 2023). Direct cytological evidence for the process of oogenesis in facultatively parthenogenetic vertebrates is lacking. Based on the observations of the genetic variability in the offspring, it is assumed that the ploidy level of the egg is restored after meiosis by a "fusion" of the nuclei of an egg and a polar body (more likely by

the suppression of the second meiotic division) (Booth et al., 2012, 2014; Booth and Schuett,

2016; Kratochvíl et al., 2020), or by doubling of the haploid egg genome leading to the total

loss of heterozygosity (Ho et al., 2023).

Among vertebrates, all-female lineages with obligate parthenogenesis occur only in

squamate reptiles (lizards and snakes). In most cases studied, their origin is associated with

hybridization and the eventual subsequent generation of polyploid clones by mating with a

male of a sexual lineage, with the xantusiid genus *Lepidophyma* being a notable possible

exception (Sinclair et al., 2010; Wynn et al., 1987; Reeder et al., 2002; Adams et al., 2003;

Brunes et al., 2019; Grismer et al., 2014; Abdala et al., 2016; Barley et al., 2021). The

mechanism of unreduced egg formation has only been studied in five lizard genera with

obligate parthenogenetic species. In the teiid genus *Aspidoscelis* (Cuellar, 1971; Lutes et al.,

2010; Newton et al., 2016) and three gecko genera *Hemiphyllodactylus, Heteronotia* and

Lepidodactylus (Dedukh et al., 2022), the ploidy level is maintained by genome doubling

prior to meiosis. During the first meiotic division, bivalents are formed between genetically

identical sister chromosomes, produced by the first of two premeiotic replication cycles. The

pairing of sister chromosomes ensures the maintenance of heterozygosity. Interestingly,

during the pachytene stage of meiosis, two populations of cells can be distinguished (Newton

et al., 2016; Dedukh et al., 2022). The first, which is much more numerous, enters the meiosis

without premeiotic duplication, fails to form proper bivalents and is arrested by the pachytene

checkpoint and driven to apoptosis. Only a small minority of oocytes undergo premeiotic

duplication and are able to enter further meiotic phases and to form unreduced eggs (Lutes et

al., 2010; Newton et al., 2016; Dedukh et al., 2022).

Premeiotic endoreplication was considered as the mechanism of parthenogenetic

reproduction also in several earlier studies of the rock lizards of the lacertid genus *Darevskia*

distributed in the area between the Lesser Caucasus Mountains and Lake Van (Uzzell, 1970;

Darevsky et al. 1985). For example, Uzzell (1970) noted: "It is possible that the second

meiotic division is suppressed in *Lacerta armeniaca*, as suggested by Darevsky (1966), but it

seems more likely that there is a premeiotic mitosis without cytokinesis." However,

premeiotic genome endoreplication was further declined and the restoration of the diploid

chromosome set by a fusion of the egg cell and the polar body nuclei (central fusion) was

again suggested on the basis of observations of pachytene and diplotene during meiosis in

adult females of the obligate parthenogenetic diploid species *Darevskia unisexualis* and

Darevskia armeniaca (Kupriyanova, 2009; Kupriyanova et al., 2021; Spangenberg et al.,

2020, 2021). Recently, Spangenberg et al. (2024) reported both non-endoreplicated and

endoreplicated oocytes in pachytene of *D. armeniaca.* However, later meiotic stages were not

examined and these authors raised questions if postmeiotic fusion could be the mechanism of

the production of non-reduced eggs (Spangenberg et al., 2024). It was suggested that oocytes

with the original ploidy level overcome a prophase I checkpoint that allows them to proceed

in meiosis (Spangenberg et al., 2020, 2024).

Here, we present evidence from the observation of chromosomes in diplotene in a

sexual and three parthenogenetic species of the genus *Darevskia*, resolving the long-standing

controversy over the mechanism of unreduced egg production in these lizards.

Material and Methods

Experimental animals

We compared oocytes at the diplotene stage between adult females of the obligate

parthenogenetic hybrid species *D. armeniaca, D. unisexualis* and *D. dahli*, and of *D. raddei*

nairensis. representing ancestral, sexual reproduction. *D. unisexualis* evolved by a cross

between a female of *D. raddei nairensis* with a male of *D. valentini* (Vergun et al., 2020;

Tarkhnishvili et al., 2020). *D. dahli* is a hybrid between a female of *D. mixta* and a male of *D.*

portschinskii (Yanchukov et al., 2022; Arekelyan et al., 2022). The origin of *D. armeniaca* is

less clear. It was reported to be the product of the hybridization of a *D. mixta* female with a

D. valentini male (Murphy et al., 2000; Freitas et al., 2019). However, based on allele sharing

at microsatellite loci, Tarkhnishvili et al. (2020) suggested that *D. armeniaca* could be the

result of a backcross between a parthenogenetic female of *D. dahli* and a male of *D. valentini*,

which inherited parthenogenesis from its already parthenogenetic mother.

The experimental animals originated from Armenia (for the list of individuals and

their localities see Supplementary Table S1). Alive-animal handling procedures were

approved by Yerevan State University according to the ethical guidelines, capture permit No.

3/29.7/1043 was issued by the Ministry of Environment of the Republic of Armenia. The

experimental procedures were carried out by an accredited person (M.A., CZ 01223) and

under the approval of the Ethical Committee of the Faculty of Science, Charles University

(UKPRF/28830/2021).

Mitotic chromosomes analyses

Mitotic chromosomes were prepared by cultivation of leukocytes from fresh peripheral blood

and then standard cytogenetic methods were applied: C-banding for visualisation of

constitutive heterochromatin and fluorescence *in situ* hybridization (FISH) with probes

detecting telomeres and 18S rDNA clusters. Telomeric FISH using a Cy3-labelled PNA

probe was performed according to the manufacturer's instructions (Telomere PNA FISH

Kit/Cy3, Dako), all other methods including the cell cultivation followed the detailed

protocols available in Supplementary Data of Altmanová et al. (2023). The 18S rDNA probe

labelled by biotin (Roche) has been prepared from genomic DNA of the slow worm *Anguis*

fragilis, its efficient hybridization on a broad phylogenetic scale was proven in other

sauropsids (Poignet et al. 2021, Altmanová et al. 2023). In both FISH analyses and C-

banding, the slides were stained and mounted in Vectashield Antifade Medium containing

4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Diplotene chromosomes preparation and analyses

Before the dissection, all experimental animals were sacrificed by blunt force trauma to the

head and then the gonads were removed. Diplotene chromosome spreads (also known as

lampbrush chromosomes) were prepared according to the protocol of Gall et al. (1991) as

described by Dedukh et al. (2022). In order to accurately count chromosomes during

diplotene and to assess correct bivalent formation, we performed FISH with the telomeric

probe under the same conditions as mentioned above for mitotic chromosomes.

Widefield and fluorescence microscopy

Microphotographs of mitotic chromosomes were captured by an Axio Imager Z2 microscope

equipped with a CoolCube 1 b/w digital camera (MetaSystems) using the MetaSystems

platform for automatic search, capture and image processing. Meiotic chromosomes after

immunofluorescence staining and FISH were analysed using Olympus BX53 equipped with

standard fluorescence filter sets and the microphotographs were captured by a CCD camera

(DP30W Olympus) using Olympus Acquisition Software. Microphotographs were finally

adjusted and arranged in Adobe Photoshop CS6 software.

Results and discussion

Cytogenetic inspection of mitotic metaphase chromosomes confirmed previous karyological

data in the studied species of the genus *Darevskia* (Kupriyanova, 2009). Females of all

species possess diploid chromosome number 2n = 38 with three microchromosomes, one of

which is the sex chromosome W (Figures 1, S1). Centromeres, the W chromosome and also

terminal regions of some other chromosomes are heterochromatic (Supplementary Figure

S2). FISH with the telomeric probe labelled only the ends of the chromosomes (contrary to

the report of Kupriyanova, 2009, in *D. armeniaca*), with a stronger signal in the terminal

region of the W chromosome in *D. r. nairensis* (Supplementary Figure S2). We found

clusters of 18S rDNA on two chromosomes in all the studied species. They are placed on a

middle-sized pair in *D. armeniaca* and *D. dahli*, on a smaller pair in *D. r. nairensis*, and on

one middle-sized and one smaller chromosome in *D. unisexualis* (Supplementary Figure S2).

Compared to sexual *D. r. nairensis*, one cluster was always larger, probably dominant, in the

parthenogens, which would explain why only one NOR has been previously reported in the

parthenogens (Kupriyanova, 2009). Because the general genome organisation is very similar

in all studied species, the apparent cytogenetic evidence of hybrid origin was documented

only in *D. unisexualis* thanks to different topology of 18S rDNA between the two parental

species chromosome sets.

The analysis of the diplotene chromosomes in the sexual species *D. r. nairensis*

revealed 19 bivalents formed by 38 chromosomes (Supplementary Figure S3). As in mitotic

chromosomes, FISH with the telomeric probe did not reveal interstitial telomeric sites and

signals were located only in the terminal part of the chromosomes. In all oocytes of the

parthenogenetic *D. armeniaca, D. unisexualis* and *D. dahli* we observed 38 bivalents,

indicating the presence of 76 chromosomes (Supplementary Figures S4-S6).

In all three obligate parthenogens, we found only oocytes with duplicated genomes in

diplotene, suggesting that premeiotic endoreplication is required for progression to later

meiotic stages. We can only speculate why the mechanism of the formation of unreduced

oocytes was misinterpreted in previous studies of the parthenogenetic lizards of the genus

Darevskia. Similar to the other parthenogenetic complexes of the hybrid origin studied so far

(*Aspidoscelis*: Lutes et al., 2010; Newton et al., 2016; Cole et al., 2020; *Hemiphyllodactylus,*

Heteronotia and *Lepidodactylus*: Dedukh et al., 2022), only a small fraction of oocytes

undergoes premeiotic duplication and enter later stages also in *Darevskia armeniaca*

(Spangenberg et al., 2024). The majority of meiotic oocytes (even well over 90%) have an

initial ploidy level, they usually do not pair chromosomes correctly and do not progress

through meiosis. It can be confusing that only a small fraction of the cells, easily missed

when only a few oocytes are examined, are doing what is essential for reproduction. Along

with a pairing of autosomes, Spangenberg et al. (2024) found that the sex chromosomes in

around half of the non-duplicated oocytes are not correctly paired at pachytene in the

parthenogenetic rock lizards. In contrast, a contact between Z and W sex chromosomes was

found in the remaining non-duplicated oocytes, but a support for the synapsis between the

homologous chromosomes for example by the visualisation of recombination foci allowing

the identification of paired chromosomes during pachytene was not provided. These authors

assumed that the sex chromosomes and autosomes are synapsed in these oocytes and that

such cells could proceed to the next phases of meiosis. Nevertheless, we found exclusive

oocytes with premeiotic endoreplication at the later stages of meiosis (diplotene), although

they form only a small fraction at earlier stages. The analysis of diplotene oocytes was earlier

performed in the parthenogenetic *D. armeniaca* (Kupriyanova, 2009). However, the obtained

chromosomal spreads were either incomplete or bivalents were intensively intermixed thus

preventing their counting.

The discovery of the mechanism of oogenesis in the parthenogenetic rock lizards has

important implications. Premeiotic endoreplication followed by the pairing and

recombination in identical chromosome copies ensures clonality, at least temporarily. In the

longer term, a loss of heterozygosity in hybrids with premeiotic endoreplication is most likely

due to occasional recombination between orthologs and gene conversion on the homeologs,

as documented in gynogenetic teleost fishes (Janko et al., 2021). Gene conversion should

occur more likely at recombination hotspots rather than being completely randomly

distributed (Lorenz and Mpaulo, 2022). On the other hand, central fusion should be

associated with a typical recombination between homologous chromosomes, and thus

heterozygosity should be preserved only in the central part of the chromosomes and lost in

the regions of the chromosome arms between a recombination site and the telomere of each

chromosome. Quite significant loss of heterozygosity was recorded in *Darevskia* in certain

microsatellite loci (Tarkhnishvili et al., 2020). It should be tested whether the distribution of

homozygous loci throughout the genomes is more consistent with conversion and random

occasional recombination between orthologs, or regular recombination of chromosomes

expected under the fusion hypothesis by genomic data.

The apparent variability of mechanisms in the obligate parthenogens of the hybrid

origin, based on the information available at the time in parthenogenetic *Aspidoscelis* and

Darevskia lizards, led to the conclusion that "the mechanisms of meiosis in vertebrate

parthenogens may not conform to a one-size-fits-all scenario" (Freitas et al., 2022). However,

it seems that we need to revise this view: while terminal fusion and postmeiotic doubling

connected with the loss of heterozygosity seem to be the mechanism of facultative

parthenogenesis (Booth et al., 2012, 2014; Booth and Schuett, 2016; Kratochvíl et al., 2020;

Ho et al., 2023), only premeiotic endoreplication is now known in obligate parthenogenetic

vertebrates of hybrid origin. The same mechanism was uncovered in the studied

representatives of eight or nine independent hybrid origins of obligate parthenogenesis: in the

teiid diploids *A. tesselata* (Lutes et al., 2010) (1) and *A. neomexicana* (Newton et al., 2016)

(2)*,* in the triploid *A. uniparens* (Cuellar, 1971) and tetraploid *A. priscillae* originating from

the laboratory cross of *A. uniparens* with a male of sexual diploid *A. inornatus* (Cole et al.,

2017) (3), in the triploid geckos *Heteronotia binoei* (Dedukh et al., 2022) (4) and

Hemiphyllodactylus typus (Dedukh et al., 2022) (5), in the diploid and triploid geckos

Lepidodactylus lugubris (Dedukh et al., 2022) (6), and - as shown in this study, in the diploid

lacertids *D. unisexualis* (7)*, D. dahli* (8), and *D. armeniaca* (9 - depending on the mode of its

origin, as it might inherited parthenogenesis from its already parthenogenetic ancestor;

Tarkhnishvili et al., 2020).

251   Our finding of exclusively endoreplicated oocytes in late diplotene, although cells

with the original ploidy levels being observed in earlier stages (Spangenberg et al., 2020,

2024), suggests that the pachytene checkpoint is conserved and active in all of the

parthenogenetic lizards studied. The conservation of the fully functional pachytene

checkpoint may indicate its importance to prevent aneuploidy in both sexual females and

parthenogenetic lineages. It seems that parthenogenesis was forced to find a way to produce

unreduced eggs while still passing through the strict control of the pachytene checkpoint. It

appears that two solutions to this challenge were found during the evolution: premeiotic

endoreplication after which all chromosomes are properly paired in hybrids, and normal

pairing followed by typical meiosis and a fusion in facultative parthenogenesis.

261   Previously, we speculated that the repeated involvement of premeiotic endoreplication

for the production of unreduced eggs in hybrids might be caused by the co-option of the pre-

existing mechanism of induced genome duplication by increased levels of apoptotic cells,

specifically by a mass of discarded oocytes with the original ploidy level arrested by the

pachytene checkpoint (Dedukh et al., 2022). This hypothesis attempts to explain why

premeiotic endoreplication occurs repeatedly and instantaneously in the F1 hybrid generation

and, at the same time, why the majority of oocytes enter meiosis without prior

endoreplication and fail to complete their development. We also emphasised that premeiotic

endoreplication - in contrast to egg/polar body fusion - opens the way to polyploidization: it

seems easier to produce unreduced oocytes after incorporation of the other haploid

chromosome set using this mechanism than under a fusion. The possible backcrossing

leading to the emergence of *D. armeniaca* and other introgressions in the *Darevskia* lizards

suggested by Tarkhnishvili et al. (2020) would be more plausible through a fertile triploid

produced by a fertilisation of a diploid egg of a parthenogenetic lineage with a haploid sperm

of a sexual species under endoreplication than under fusion, as already pointed out by these

authors. It should be noted that the observed allele sharing could be the result of a gene flow

from the parthenogenetic to the sexual population as known in other asexual complexes

through a polyploid: in some teleost hybrid complexes, triploids produce haploid recombined

gametes although their diploid ancestries were gynogenetic (Alves et al., 2001; Goddard et

al., 1998; Dedukh et al., 2023). However, there is still no direct evidence for fertility of

triploid females in *Darevskia* (only for well-developed reproductive organs in them), and no

all-triploid parthenogenetic populations have ever been found in this genus. Nevertheless, the

finding of tetraploid individuals (Danielyan et al., 2008), probably resulting from a cross between

a triploid and a diploid sexual individual, and triploid females showing signs of previous clutch

laying in the field suggest that at least some triploid females could be fertile (reviewed in

Tarkhnishvili et al., 2020).

In summary, we emphasise that only premeiotic endoreplication has been documented

as a mechanism of the production of unreduced eggs in obligate parthenogenetic vertebrates

of hybrid origin to date. It seems that evolution has not been so creative in finding ways to

escape sexual reproduction in them, always maintaining the pachytene checkpoint and co-

opting the same developmental mechanisms, the premeiotic endoreplication, over and over

again.

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formal analyses: D.D., M.A.; visualisation: D.D., M.A.; resources: D.D., M.A., L.K., R.P.,

M.A.; data curation: D.D., M.A., L.K.; funding acquisition: D.D., M.A., L.K; writing -

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Competing interests

The authors declare no competing or financial interests.

Supplementary material

Table S1, figures S1-S6.

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Figure 1. Mitotic and meiotic chromosomes of the asexual species *Darevskia dahli*. A) Karyotype with 2n = 38 is widely shared among *Darevskia* lizards. B) Identification of the W sex chromosome by visualization of constitutive heterochromatin which is absent on the pair of microchromosomes (labelled as m). C) Fluorescence *in situ* hybridization (FISH) with the telomeric (TTAGGG)$_n$ probe (red) revealed a standard distribution in the terminal regions of all chromosomes. D) The detection of 18S rDNA clusters (green) showed a different amount of accumulation on homeologs. In contrast, the signals on two chromosomes of different sizes, providing evidence of a hybrid origin in the otherwise conserved karyotype of *D. unisexualis* (box). E) All oocytes in the diplotene stage have double the number of chromosomes (i.e., 38 bivalents = 76 chromosomes) compared to the mitotic nuclei, documenting past premeiotic endoreplication. Telomeric FISH was applied to facilitate the counting of chromosomes/bivalents. Note that each detail shows one or more bivalents. The asterisk labels a small bivalent with strong heterochromatic region indicating putative WW bivalent. Scale bar = 10 µm. Complete sets of results for all species studied here are in the Supplementary Material.