# OOGENESIS IN UNISEXUAL WHIPTAIL LIZARDS

# (GENUS *ASPIDOSCELIS*)

**by**

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**OOGENESIS IN UNISEXUAL WHIPTAIL LIZARDS (GENUS *ASPIDOSCELIS*)**

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## Abstract

Parthenogenesis is a reproductive mode that does not require males. Though theoretically advantageous, its rarity among metazoans suggests otherwise. Paradoxically, some unisexual species within the genus *Aspidoscelis* appear to be thriving by reproducing through obligate parthenogenesis. Formed via hybridization between different bisexual species, these lizards apparently reap the benefits of both hybrid vigor and higher reproductive potential. Previous studies have demonstrated the high degree of heterozygosity between generations, which contributes to the success of these lineages; a loss of heterozygosity would likely be accompanied by a decrease in fitness. How meiosis is modified in these animals is unknown. The predominant hypothesis suggests that endoreplication takes place prior to meiosis, resulting in a two-fold increase in DNA; however this has not been definitively shown. Further, the downstream modifications that result in a heterozygous gamete have merely been speculated upon.

In the following thesis, meiosis within three parthenogenetic species is investigated. Quantification of DNA in germinal vesicles of the diploid parthenogen *A. tesselata* and the bisexual species *A. gularis* demonstrated that the parthenogen contains two-fold more DNA, despite equivalent somatic cell ploidy. Chiasmata were present on diplotene-stage chromosomes in both species, indicating that recombination is not bypassed. Additionally, synaptonemal complexes were found during pachytene in each species. Maintenance of heterozygosity is highly dependent on chromosome pairing in the parthenogen. Pairing between homologous chromosomes would result in a decrease; whereas pairing between identical (sister) chromosomes resulting from the additional DNA doubling event would preserve heterozygosity. Using homolog specific FISH

probes in the parthenogen *A. neomexicana*, sister chromosome pairing is revealed. Thus, sister chromosome pairing after an additional DNA doubling allows for maintenance of heterozygosity in *Aspidoscelis* parthenogens. Secondly, four self-sustaining lineages of a new tetraploid species were generated from the mating between the diploid bisexual species *Aspidoscelis inornata* and triploid parthenogen *A. exsanguis.* The identity of these hybrids was confirmed by genotyping analysis. Females retain the ability to reproduce parthenogenetically through the doubling mechanism described in diploid species. These tetraploids have demonstrated how ploidy elevation hypothetically occurs in natural parthenogens. The mechanisms described in this thesis may be utilized in other parthenogens. Recent findings and future directions based on this work are presented.

## Acknowledgements

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# **Chapter 1:** Introduction

## An Evolutionary Conundrum

In the dry and arid southwestern regions of the U.S. and northern Mexico, throughout disturbed desert areas uninhabitable by many native animals, an evolutionary conundrum is taking place. Within some of these environments, lizards of the genus *Aspidoscelis* appear to be thriving – but not all individuals within *Aspidoscelis* are created equal. The parthenogenetic, or unisexual, species within the genus are primarily responsible for this inhabitation. And although sexual reproduction prevails among most multicellular organisms, there is an obvious discrepancy in the *Aspidoscelis* genus.

Though uncommon, parthenogenesis occurs in a subset of organisms, and some such species adapt to harsh environments better than related bisexual counterparts. As one extreme example, the *Bdelloid* rotifers have apparently survived for millions of years without sex. How are rotifers and other *Aspidoscelis* parthenogens successful, and what sets them apart from other animals in which asexual reproduction has never been reported? Perhaps more importantly, why is parthenogenesis rare, even among reptiles? Unisexual *Aspidoscelis* species arose from interspecific hybridization between closely related sexual species, however, unlike other hybrids, these animals are capable of escaping sterility. How this is achieved is not well understood.

### *Aspidoscelis*

A breakthrough in herpetology came from the Caucasus region of Eurasia in 1958, when Darevsky described the first known unisexual lizard species in the genus *Lacerta (Darevsky, 1958)*. This fueled the search for other parthenogenetic species, and led to its

discovery in *Aspidoscelis*. The first publications reporting all-female *Aspidoscelis* lineages arrived in 1962, after initial observations noted an absence of males (Duellman and Zweifel, 1962; Maslin, 1962; Wright, 1993). The genus was originally grouped with South American *Cnemidophorus* species until mitochondrial data revealed significant variation that led to the separation of these two regions *(Reeder et al., 2002a)*. Thereafter the genus has been referred to as *Aspidoscelis.* To date, the genus consists of at least 50 recognized species, of which approximately one-third are unisexual.

*Aspidoscelis* parthenogens were formed by hybridization between bisexual progenitors. In some cases, the parentage is assumed based on morphology, or confirmed by molecular techniques. Noted for harboring high levels of heterozygosity, the parthenogen contains several distinct alleles which can be used to identify parentals. Indeed, parentage has been affirmed for several lineages by genomic or allozyme analysis. Inherent to the hybridization event is the convergence of two distinct genomes and a high correlation with the phenomenon known as *hybrid vigor*. Similarly, *Aspidoscelis* parthenogens apparently benefit from the union of two (or in some cases, more) separate genomes. Unlike other hybrids, which commonly experience reduced fertility, successful *Aspidoscelis* lineages are fertile and maintain high levels of heterozygosity at tested loci (Neaves and Gerald, 1968a). Preservation of heterozygosity from generation to generation is taken to an extreme, as these parthenogenetic species reproduce in a clonal manner (Cuellar, 1976; Cuellar and Smart, 1977). This high conservation of heterozygosity appears to be linked with the meiotic aberration that allows this species to reproduce clonally.

Whiptails are among the most widely researched lizards, after only *Sceloporus* and *Anolis.* Studied not only for an unusual reproductive biology, their ubiquity in North America makes them amenable to the study of natural unisexual populations, including behavioral aspects, physiology, and population genetics, among other interests (Hernández-Gallegos et al., 2003; Hotchkin and Riveroll, 2005; O'Connor et al., 2011).

## Phylogeny

The plasticity of speciation within *Aspidoscelis* has long been the bane of herpetologists who have attempted to classify it (Wright, 1993). Parthenogenetic combinations can become very complicated. The current phylogenetic consensus has divided the genus into five groups: *A. cozumela*, *A. deppii*, *A. sexlineata*, *A. tesselata*, and *A. tigris*. The latter three are the source of the majority of parthenogens (Reeder et al., 2002a). As with most obligate parthenogenetic vertebrates, diploid *Aspidoscelis* parthenogens arose from hybridization between two different bisexual species, and triploid lineages arose via addition of a sperm genome from a bisexual male onto the diploid parthenogen oocyte (Cole, 1979; Lowe and Wright, 1966; Neaves, 1969a; Neaves and Gerald, 1968a). One of the most commonly studied parthenogens, *A. tesselata*, resulted from the mating between *A. tigris marmorata* and *A. gularis septemvittata*, (Fig. 1). Both of these parental species are extant, which allows for comparative analyses between parthenogen and parentals, and can answer important evolutionary biology questions. On the contrary, triploid *A. exsanguis*, *A. opatoe*, *A. uniparens*, and *A. velox* arose from a theoretical intermediate which no longer exists, and although the bisexual species persist, the inability to sample the intermediate occludes several meaningful

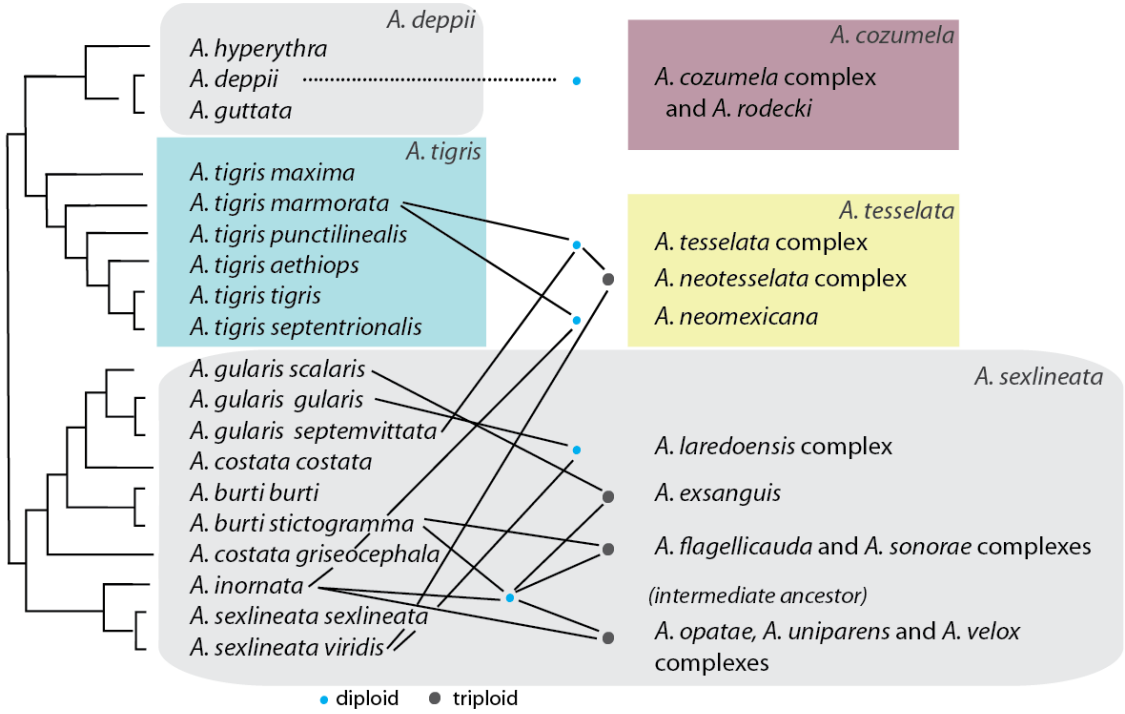



**Fig. 1. Phylogeny of a subset of *Aspidoscelis* bisexual lizards (left) and resulting hybrids, i.e. unisexual species, (right).** Adapted from (Reeder et al., 2002a).

experiments. For example, using populations of *A. tesselata* and *A. tigris marmorata* for mitochondrial DNA restriction analyses revealed less than 1% nucleotide divergence between these two groups as well as among individuals of the *A. tesselata* population, indicating that (a) *A. tigris* is the maternal parent of *A. tesselata*, and (b) the hybridization event that resulted in this population occurred relatively recently (Iii et al., 1989). These findings appear consistent across *Aspidoscelis*; the number of hybridization events that created most, if not all, existing parthenogenetic species is estimated to be greater than one, but still relatively few (Iii et al., 1989; Moritz et al., 1992; Moritz et al., 1989b).

Interestingly, while parthenogens have risen within the *A. sexlineata* group, no such phenomenon seems to occur in *A. tigris*, which apparently requires hybridization

with the *A. sexlineata* group for parthenogen formation. These *A. tigris* / *A. sexlineata* parthenogens comprise a separate group, *A. tesselata*, whereas unisexual lineages created within the *A. sexlineata* clade retain their group classification. In practice, each population of hybrids is considered a species, although this is not without debate; a suggestion to designate *each* hybridization event has been put forth, however it is not very practical and therefore is not widely used (Cole, 1990; Frost and Hillis, 1990).

## Ploidy

Although bisexual species in the *Aspidoscelis* genus are strictly diploid, unisexual whiptails do not appear to be constrained to two genomes. Homolog pairing in the bisexual probably plays a role in this restriction, as odd-ploidy animals rarely reproduce sexually [see exception (Stock et al., 2012)], but presumably, the mechanism of meiosis in unisexuals circumvents the traditional pairing process. As illustrated in Fig. 2, triploid unisexual species abound in the genus: of the eleven documented unisexual species in *Aspidoscelis*, seven are triploid (Vrijenhoek et al., 1989). This suggests that perhaps triploids have an advantage over diploids, especially when considering that a diploid intermediate would have co-existed for some time with the triploid. Muller's ratchet – an evolutionary hypothesis which suggests that unisexuals accrue detrimental alleles – may play role, as three genomes would increase gene redundancy. This might alleviate the effect of any mutations, thereby allowing a triploid species to outcompete a diploid. It is as yet unknown what other differences exist between a diploid and triploid. Is the mechanism of meiosis conserved in diploid and triploid unisexuals? Do other advantages

exist, such as increased physiological plasticity, which might favor a triploid genome in the harsh environments of the southwestern U.S.?

Remarkably, ploidy does not culminate with three genomes; two female tetraploid *Aspidoscelis* have been found in nature; in one case, the animal appeared to be sterile, and in the other, fertility was apparent, but validation was unfortunately not possible (Hardy and Cole, 1998; Neaves, 1971). The viability of a tetraploid species is interesting in its own right; however, more confounding may be the absence of these species in a genus in which viability is clearly possible. The ratchet-supporting hypothesis that 'more is better' does not appear to apply at the tetraploid level in *Aspidoscelis*, and several more questions emerge: How are four sets of chromosomes disadvantageous? Are such animals fertile? Can meiosis continue with so much DNA that presumably necessitates pairing, etc., and other processes that are normally managed with only two sets? The evolutionary biology and history of these animals are fascinating, and answers to these questions will undoubtedly be enlightening.

## Geography

While *Aspidoscelis* parthenogens predominantly occupy the Southwest, bisexual species within the *A. sexlineata* group are ubiquitous throughout the U.S. and Mexico, distributed as far as the East Coast of the U.S. (see Fig. 2). A. *tigris*, on the other hand, is distributed primarily in the West, with extensions into Northern Oregon. The overlap between these two territories correlates with the prevalence of parthenogen inhabitation. There is an obvious lack of parthenogen *A. sexlineata* dispersal, despite its overall distribution and higher propensity to form viable inter-specific hybrids, e.g. *A. exsanguis*

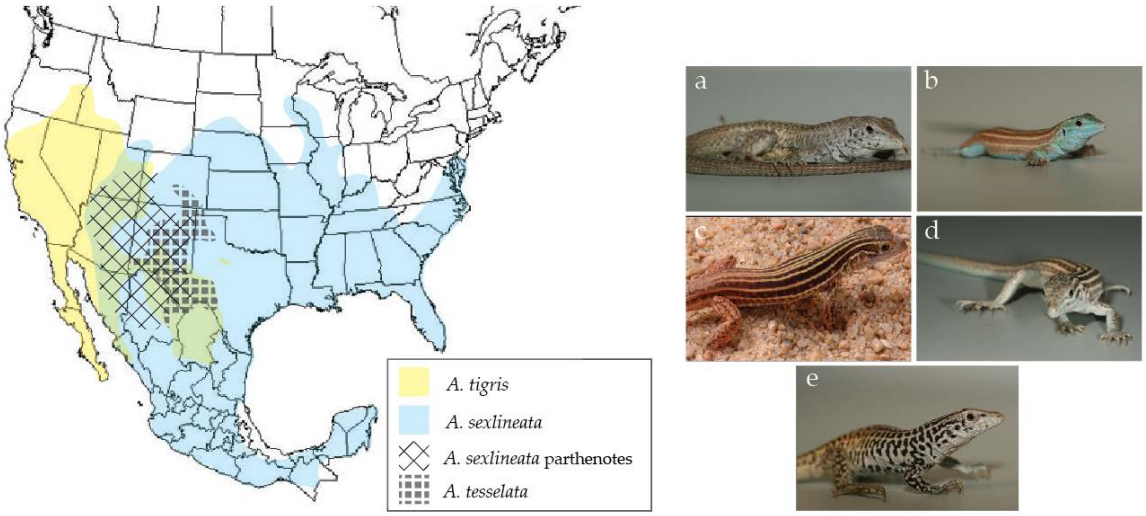



**Fig. 2. Geographic distribution of two *Aspidoscelis* parthenogenetic species and their bisexual parental species. (a)** Bisexual *A. tigris.*Bisexual *A. sexlineata* group: **(b)** *A. inornata* and **(c)** *A. gularis.* Parthenogenetic *A. tesselata* group*:* **(d)** *A. neomexicana*, hybrid of *A. tigris* and *A. inornata* **(e)** *A. tesselata*, hybrid of *A. tigris* and *A. gularis* Adapted from (Wright, 1993).

and *A. uniparens*. This can probably be explained by two observations. First, species within the *A. sexlineata* clade lack considerable sympatry, decreasing the likelihood for interspecific interactions (Wright, 1993). Secondly, successful asexual lineages appear to originate from a single or few hybridization events (Parker and Selander, 1976). Illustrating this is the observation of hybrids at the interface between bisexual species, the majority of which have been sterile (Dessauer et al., 2000; Taylor et al., 2001; Walker et al., 1990). Thus, hybridization appears to correlate with parthenogenesis, but it is not sufficient to create a fertile lineage.

## Meiosis

### Overview

A highly dimorphic mechanism used to generate gametes, meiosis is largely conserved within each sex and provides the mechanism by which sexual reproduction can occur (see Fig. 3). By halving the genome and creating 1C gametes, ploidy can be restored upon union of sperm and oocyte.

At the onset of meiosis is an S-phase that, karyotypically, is indistinguishable from that of mitosis, but is actually unique in several aspects. First, the pre-meiotic S-phase is longer: by a factor of 3 in yeast, and in mammals not as drastic, but still significant (11.5 hour compared to 10 hour in somatic cells) (Kofman-Alfaro and Chandley, 1970; Wartenberg et al., 1998). The reason for this increase is unknown, although in yeast it may be an artifact of nutrient starvation required for sporulation rather than a meiosis-specific effect (Blitzblau et al., 2012). Second, the meiotic replication machinery has novel factors included, distinct from mitosis. CLB5 mutants were initially characterized for a mild mitotic S-phase defect and failure in meiotic progression, but later shown – along with another cyclin, CLB6 – to be essential for normal meiotic S-phase (Epstein and Cross, 1992; Stuart and Wittenberg, 1998).

Homologous chromosomes resulting from S-phase subsequently undergo pairing, a process characterized by transient interactions between chromosomes, both homologous and nonhomologous. Clustering of telomeres to the centrosome (spindle pole body) of the nuclear periphery effectively reduces the search to two dimensions. Interestingly, homologous domains have been observed to remain associated longer than nonhomologous regions. The mechanism behind this double strand break-independent phenomenon has been elusive; however, recent findings in *Schizosaccharomyces pombe* indicate that cis-acting noncoding RNAs play an important role in this process (Ding et

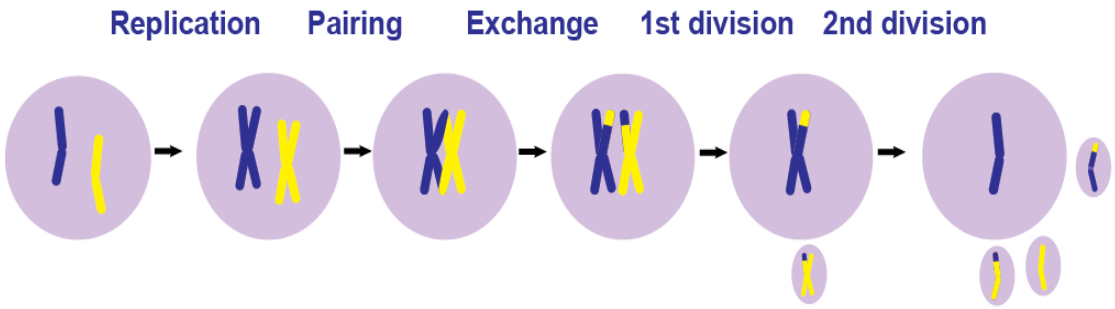



**Fig. 3. The basic process of meiosis, depicted as in an oocyte.** Premeiotic S-phase creates a 4C nucleus. Homologous chromosomes undergo recombination and after two cell divisions, four genetically distinct cells result, comprised of one oocyte and three polar bodies.

al., 2012). Pairing initiates homolog interactions, but in most organisms, premature chromosome segregation is prevented by the production of double strand breaks (DSBs) followed by formation of the synaptonemal complex (SC). DSBs are universally created by the endonuclease SPO11; mutants for the highly conserved protein exhibit extensive amounts of aneuploidy. Visible by electron microscopy as a ladder-like structure, the 200 nm wide SC is a proteinaceous lattice that aids in holding the homolog along its length, like a zipper. Early SC formation correlates with sites of recombination, and crossover sites are suspected to be associated with recombination nodules, SC-associated proteinaceous foci that measure approximately 100-nm in diameter. However, the strongest indication of a recombination site is the chiasma, the resulting physical attachment after a reciprocal crossover which is visible by light microscopy in many organisms.

Cell divisions are the final hallmarks in meiosis that will be introduced here. While the second division is essentially identical to that of mitosis, it is the first – a reductional division – which is unique to reproduction, by separating homologs rather than sister chromatids. Cohesion between chromatids is essential for reductional division, and is

accomplished though the meiosis-specific cohesin Rec8. Rec8$^{-/-}$ cells undergo equational division and, interestingly, continue to complete the second division as well (Molnar et al., 1995; Watanabe and Nurse, 1999). Another factor involved in proper chromosome segregation is the orientation of the kinetochores, the proteinaceous structures that assemble on each chromosome and interact with microtubules to physically separate chromosomes (for reviews, see (Santaguida and Musacchio, 2009; Watanabe, 2012)). In mitosis, kinetochores are located at opposite ends of the centromere, and bi-orientation is favored (microtubules emanating from opposite poles), making segregation of chromatids favorable. In contrast, during meiosis I, sister chromatids are to remain associated. For a successful first division, chromatids should be mono-oriented instead, and the bivalent should be bi-oriented. Facilitating this is a unique fusion of the kinetochore structure within each chromatid pair, which favors their mono-orientation. Additionally, and perhaps more importantly, the tension created from bi-orientation of the bivalent favors its formation and stability.

Meiosis is unusual in having two consecutive divisions without an intervening interphase. How is cell division number controlled? This is as yet unknown, although one hypothesis suggests that replication licensing is correlated with the presence of sister chromatid cohesion (Wilkins and Holliday, 2009). Because the first division separates homologous chromosomes, chromatids are left intact, thereby signaling progression into sister chromatid separation without a preceding S phase. Mechanisms that dictate division number may also prove to be significant to parthenogenesis.

Given the high conservation of meiosis, one might assume that the processes therein are disrupted only in parthenogens and pathological conditions. Surprisingly, several

sexually-reproducing organisms successfully generate haploid gametes in spite of obvious meiotic aberrations. For example, while recombination is required in most organisms, all chromosomes in male *Drosophila*, as well as the fourth in females, waive this mechanism; rather, a DSB-independent process unites homologs to prevent chromosome nondisjunction (Harris et al., 2003). Another example, *Schizosaccharomyces pombe,* undergoes recombination but without the characteristic tripartite synaptonemal complex. Seemingly analogous structures known as linear elements can be visualized between homologs via electron microscopy, although these are probably not required for normal gamete formation (Bahler et al., 1993; Wells et al., 2006).

## Routes to a 2C gamete

An additional DNA doubling within a cell is conceptually very easy: two rounds of replication, a failed cytokinesis, or reentry into G1 are among a few possibilities. However in reality, a cell with exactly twice the DNA content is rare. Cells are attuned to the DNA content required; perhaps more importantly, the effects of polyploidy could be disastrous for a species in the long-term (Fujiwara et al., 2005). Paradoxically, polyploid cells exist naturally, and may even be necessary for normal physiological function. For example, the *Drosophila* germline generates oocyte-nourishing polyploid cells by utilizing endoreplication, a special cell cycle in which multiple rounds of DNA replication occur. Germ cells in this organism divide within a cyst from a single progenitor, after which only one of the resulting 16 cells differentiates into the oocyte. The remaining cells differentiate into nurse cells and endoreplicate 10-12 times to

generate >1000C nuclei (Dej and Spradling, 1999). This mechanism is not restricted to invertebrates. It is used during embryonic development in mammalian trophoblasts and in the red blood cell precursors, megakaryocytes [see (Edgar and Orr-Weaver, 2001) for endoreplication review].

As it pertains to *Aspidoscelis*, how can 2C gametes consistently be generated? The following hypothetical scenario, using endoreplication as a mechanism, is based on the fact that each (diploid) parthenogenetic species is a hybrid of two different sexual species. Consequently, the parthenogen essentially contains two different genomes, and the resulting genetic diversity likely extends to meiotic proteins (Neaves, 1969a; Neaves and Gerald, 1968a). Let us assume that the temporal expression of meiosis-specific cyclins varies between sexually-reproducing *Aspidoscelis* species such that cyclins are expressed in different parts of premeiotic S phase. The parthenogenetic species, by nature of their formation, would have inherited both expression patterns, and would express both cyclins, each at a different time of premeiotic S phase. Such a minor variation may be sufficient to initiate and complete two rounds of DNA replication. However, one inconsistency with this hypothesis is that hybridization between bisexual parental species would be sufficient for establishing parthenogenesis, and this is not observed. Mating experiments between known parental species have yielded infertile hybrids (Cole et al., 2010). Therefore, it would strongly suggest that a mutation is required in addition to the hybridization event (Fig 4). Another problem with the endoreplication hypothesis, which essentially requires reentry into G1, is that it conflicts with the idea put forth by Wilkins and Holliday to explain the existence of two meiotic divisions (Wilkins and Holliday, 2009).

Other mechanisms could account for a 2C gamete. Failed cytokinesis, through a variety of mechanisms, could occur at any of the oogonial or meiotic divisions and result in twice the DNA content. A contractile ring that is incorrectly positioned or poorly constructed, can yield unevenly distributed chromosomes or cause complete failure of furrow ingression (Lacroix and Maddox, 2012). A return to the cell cycle from meiosis can potentially explain a 2C gamete – and there is precedence for this phenomenon. *Saccharomyces cerevisiae* cells that have initiated meiosis can return to vegetative growth if supplied with nutrient-rich media (Sherman and Roman, 1963; Simchen et al., 1972). Thus, there are numerous cytological and genetic alternations in meiosis that can theoretically generate a parthenogen. Uncovering the biologically relevant mechanism(s) will be the challenge.

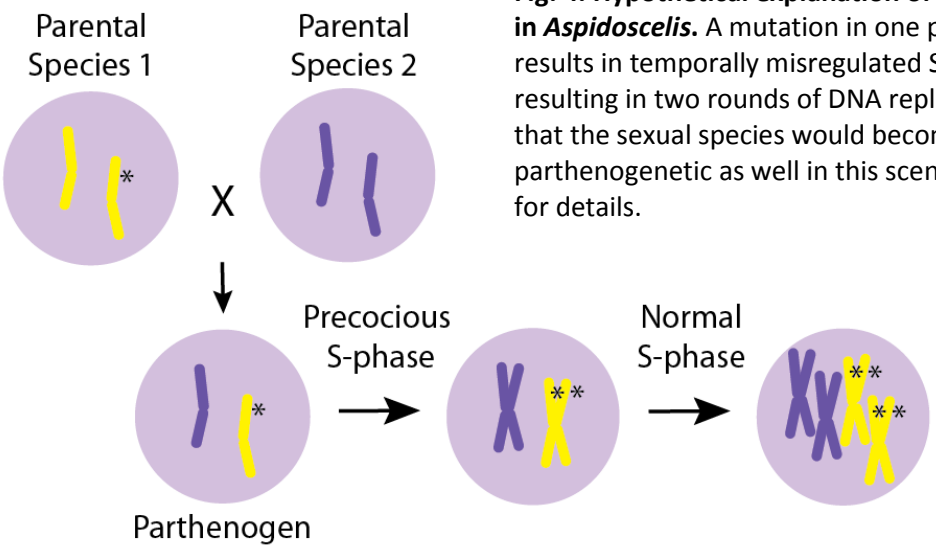



**Fig. 4. Hypothetical explanation of endoreplication in *Aspidoscelis*.** A mutation in one parental species results in temporally misregulated S-phase, resulting in two rounds of DNA replication. Note that the sexual species would become parthenogenetic as well in this scenario. See text for details.

## Meiosis in most hybrids

The textbook example of hybridity – the mule – with all its inherent strengths, is also subject to a fate common in hybrids: loss of fertility (Taylor and Short, 1973; Wodsedalek, 1916). Across species, the underlying cause is the same: divergence between two parental genomes; however, mechanisms responsible for sterility or infertility vary considerably, and the performers involved may be DNA, RNA, or protein. Research in the past few decades has vastly expanded on basic hypotheses first proposed in the early twentieth century.

Although it may seem reasonable to assume that genetic divergence directly abrogates chromosome pairing, in reality, this explains only a few known cases of hybrid incompatibility (Coyne and Orr, 1998). Chromosome pairing deficiencies affect both sexes, and thus cannot explain the high incidence of incompatibility in the heterogametic sex (Haldane's rule). A more applicable hypothesis, suggested in the 1930s and 1940s, would later become known as the Dobzhansky-Muller model (discussed in (Coyne and Orr, 1998)). According to this model, multiple loci – which are compensatory within each parental species – yield irreconcilable combinations in the hybrids. These antagonisms may manifest as loss of function (Mihola O Fau - Trachtulec et al.) or gain of function (Bayes and Malik, 2009; Long et al., 2008). The first known hybrid incompatibility gene among vertebrates is PRDM9, a H3K4 trimethylase required for recombination in mice and humans (Baudat et al., 2010; Parvanov et al., 2010). Mutations in this DNA-binding protein result in sterility, and the nature of hybrid incompatibility stems from the variability in the zinc finger domain.

## Parthenogenesis

Superficially, parthenogenesis appears to be advantageous: each individual within a species is capable of reproduction, which circumvents a significant disadvantage dealt to sexually-reproducing species: males (Maynard Smith, 1971). Bisexual species require energy for mate searching and mating, and a male's inability to generate offspring decreases the fecundity of the species. Because each individual within a parthenogenetic species is capable of reproduction, the population can expand more quickly. Theoretically, a parthenogenetic population would increase by a factor of two in each generation, exponentially outnumbering a bisexual species, assuming all other factors are equal (Fig. 5). Unisexuals also have an apparent advantage in that favorable genotypes remain intact over time. Bisexual species, on the other hand, are affected by the recombinational load that may potentially break up advantageous combinations. However, in reality, sexual eukaryotes overwhelmingly outnumber parthenogens. Additionally, parthenogens (as well as hermaphrodites) rarely comprise more than a collection of species and are typically present at the terminal nodes of phylogenetic trees, suggesting that these species are evolutionarily lackluster and result in dead-ends. Therefore, sexuality must harbor one or more advantages that compensate for deceased fecundity and recombinational load.

## Hypotheses for the predominance of sexual reproduction

The focus will be on concepts and predominant theories within the field. For a comprehensive summary of theories, see (Chao and Tran, 1997; de Visser and Elena, 2007; Muller, 1932).

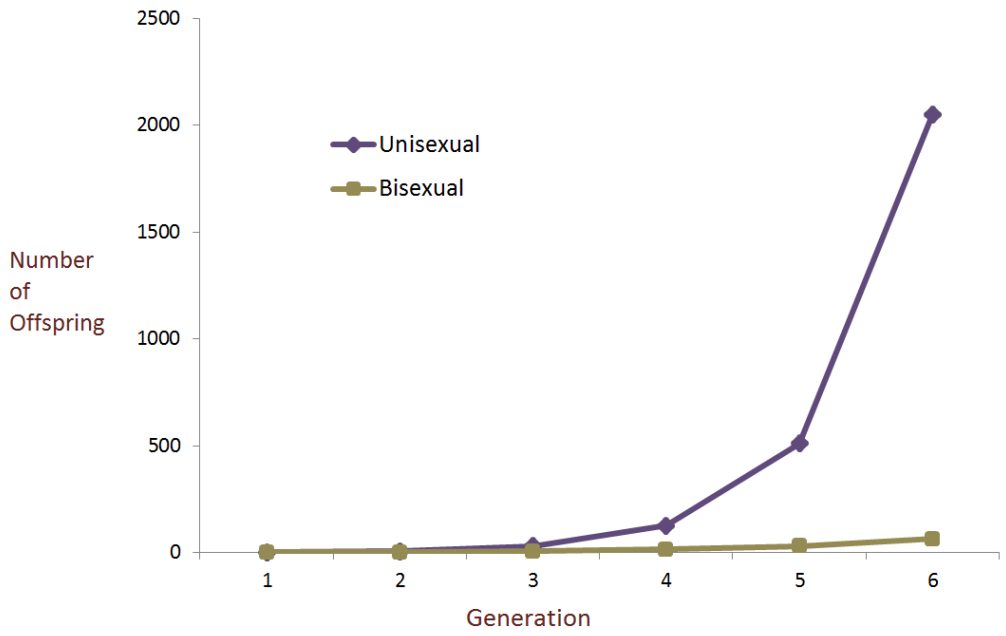



**Fig. 5. The two-fold cost of sex.** Theoretical population expansion in unisexual and bisexual species, assuming four offspring per female individual and neutral contribution from the male.

## Fisher-Muller hypothesis

Unlike parthenogenesis, sexual reproduction is a way in which genetic information from individuals within a species can be combined, thereby accelerating the formation of favorable genotypes. In a parthenogenetic species, such favorable combinations would need to arise independently within the same lineage and would thus require more time (Fig. 6a). Fisher and Muller independently proposed that sexual reproduction could be favored over asexual reproduction due to a faster combination of favorable alleles (Fisher, 1930; Muller, 1932).

## Muller's ratchet

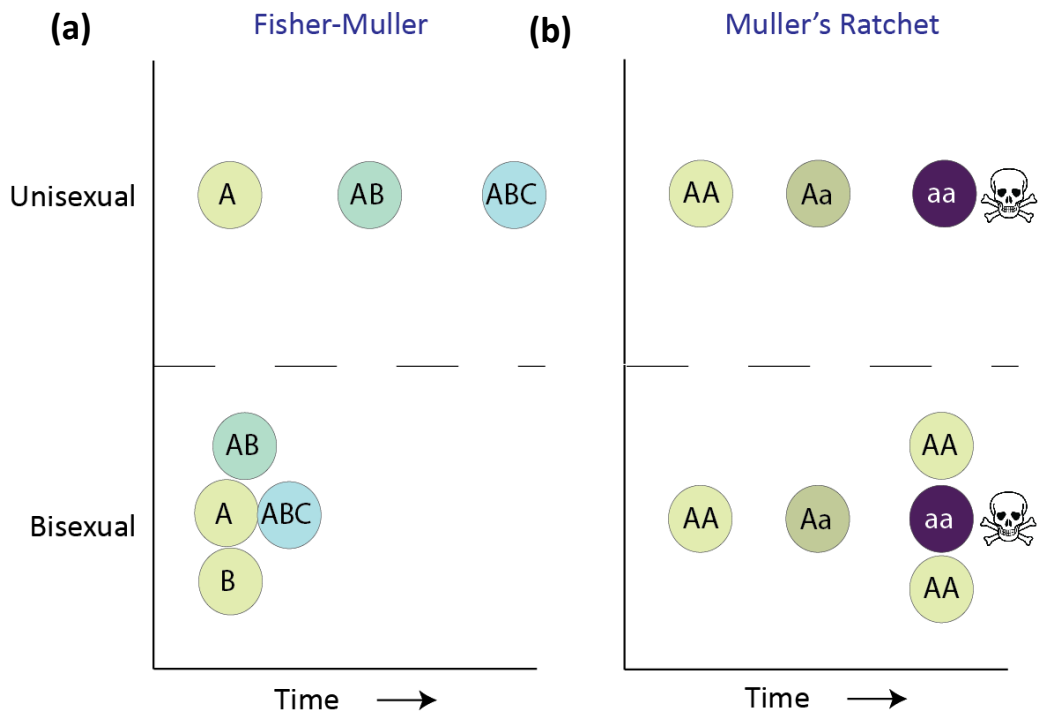
Extrapolating from the Fisher-Muller hypothesis is Muller's ratchet. Because most unisexual species reproduce as genetic copies of themselves, they are essentially locked into their genotype. Inheritable genetic variability predominately arises from spontaneous germline mutations. While most mutations would likely be neutral and not affect the fitness of the animal, those of a deleterious nature would inevitably arise over time (Fig. 6b). A unisexual species would have no means by which to purge such a mutation, creating an irreversible "click" in the ratchet. Eventually, the ratchet would reach a point in which the fitness of the animal was severely decreased, thereby lowering its fitness as a species. Hemiclonal frogs of the species *Rana esculenta* provide support for this model. These sexually-reproducing amphibians are hybrids (genotype RL) produced from two bisexual species (genotypes RR and LL). Offspring inherit the R genome clonally – without net genetic recombination – and the other genome sexually, yielding a RL' genotype. Crosses between RL' offspring within a population to produce RR animals typically leads to embryo lethality; however, crosses between RL offspring of a different parental origin (that produce RR' individuals with genetically distinct R genomes) result in healthy tadpoles (Vorburger, 2001). This suggests that deleterious alleles may have accumulated in the R genome since the hybridization event that formed *R. esculenta*.

## Red Queen hypothesis

One of the strongest hypotheses to describe the advantages of sexual reproduction, in large part due to recent studies, is the Red Queen. The name is derived from an event in Lewis Carroll's *Through the Looking Glass* in which the protagonist, Alice, tries to

race away from the Red Queen, but instead finds that, for all her efforts, she has merely remained in the same spot. Such an analogy has been used in evolutionary biology: species expend large amounts of effort simply to remain competitive with the surrounding co-evolving species (van Valen, 1973, 1974). Parasites in particular are used to illustrate this phenomenon, as they typically infect common host genotypes, giving selective advantage to rare varieties (Dybdahl and Lively, 1998; Neiman and Koskella, 2009). Therefore, unisexual species, due to their genetic invariance, would be at a disadvantage compared to their bisexual, and inherently diverse, counterparts (Fig. 6c).

Several studies have demonstrated the correlation between parasitism and reproductive mode of the host species. In the New Zealand freshwater snail, *Potamopyrgus antipodarum*, sexual varieties co-inhabit the shallow regions of lakes with parasitic *Microphallus* sp, whereas asexual forms are predominately found in deeper, *Microphallus*-sparse regions (Jokela and Lively, 1995; King et al., 2009). Vertebrates show similar trends. Wild-caught geckos of the species *Heteronotia binoei* display higher rates of mite infestations compared to a closely related sexual species (Moritz et al., 1991). In an analysis of 619 lizards, 51% of parthenogenetic individuals contained mites compared to 2% of the bisexual species with a mean infestation of 21.64 and 0.59 per individual, respectively. Higher mite infestations were verified by Shine's group (Kearney and Shine, 2004). To directly test the cause-effect relationship between genetic variability and co-evolution with parasites, Curtis Lively's team grew strains of *C. elegans* with *S. marcescens*, a bacterium that produces a potentially lethal infection in the worm. *C. elegans* is capable of either self-fertilization or outcrossing, and particularly useful selfing-restricted mutants have been generated. To simulate co-evolution and



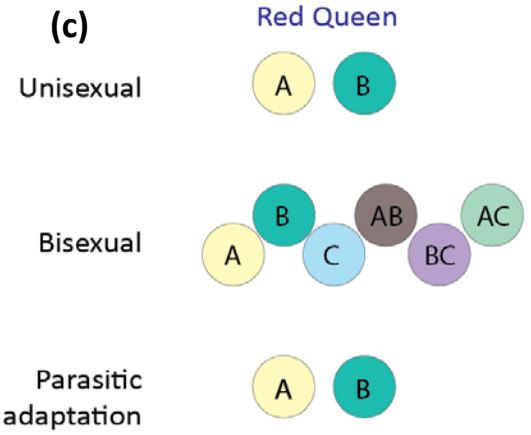




**Fig. 6. Hypotheses for prevalence of sex**. **(a)** Fisher-Muller suggests that advantageous alleles (ABC) will marry more quickly in bisexual species. **(b)** In Muller's ratchet, slightly deleterious alleles (Aa) accumulate until lethal (aa), but unlike some bisexual species, unisexuals cannot purge the intermediates. **(c)** Parasites adapt to the unisexual alleles A and B, but the genetic variation in bisexuals increases their resistance in the Red Queen hypothesis.

select for the most harmful parasites, bacteria were harvested from dead worms and recultured with survivors. In the absence of the parasite, wildtype populations of *C. elegans* outcrossed at a rate of 20%; however in its presence, outcrossing escalated to more than 70% over a period of 30 generations. Further, obligately selfing strains of *C.*

*elegans* died out within 20 generations (Morran et al., 2011). Taken together, these studies suggest that outcrossing is essential in order to compete with parasitic species.

Notably, the long-lived *Bdelloid* rotifers have recently been found to escape fungal parasites with an unusual ability to desiccate and blow away (Wilson and Sherman, 2010). This may partly explain how this species is able to overcome the Red Queen effect. However, Meselson and colleagues have shown that despite the presence of this defense mechanism, large amounts of horizontal gene transfer have occurred in rotifers, including a gene of bacterial origin which is capable of expression in the rotifer genome (Gladyshev et al., 2008).

### Unisexuality across species

From microscopic invertebrates such as the rotifer to the largest lizard in the world, the Komodo dragon (*Varanus komodoensis*), observations of parthenogenesis have been found in a wide range of animals. The variation of meiosis across species rivals the variation of the organisms themselves. At the most extreme, meiosis is essentially abolished as suspected in rotifers, and oocytes are produced via a mitosis-like mechanism (HSU, 1956). Despite extensive studies, meiotic genes have yet to be found, suggesting that parthenogenetic rotifers have survived without meiosis to such an extent that these genes have diverged. However, in general, most organisms have retained key aspects of meiosis, which include, but are not limited to, the expression of meiotic genes, pairing of homologous chromosomes, and a reductional division that separates homologs.

Additionally, ploidy restoration to equate that of a somatic cell is essential for successful parthenogenesis.

## Polar body fusion

Many known examples of asexual reproduction apparently occur via fusion of the haploid oocyte and a polar body. Interestingly, the genotype of the parthenogenetic oocyte will differ drastically depending on the type of fusion in meiosis. High levels of heterozygosity are retained from central fusion, merging of the oocyte with a polar body from the first division (Fig. 7). Based on microsatellite analyses, this mechanism appears to occur in, among others, the queen fire ant *Wasmannia auropunctata*, the cape honeybee *Apis mellifera capensis*, and some strains of *Drosophila* (Baudry et al., 2004; Fuyama, 1986; Rey et al., 2011). On the other hand, terminal fusion – that between the oocyte with its polar body from the second division – will result in high levels of homozygosity (Fig. 7). The snake *Epicrates maurus* and the termite *Reticulitermes virginicus* have been observed to undergo such fusion, and this mechanism may be responsible for facultative parthenogenesis in the Komodo dragon and the Hammerhead shark (Booth et al., 2011; Vargo et al., 2012; Watts et al., 2006).

It is important to note the apparently high incidence of fusion in ovaries of the aforementioned animals. One critical question is whether fusion is exclusive to parthenogens or a general occurrence during oogenesis. There are exceptions to the fusion rule, such as the *Bdelloid* rotifers. And while genetic analysis can merely suggest that polar body fusion has occurred, only cytological investigation can distinguish one

mechanism among others. In the cyclical parthenogen *Daphnia pulex*, for example, it had been postulated that diploid oocytes formed by apomixis, the mitotic-like division characteristic of the rotifers. However, Hiruta and colleagues demonstrated that meiosis I proceeds normally until the first division when anaphase aborts and chromosomes reunite, a process termed abortive meiosis (Hiruta et al., 2010). Possibly, abortive meiosis is more common than previously thought, as it could account for the high levels of heterozygosity observed in cases of presumed central fusion. Further cytological analysis in those animals can positively distinguish between central fusion and abortive meiosis. Likewise, terminal fusion may actually result from an abortive second division. Although one can argue that such a distinction is unnecessary as long as the genetic outcome is known, an understanding of the cellular and molecular mechanisms is crucial in order to explain the rarity of parthenogenesis.

### Premeiotic doubling

Premeiotic doubling of DNA solves the problem of ploidy loss: cells begin with twice as much DNA as a sexually-reproducing species and end with twice as much (diploid). This mechanism also appears common among hybrids; chromosomes that would not normally pair due to sequence dissimilarities instead match up identical chromosomes that resulted from the doubling. Meiosis then apparently proceeds in a quasi-normal manner, and after two divisions, the resulting daughter cells are genetically identical to each other and to the mother cell. The grasshopper *Warramaba virgo,*the brown alga *Ectocarpus, Poeciliopsis,* and several species of amphibians and reptiles,

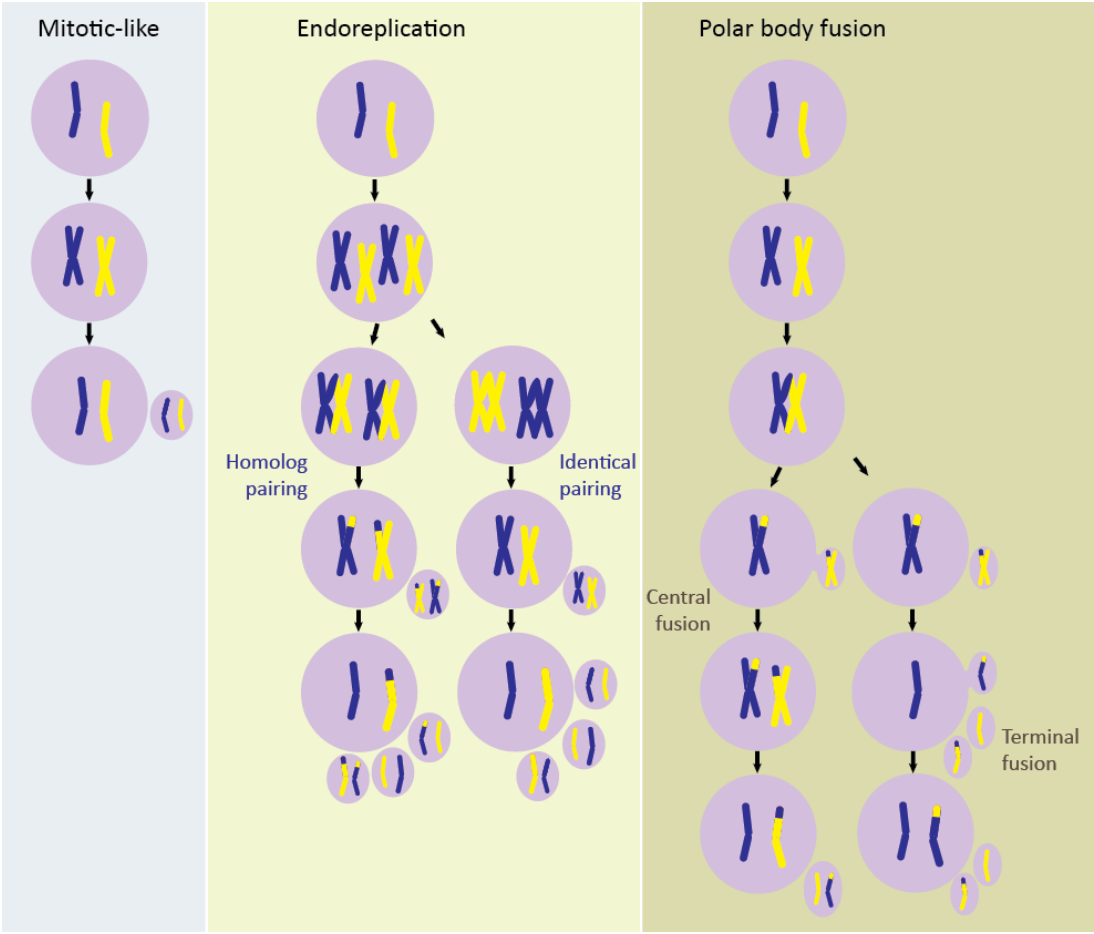



**Fig. 7. Theoretical mechanisms of parthenogenesis.** A mitotic-like division is presumed to take place in Bdelloid rotifers, resulting in maintenance of heterozygosity. It would manifest cytologically by absence of homolog pairing and generation of one or two polar bodies. Endoreplication is characterized by an additional chromosome doubling prior to meiosis. The nature of chromosome pairing would dictate the resulting genetic outcome. Polar body fusion may occur with the first or second polar body and, as in endoreplication, would generate different degrees of heterozygosity depending on the exact nature of the fusion.

including *Aspidoscelis*, are among the organisms in which premeiotic doubling is suspected due to the observed DNA content elevation during meiotic prophase (Bothwell et al., 2010; Cuellar, 1971; White et al., 1963). Prior to our research, premeiotic doubling, and downstream events such as chromosome pairing, had yet to be definitively demonstrated. DNA elevation has been better characterized in the planarian; in some species, the entire germline is elevated by two-fold (Benazzi, 1963).

## Obligate and facultative parthenogenesis

In a Nebraska zoo in 2001, a single newborn hammerhead shark changed the way scientists view reproduction. No males of the same species were housed in the enclosure. It was later confirmed that the pup was produced parthenogenetically by one of three females housed in the aquarium, making it the first confirmed example of parthenogenesis in a shark (Chapman et al., 2007). The combination of a controlled environment (the zoo) and an increased interest in microsatellite analysis has allowed researchers to positively identify cases of sporadic parthenogenesis in recent years. Incidents such as this one demonstrate the reproductive plasticity of animals once thought to be strictly sexual. And it is highly likely that other examples will be revealed in the coming years.

Parthenogenetic species can be subdivided based on their dependence to parthenogenesis, and range from full dependence (obligate) to seasonal (seasonally facultative) to sporadic incidences (facultative). In general, obligate parthenogens retain heterozygosity from generation to generation, whereas facultative organisms, both seasonal and sporadic, appear to lose heterozygosity. Therefore, it is quite likely that the molecular and cellular mechanisms between facultative and obligate parthenogens are distinct.

It is known from facultative parthenogens that some bisexual species can transition to parthenogenesis, but can obligate parthenogens transition to sexuality? In the case of animals that undergo premeiotic doubling, fertilization from a closely related bisexual species can occur, but this is followed by ploidy elevation from the sperm DNA, i.e. a triploid produced from a diploid mother. Therefore, depending on semantics, sexual

reproduction can occur; but true sexual reproduction, in which oocyte DNA content is halved, has yet to be found. Generally, an indicator of cryptic sex within a parthenogenetic population is the presence of fertile males. However, this can be misleading, as demonstrated in the *Darwinulid* ostracods, a controversial ancient asexual clade comprised predominantly of females, but in recent years found to contain a few conspicuous males. While initially exposed as a "sex scandal," it has since been confirmed that males can arise from mutations in the sex determination pathway (Schon et al., 2009). It is currently unknown whether these males are capable of passing on their genes. Schultz noted the correlation between interspecific hybridization, polyploidy, and sexuality: if a parthenogenetic male could produce unreduced sperm and fertilize an unreduced oocyte, the resulting tetraploid could be capable of normal pairing and sexual reproduction could theoretically be restored (Schultz, 1969). This appears to be the case in the minnow *Leuciscus alburnoides*, in which males are fertile and produce diploid sperm (Alves et al., 2001). Thus, even if parthenogenetic species are evolutionary short-lived, they may play an important role in the evolution of sexual species.

## Sperm dependence in unisexual lineages

Many unisexual species, predominantly fish and amphibians, require sperm for egg activation. Some, including *Ambystoma* and *Poeciliopsis*, may even incorporate paternal chromosomes or the entire genome while preserving the maternal complement, utilizing mechanisms known as kleptogenesis and hybridogenesis, respectively. On the other hand, sperm-independence is prevalent among invertebrate unisexuals as none of the putative ancient lineages (rotifers and ostracods) require fertilization (Schurko et al., 2009). The

only exception among vertebrates is Squamata (lizards and snakes), which apparently undergoes *de novo* centrosome synthesis and oocyte activation (Neaves and Baumann, 2011a). Although poorly understood among vertebrates, insights have been made in the arthropods *Drosophila* and *Bacillus*. *De novo* formation of functional centrosomes occurs in the majority of oocytes within a strain of *Drosophila mercatorum* and may explain the parthenogenetic development observed in 5% of oocytes (Kramer and Templeton, 2001; Riparbelli and Callaini, 2003). And in the stick insect *Bacillus*, oocytes bypass the requirement for centrioles during spindle formation, thus assembling components exclusively from maternal origin (Marescalchi et al., 2002). Therefore, a similar mechanism may exist among vertebrates.

Sperm dependence adds to the confusion regarding speciation in unisexual lineages as it necessitates a close relationship with a related sexual species that acts as a sperm donor (Schlupp, 2005). Because fertilization proteins such as the zona pellucida evolve rapidly (Swanson and Vacquier, 2002), the unisexual and bisexual species are linked, constraining the unisexuals in regards to habitat, behavior, etc. In some examples, the unisexual species can be fertilized by another bisexual species, thereby allowing expansion of their habitat (Choleva et al., 2008). Because squamates are the only known vertebrates that display sperm-independent parthenogenesis, they arguably constitute independent lineages and can comprise a species, whereas the sperm-dependence of other vertebrates occludes this possibility (Cole, 1990; Frost and Hillis, 1990). However, some ichthyologists maintain that requirements for sperm activation, especially in gynogens (which do not incorporate paternal DNA), are overshadowed by other species-defining characteristics, such as morphological, ecological, and genetic novelty (Schlupp, 2005).

## Correlation with interspecific hybridization and polyploidy

The correlation between interspecific hybridization and obligate parthenogenesis is striking, especially among vertebrates (Neaves and Baumann, 2011a; Vrijenhoek et al., 1989). Of approximately 80 known taxa of obligate vertebrate parthenogens, only two – both lizard species – have been apparently generated via nonhybrid mechanisms (Sinclair et al., 2010). Obligate parthenogenesis appears to be a means by which hybrids circumvent sterility caused by improper chromosome pairing of homologs. By doubling DNA and allowing identical chromosomes to pair, or dividing through a mitotic-like process, oocytes bypass homolog pairing. Importantly, however, hybridization is not sufficient to create parthenogenetic lineages, as sterile hybrids have been found within asexual-rich genera (Hardy and Cole, 1998; Neaves, 1971). Likewise, hybrids generated under laboratory conditions using presumed parental species have also been sterile, with few exceptions (Cole et al., 2010; Mavarez et al., 2006; Schultz, 1973a). Then why are so many parthenogens of hybrid origin? The answer may lie in the highly heterozygous makeup inherent to interspecific hybrids. As exemplified by the horse-donkey cross, mules are superior to their parental species in both strength and endurance. This process, termed heterosis, or more commonly 'hybrid vigor', is observed across the animal and plant kingdoms (Baack and Rieseberg, 2007; Chen, 2010). Estimates indicate that hybridization has occurred in approximately 10% of animals and 25% of plants, suggesting that it is a significant driving force in speciation (Mallet, 2005, 2007a).

Experiments to test heterosis in lab-synthesized hybrids have yielded conflicting results. In the hemiclonal frog, *Rana esculenta*, F1 hybrids generated in the laboratory exhibited higher survival rates leading up to metamorphosis, faster development rates,

and shorter times to metamorphosis than either parental species (Hotz et al., 1999). This was higher than natural lineages, indicating that the hybridization event itself may have initial advantages. On the other hand, *Poeciliopsis* hybrids exhibited a higher percentage of deformities compared to naturally-formed counterparts, suggesting that the strongest hybrids persist to generate robust lineages (Wetherington et al., 1987a). And in *Heteronotia*, parthenogenetic species displayed equal or inferior attributes compared with sexual counterparts in regards to burst speed and parasitic load (Kearney and Shine, 2004). However, they also displayed greater average head width and greater mass at hatching. Likewise, natural populations of *Poeciliopsis* exhibit greater heat tolerance than their sexual counterparts (Bulger and Schultz, 1982). Taken together, this suggests that phenotypic advantages may be selected for in the hybrids, which allow them to establish their own ecological niche.

Like hybrids, polyploids comprise a large fraction of parthenogens. Approximately two-thirds of vertebrate parthenogens are polyploid (Vrijenhoek et al., 1989). For some hybrids, with numerous examples in plants, polyploidy allows for successful homolog pairing and a persistence of sexual reproduction. In these examples, autopolyploidy (ploidy elevation within one species) may coincide with hybridization. For others, including many vertebrates, a frequently observed phenomenon is allopolyploidy (different parental genomes comprise the extra ploidy levels); in fact, some parthenogenetic diploids are outnumbered by their triploid relatives. The advantages for polyploidy in these species is unknown, but elevated DNA content may result in greater phenotypic variability needed for niche establishment. Alternatively, the extra genome, which is highly redundant, may lighten the load on Muller's ratchet.

## Mitosis vs. Meiosis

Parthenogens reproduce either through a mitotic mechanism (often referred to as apomixis) or through a modification of the meiotic program (see Table 1). Among vertebrates, there are no known examples of mitotically produced gametes, and all known vertebrates have retained the basic meiotic program in some way. This may be a reflection of a specie's longevity. Organisms such as rotifers and Darwinulid ostracods, which are thought to reproduce mitotically, are regarded as ancient lineages, existing for approximately 35 and 70 million years, respectively (Judson and Normark, 1996). In these animals, evidence for meiotic genes is sparse, indicating that these genes may have devolved after years of disuse. However, the meiotic characteristics that influence reproduction in these ancient lineages remain unknown.

The extent to which ancient asexuals retain properties of oogenesis may have important implications in aging. Angelika Amon's group recently reported a correlation between meiosis and replicative life span in yeast, demonstrating that mitotically-dividing strains yielded lower survival rates compared to those that underwent sporulation (Unal et al., 2011). Presumably, cellular damage is repaired during this process. The group also showed that expressing the meiosis-specific transcription factor *ndt80*, life span could be restored in aged cells without the process of meiosis. The significance of this mechanism in higher organisms is unknown. Do ancient asexuals retain any aspects of oogenesis that reset the replicative lifespan of their offspring? Is there another mechanism by which they achieve this rejuvenation? Because rotifers undergo egg formation, we know that at least some oogenesis processes must be retained.

| | Clade | Asexual mode | Modifications to meiosis | Offspring heterogeneity* | Sperm needed? | Additional info | References |
|---|---|---|---|---|---|---|---|
| Invertebrates | ***Bdelloidea*** (rotifer) | Ob, Fac | Thought to be a mitotic-like process | Identical to mother | N | *Bdelloidea* is an Order that includes several ancient unisexual Families | (Birky and Gilbert, 1971) |
| | ***Ostracoda*** | Ob | Probably mitotic-like | Identical | N | Status as ancient lineage is debated due to the discovery of males | (Martens et al., 2003; Smith et al., 2006) |
| | ***Dugesia*** (planarian) | Ob | Ploidy of entire germline doubles | Identical | N | *Dugesiidae* is a Family consisting of parthenogenetic and sexual lineages | (Benazzi, 1963) |
| | ***Daphnia*** | Fac – seasonal | Failed cytokinesis at first meiotic division | Identical | N | | (Hiruta et al., 2010) |
| | ***Potamopyrgus*** (snail) | Ob | Unknown | Identical | N | 2 lineages estimated to be near 500,000 years old | (Neiman et al., 2005; Weetman et al., 2002) |
| | ***Timema*** (walking sticks) | Ob | Unknown | Identical | N | Females capable of asexuality through homozygosing mechanism | (Schwander and Crespi, 2009) |
| Vertebrates | ***Poeciliopsis*** (fish) | Ob | Premeiotic doubling **1)** sperm DNA incorporated **2)** or not | **1)** maternal identical **2)** identical | Y | Diploid species incorporate paternal DNA, triploid do not | (Cimino, 1972; Lampert et al., 2007; Schultz, 1969) |
| | ***Sphyrna*** (shark) | Fac – sporadic | Unknown | Homozygous | ? | Evidence for sperm dependence unclear | (Chapman et al., 2007; Holtcamp, 2009) |
| | ***Ambystoma*** (salamander) | Ob | Premeiotic doubling. Paternal chromosomes may be included (see text) | Identical | Y | Regarded as the oldest lineage of vertebrates (~5 million years old). | (Bi and Bogart, 2010; Bogart et al., 2007; Hedges et al., 1992; Lampert and Schartl, |
| | ***Aspidoscelis*** | Ob | Doubling prior to meiosis | Identical | N | Comprised of bisexual and unisexual species | (Cuellar, 1971; Lutes et al., 2011; Lutes et al., 2010) |
| | ***Heteronotia*** (gecko) | Ob | Probably similar to *Aspidoscelis* | Identical | N | Genus comprised of bisexual and unisexual | (Moritz et al., 1989a) |
| | ***Varanus*** (Komodo ) | Fac – sporadic | Unknown, possible polar body fusion | Homozygous | N | | (Watts et al., 2006) |
| | ***Meleagris*** (turkey) | Fac – sporadic | Unknown | Possible homozygous | N | All embryos died; Offspring gender was male in all cases | (Olsen and Marsden, 1954) |

**Table 1**: **Characteristics of various unisexual animals.** Ob = Obligate, Fac = Facultative. *as demonstrated through genetic analysis, barring spontaneous mutations.

By looking at the extent to which meiotic genes have diverged, we may gain insights into the advantages of key meiotic genes, independent of gamete formation.

## The vertebrate ovary

The body is highly resourceful, and several organs can be equated to a highly efficient factory, producing high volumes of their specialty – blood, filtered urine, or sperm, for example. On the other hand, the vertebrate ovary can be likened to a nursery. Relatively inefficient, but with high quality control, the ovary houses very few oocytes that will be used in fertilization. The amount of energy invested into each useful cell is relatively high compared to other organs. Indeed, there is a high rate of follicular atresia in the ovary such that women, upon reaching puberty, contain only a portion of the oocytes with which they began. The vertebrate ovary consists of two basic types of cells: (1) germ cells that will rise to become oocytes, and (2) somatic cells that either provide support to the organ or nourishment to the germ cells. Most oocytes arrest in the primordial state and reside in the ovary cortex in the diplotene stage of meiosis. Through an unknown mechanism, some oocytes selectively grow larger than neighboring germ cells. These large diplotene-stage oocytes resume the cell cycle after a hormonal response from luteinizing hormone and undergo germinal vesicle breakdown before arresting again prior to fertilization (for review, see (Zhang, 2012)).

Molecular analysis in the vertebrate ovary is best described in mouse, and to a lesser extent in chicken. In higher vertebrates, oogonia divide mitotically until meiosis is initiated via a wave-like mechanism involving retinoic acid, which acts as a morphogen

(Bowles et al., 2006; Bullejos and Koopman, 2004). The speed with which meiosis is initiated may be facilitated by cytoplasmic bridges between oogonia. These connections are created by incomplete cell divisions and intimately bind groups of oogonia descended from a single progenitor. Just prior to meiosis-specific gene expression, DNA replication takes place: at approximately 12.5 dpc in mouse (Lima-De-Faria and Borum, 1962) and embryonic day 14.5 in chicken (Callebaut, 1967). A widely favored – and highly controversial – hypothesis is that all oogonia within mammals initiate meiosis well prior to sexual maturity. Suggesting that infertility is nonreversible in women, this topic has important health implications. Several years ago, neo-oogenesis, the reprogramming of somatic cells into oocytes, had been claimed in adult mammals (Johnson et al., 2005; Johnson et al., 2004); however, it has since been refuted (Eggan et al., 2006).

### Oogenesis in reptiles and other lower vertebrates

Physiologically similar to the higher vertebrates, oogenesis in lower vertebrates varies in several aspects. Perhaps of most importance is that lower vertebrates continually engage a subset of oogonia to enter meiosis, beyond birth and into adulthood (Andreuccetti et al., 1990; Nakamura et al., 2010). This process takes place in the cortex of the germinal bed, an oocyte reserve attached to the dorsal side of the ovary. As a result of germ cell differentiation, germinal beds are absent in adult chickens and mammals. On the other hand, ovaries among lower vertebrates evoke only a fraction of oogonia to initiate meiosis at any given time, leaving a population of mitotic germ cells in the adult (Andreuccetti et al., 1990; Arronet, 1973; Guraya, 1989). One known exception is the

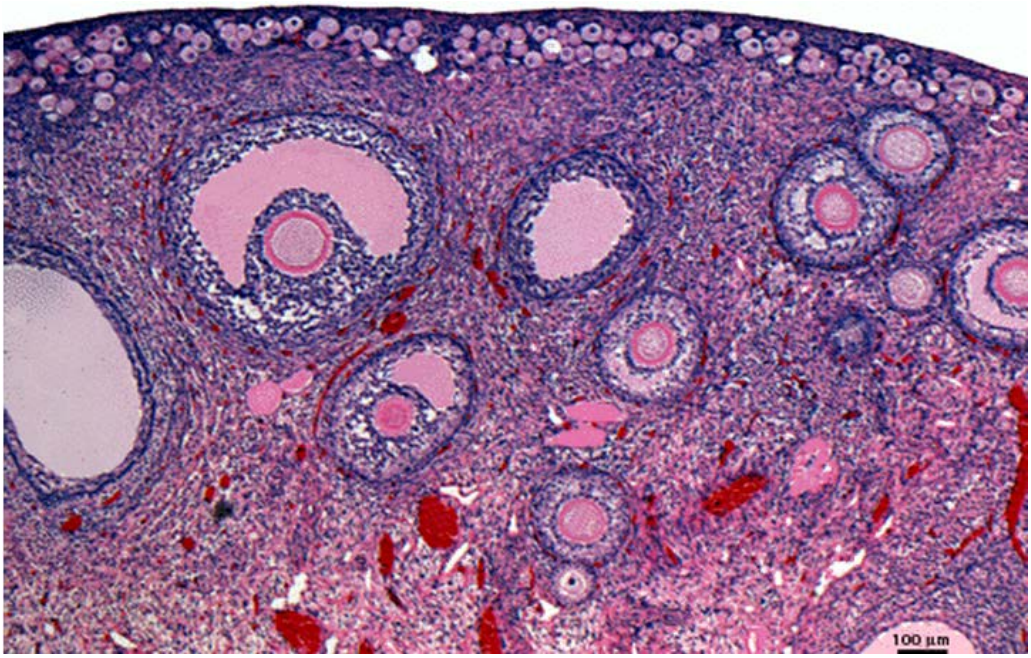



**Fig. 8. Cortex of mammal ovary.** A small number of oocytes progress through meiosis. Several primary and secondary follicles can be seen in this section. Note the large number of primordial oocytes on the periphery of the tissue (Hill, 2011).

tuatara, *Sphenodon punctatus*, whose oogonial population appears to completely commence meiosis prior to hatching.

And just as differences exist between the two groups of vertebrates, some characteristics vary greatly even among amphibians, fish and reptiles. Follicle number and follicle size are two such examples. In many lizard species, a single ovulated egg is common, whereas *Xenopus laevis* produces hundreds of follicles. Egg size ranges from 0.5 mm in the diminutive Southeast Asian cyprinid fish, *Paedocypris*, to 30 cm in the enormous whale shark, *Rhincodon typus* (Compagno, 1984; Kottelat et al., 2006). The follicle size prior to vitellogenesis also differs, and has practical applications in experimental biology. Larger oocytes are found in egg-laying animals, allowing for easier

visualization of nuclei and chromosomes. Thus, some lower vertebrates are more amenable to chromosome dynamics studies.

Important similarities exist among most vertebrates, including the round shape of the nuclei and the clear cytoplasm of young oocytes. In fact, many of the mechanisms within the oocyte are conserved. The histology of the primary, secondary and tertiary follicles is strikingly similar between animals. And as in higher vertebrates, intercellular bridges between oogonia have been found in lizards, suggesting that reptilian oogonia undergo synchronous development as well (Filosa and Taddei, 1976). It should also be noted that meiosis initiates during embryogenesis in many lower vertebrates, as is the case in mouse and chick (Guraya, 1989).

As it pertains to parthenogenetic vertebrates, what modifications might be expected in oogenesis? Unless a mitotic-like mechanism occurs, which would involve a single cell division, ploidy must increase during oogenesis. At what stage might this occur? One hypothesis suggests that cells double at the onset of meiosis; however, there is no precedence for this. On the contrary, one study of parthenogenetic loaches suggests that the ploidy increase may occur long before meiosis. In the study, the research group took advantage of sex-reversal capabilities in loaches and generated males from unisexual female lineages. The advantage is that males produce greater numbers of germ cells, making cells easily amenable to ploidy analysis. Interestingly, they found that a subset of spermatogonia contain enlarged nuclei, consistent with the hypothesis that ploidy increases long before meiosis (Yoshikawa et al., 2009). Ploidy increase prior to meiosis is not exclusive to fish. Surprisingly, it is not even exclusive to unisexual species. In some sexually-reproducing snakes, mitotically-replicating oogonia undergo an additional DNA

doubling to produce 8C cells and subsequently divide twice to restore the diploid state (Becak et al., 2003). This illustrates the fluctuation in oogonial mitosis and suggests that if meiosis is modified by an additional doubling event, it may occur well prior to meiosis in *Aspidoscelis* and other unisexual lineages.

### *Oryzias* hybrids

Comparing *Aspidoscelis* with other hybrids may prove informative not only to the reproductive biology of lizards, but also of vertebrates in general. Unfortunately, many hybrids are not viable, occluding studies with model organisms such as *Danio*. However, in the genus *Oryzias* (common name ricefishes), approximately 30 species exist, and many combinations readily form viable offspring (Iwai et al., 2011). Particularly useful is the cross between *O. latipes* and *O. curvinotus*, which develops normally, but undergoes an aberrant meiosis. Both sexes are affected, but in vastly different ways. Male spermatogenesis lacks cell divisions. The majority of sperm-like cells are enlarged, display little movement, and contain 4C DNA content (Shimizu et al., 1997).

In the female hybrids, on the other hand, most oocytes do not proceed through meiosis. Instead, these cells arrest during zygotene, the pairing stage in meiosis. Interestingly, it appears that a small number of oocytes are able to successfully complete meiosis and generate diploid eggs (Iwai et al., 2011). The authors suggest endomitosis as the mechanism responsible for ploidy elevation, however the exact pathway remains to be found (Shimizu et al., 2000). The study also suggests that oogenesis contains more checkpoints to prevent progression of oocytes with unpaired chromosomes compared with spermatogenesis. It is as yet unknown whether such ploidy elevation is exclusive to

hybrids or a common occurrence among *Oryzias*. These phenomena may have widespread implications in other vertebrate hybrids.

### Unsuccessful *Aspidoscelis* hybrids

While many robust parthenogenetic lineages exist in *Aspidoscelis*, it is the analysis of unsuccessful crosses that may provide mechanistic clues into parthenogenesis. Although molecular tools are not as readily available in *Aspidoscelis* as in *Oryzias*, histological analyses have contributed significant observations. For example, gross examination of the sterile tetraploid hybrid *A. sonorae* / *A. tigris* revealed small ovaries, empty or fluid-filled follicles, and an overall disorganization of the germinal bed (Hardy and Cole, 1998). Additionally, the follicles displayed an obvious lack of vascularization. Surprisingly, the mesonephric tubules were similar in size to the paternal, rather than maternal, species. Indeed, male features may be common to female hybrids; an apparent female formed from an *Aspidoscelis inornata arizonae* and *A. tigris marmorata* exhibited male-like features, including a mesonephros that had epididymal morphology (Cole et al., 2010). This phenotype was especially perplexing because *A. inornata* and *A. tigris* are the parental species of the successful parthenogenetic lineage *A. neomexicana*, although the maternal and paternal species were reversed in this cross (Fig. 1). Even more confusing was the *A. tigris* / *A. tesselata* cross, which showed no propensity toward maleness but likewise yielded empty follicles (Taylor et al., 2001). These observations support the notion that parthenogenetic lineage establishment is rare. Also, the ease with which hybrids can be generated is more successful than in *Oryzias* where certain crosses exhibit severe aneuploidy defects in embryogenesis (Sakai et al., 2007).

## Why study parthenogenesis? And why use lizards?

The first question can be divided into two components: **(1)** Why does sexual reproduction predominate? and **(2)** What prevents parthenogenetic species from spontaneously arising? The first question is one that perplexed Darwin himself, and it stands as one of the most outstanding in evolutionary biology (Charlesworth, 2006). Meiosis, and essentially sexual reproduction, is intimately correlated with the earliest known eukaryotes, and no known examples have arisen without it. Thus, sex is a defining attribute of eukaryotes. To fully analyze its importance we must turn to those animals in which sex has been lost. As with many scientific problems, it is advantageous to study an organism that has taken the research topic to an extreme. In this case, the Bdelloid rotifers and Darwinulid ostracods, which have seemingly reproduced without sex for millions of years, are ideal organisms for study. By learning how these species have survived, and even thrived, we can understand why the majority of species cannot.

The second question, concerning the ease of parthenogenetic transition, is one best suited for the developmental biologist. Obviously, barriers limit the prevalence of asexual reproduction; otherwise parthenogenetic individuals would continually arise. Imprinting and sperm-dependent fertilization are among the explanations for absence of spontaneous asexuality in vertebrates. However, even among reptiles, which do not appear to have such fertilization requirements, the prevalence of asexuality is extremely low. To address this topic, the ideal organism of study would be a clade in which numerous parthenogenetic lineages have arisen relatively recently. While several parthenogens fall in this category, parthenogenetic whiptail lizards are excellent study animals because they will yield information regarding vertebrate oogenesis. Additionally, closely related

bisexual species, i.e. the parental species, can be utilized in control experiments. Thus, in our studies, we will gain unique insights into reptilian reproduction, parthenogenesis, and vertebrate oogenesis.

## Conclusion

For many species, parthenogenesis appears to be a short-lived fate. Many obstacles, including an inability to purge deleterious mutations and parasitic susceptibility, apparently contribute to their demise. Exceptional parthenogens have been found – namely the Bdelloid rotifers and Darwinulid ostracods – which appear to have persisted for millions of years without sex. As it clearly harbors disadvantages, mechanisms such as imprinting and paternal centrosome inheritance provide measures to ensure inheritance of the paternal genome. However, even within genera that do not possess these barriers, the prevalence of parthenogenesis is still quite low. To find other preventative measures that may be in place, we must study organisms in which parthenogenesis has recently, and relatively frequently, arisen.

*Aspidoscelis* is an ideal organism for these studies. Within the genus, parthenogenetic species – formed from the hybridization between different bisexual species – exist alongside their parent species, many of which are known. As a vertebrate, knowledge of oogenesis can be related to higher vertebrates, including mammals. Also, parthenogenesis in these reptiles is sperm-independent, allowing for feasibility of husbandry. Lastly, the large, clear germinal vesicles within these animals serve as an excellent resource for the study of chromosome dynamics in meiosis.

Meiosis is a conserved process within each sex that halves chromosome number and upon fertilization, ploidy is restored. Despite the apparent conservation, several organisms reproduce sexually with obvious aberrations, demonstrating a moderate amount of plasticity to the mechanism. In unisexual lineages, meiosis must be modified such that 2C gametes are produced. Possible ways in which this could theoretically be achieved include a mitotic-like division, two rounds of DNA replication, and an aborted cell division. In most parthenogens, the cellular mechanisms have yet to be fully understood.

Many hybrids are sterile, illustrating the disruption created by the union of distinct genomes, not only in the germline, but also in somatic cells. For parthenogens in which reproduction has been studied, reproduction appears to manifest as a modification of the normal meiotic program, proceeding in a quasi-normal manner. However, the number of organisms for which it is well studied is few.

In the following chapters, two studies in *Aspidoscelis* will be presented. Chapter 2 describes the cellular mechanism responsible for parthenogenesis in *A. tesselata* and *A. neomexicana* and the consequences of the meiotic modification. Chapter 3 investigates meiosis in a new, fertile, tetraploid hybrid generated in the laboratory from a cross between a triploid parthenogenetic mother and a male from a bisexual species. Chapter 4 places these studies in context within the field and provides future prospects for the study of parthenogenesis.

# **Chapter 2:** Sister chromosome pairing maintains heterozygosity in parthenogenetic lizards

## Summary

Although bisexual reproduction has proven to be highly successful, parthenogenetic all-female populations occur frequently in certain taxa including the whiptail lizards of the genus *Aspidoscelis*. Allozyme analysis revealed a high degree of fixed heterozygosity in these parthenogenetic species (Neaves, 1969b; Neaves and Gerald, 1968b) supporting the view that they originated from hybridization events between related sexual species. It has remained unclear how the meiotic program is altered to produce diploid eggs while maintaining heterozygosity. Here we show that meiosis commences with twice the number of chromosomes in parthenogenetic versus sexual species, a mechanism that provides the basis for generating gametes with unreduced chromosome content without fundamental deviation from the classic meiotic program. Our observation of synaptonemal complexes and chiasmata demonstrate that a typical meiotic program occurs and that heterozygosity is not maintained by bypassing recombination. Instead, fluorescent *in situ* hybridization probes that distinguish between homologs reveal that bivalents form between sister chromosomes, the genetically identical products of the first of two premeiotic replication cycles. Sister chromosome pairing provides a mechanism for the maintenance of heterozygosity, which is critical for offsetting the reduced fitness associated with the lack of genetic diversity in parthenogenetic species.

## Introduction

True parthenogenesis, characterized by the complete absence of male contributions, has been described for various species of reptiles including whiptail lizards, geckos, blind snakes and rock lizards (Vrijenhoek et al., 1989). Whiptail lizards of the genus *Aspidoscelis,* formerly part of the genus *Cnemidophorus* (Reeder et al., 2002b), are mostly native to the Southwestern United States and Mexico, and about one-third of the more than 50 species reproduce by obligate parthenogenesis.

Morphological, karyotypic and biochemical studies provided strong evidence for hybrid origins of all parthenogenetic *Aspidoscelis* species examined (Dessauer and Cole, 1986; Lowe and Wright, 1966; Neaves, 1969b). While hybridization between individuals from distinct species can explain the initially high degree of heterozygosity across the genome, allozyme analysis demonstrated surprising persistence of heterozygosity over many generations in several parthenogenetic lineages of *Aspidoscelis*, including *A. tesselata and A. neomexicana* (Dessauer and Cole, 1986). The observation that individuals within a parthenogenetic species can exchange skin grafts (Cordes and Walker, 2003, 2006; Cuellar and Smart, 1977; Maslin, 1967) and biochemical studies on several lab-reared lineages (Dessauer and Cole, 1986) further supported genetic uniformity.

The mechanism that underlies clonal reproduction and fixed heterozygosity has been the topic of much speculation. Most variations of the meiotic program that would produce diploid oocytes by skipping a division or by fusion of a haploid oocyte with a polar body cannot account for fixed heterozygosity unless recombination between homologs is suppressed. Based on the exclusion of alternative models and the observation of large

numbers of chromosomes in two oocytes from *A. uniparens* (Cuellar, 1971), premeiotic endoreplication of chromosomes was proposed as the most likely mechanism to produce mature oocytes that carry the complete complement of somatic chromosomes and maintain heterozygosity (Neaves, 1971). To test this hypothesis we set out to quantify the DNA content in oocytes of the diploid parthenogenetic species *A. tesselata* and the sexually reproducing control *A. gularis*. Extant *A. gularis* and *A. tigris* are closely related to the individuals that hybridized to generate the founding specimen of *A. tesselata* (Dessauer and Cole, 1989; Neaves, 1969b; Neaves and Gerald, 1968b; Parker and Selander, 1976).

## Results

### Chromosomal doubling occurs in oocytes from parthenogenetic *A. tesselata*

We isolated germinal vesicles (GVs), the oocyte nuclei, and visualized 4',6-diamidino-2-phenylindole (DAPI) stained chromosomes by two-photon microscopy. Bivalents were readily observed in GVs from *A. gularis* and *A. tesselata*, and their morphology was consistent with the diplotene stage of prophase I (Fig. 1a, b). Visual inspection of three dimensional reconstructions of seven *gularis* and five *tesselata* GVs revealed a larger number of bivalents in *tesselata* GVs compared to *gularis*. Ambiguities in identifying the boundaries of individual chromosomes prevented accurate counting of bivalents at this stage. Instead, we quantified the volume occupied by chromosomes in each GV as an indirect measure of DNA content (Fig. 2c; Table 1). Unlike measurements of fluorescence intensity, this approach is robust against changes in

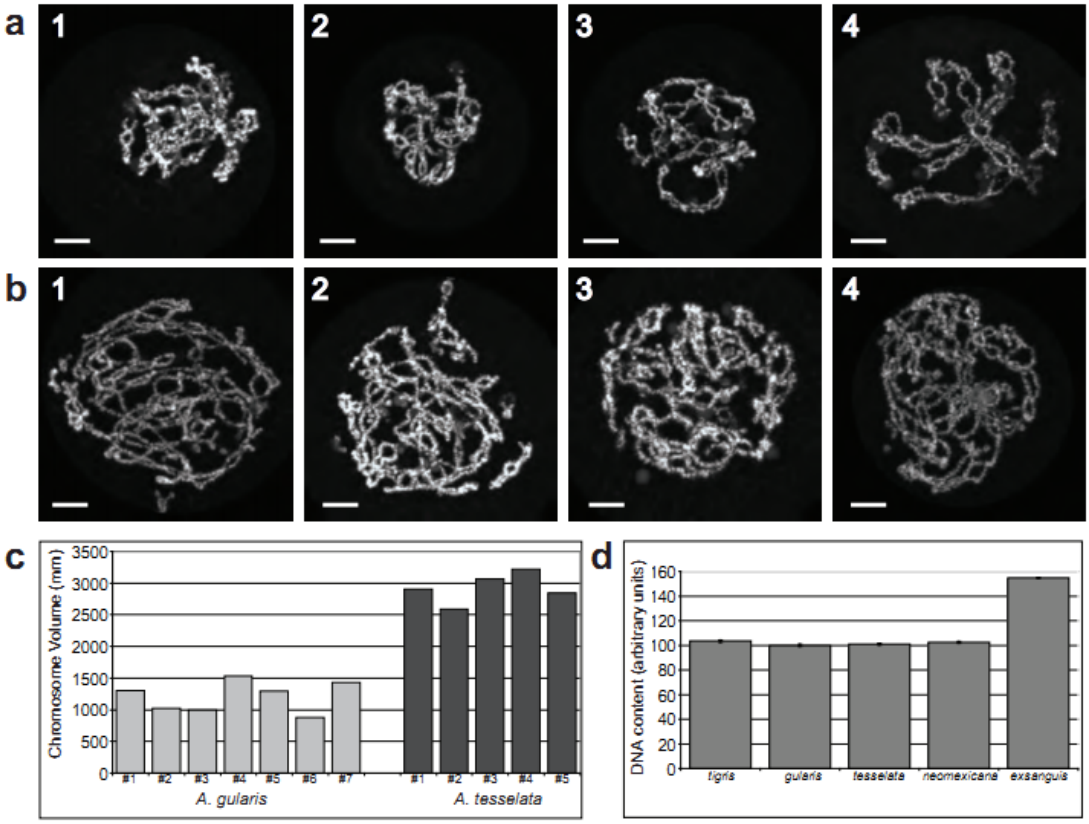



**Figure 1. Oocytes from parthenogenetic *A. tesselata* contain twice the amount of chromosomal DNA compared to sexual *A. gularis*. (a)** DAPI-stained chromosomes in germinal vesicles (GVs) from *A. gularis*. 3D projections of four GVs are shown, details on size and quantifications are available as Table 1. Scale bars correspond to 10 µm. **(b)** GVs from *A. tesselata*. **(c)** Quantification of chromosome volumes. **(d)** Quantification of DNA content in somatic cells by flow cytometry. Fluorescence intensities from biological triplicates of ~50,000 cells were averaged and normalized against samples from *A. gularis* which was set at 100 to facilitate comparison.

Table 1. DNA Quantification

| GV | Diameter (µm) | Chromosome Volume (µm$^3$) | Chromosome Volume Ratio |
|---|---|---|---|
| gularis #1 | 71 | 1304 | 1.00 |
| gularis #2 | 63 | 1023 | 0.79 |
| gularis #3 | 71 | 995 | 0.76 |
| gularis #4 | 77 | 1535 | 1.18 |
| gularis #5 | 91 | 1296 | 0.99 |
| gularis #6 | 57 | 878 | 0.67 |
| gularis #7 | 65 | 1430 | 1.10 |
| tesselata #1 | 77 | 2901 | 2.23 |
| tesselata #2 | 81 | 2585 | 1.98 |
| tesselata #3 | 99 | 3069 | 2.35 |
| tesselata #4 | 62 | 3216 | 2.47 |
| tesselata #5 | 99 | 2844 | 2.18 |

staining efficiency or laser intensity fluctuations. *A. tesselata* chromosomes occupied 2.24 +/-0.18 fold the volume of the averaged *A. gularis* samples. While indicative of a two-fold increase in the DNA content of the prophase oocyte in the parthenogenetic

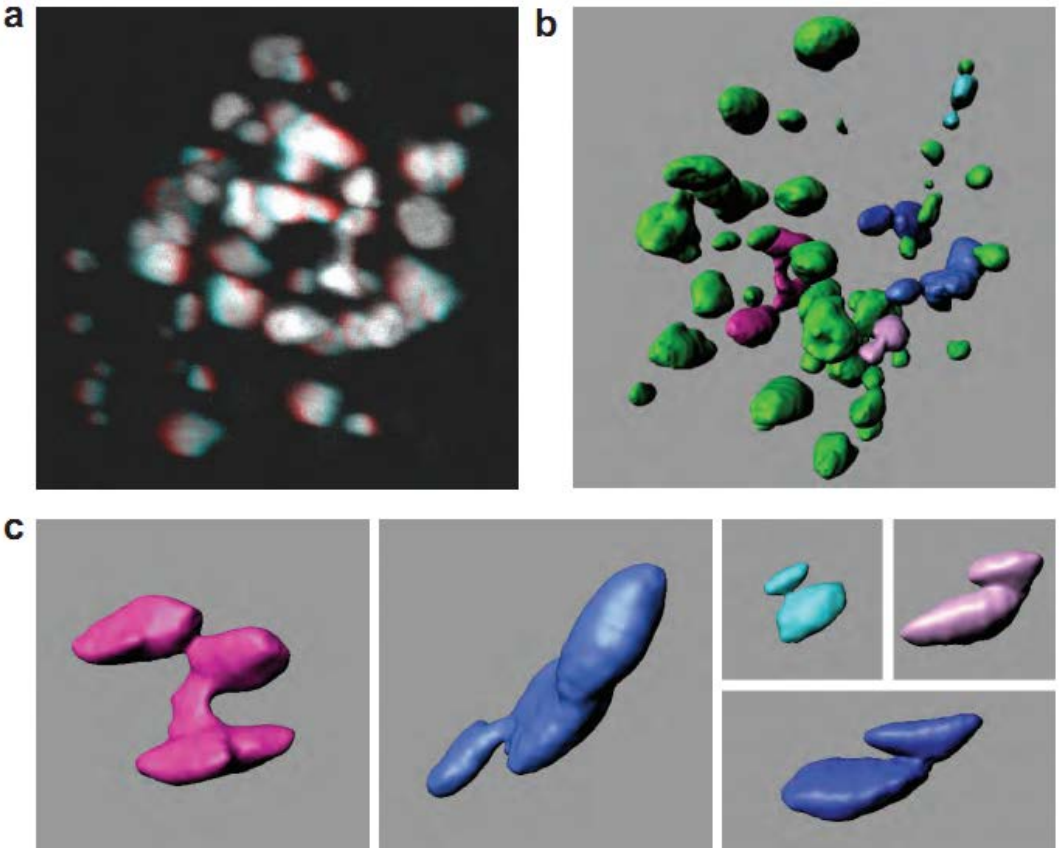



**Figure 2. Visualization of highly condensed bivalents from *A. tesselata* GVs in late prophase of meiosis I.** DAPI-stained samples were imaged by two-photon microscopy using a Carl Zeiss long working-distance C-Apochromat 40x N.A. 1.1 objective. **(a)** Stereo 3D projection of the chromosomes from a late prophase GV (265 µm diameter). Scale bar corresponds to 5 µm. **(b)** Iso-surface rendering of the chromosomes shown in (a) using the software package IMARIS 6.3 (Bitplane). An animation of the rendered chromosomes is available as download as Suppl. Fig. 1d. In this sample 35 bivalents were readily identifiable as isolated objects. **(c)** One object corresponding to three bivalents in close proximity and four objects corresponding to two bivalents each brought the total number of bivalents to 46, consistent with the number expected if premeiotic endoreplication had occurred. A minimum of 40 clearly discernable bivalents were counted in two additional *A. tesselata* GVs of similar size. Physical proximity between some bivalents prohibited definitive identification of all 46 bivalents in these samples.

species, differences in genome size could also account for the increased chromosome volume.

Although somatic cells from *A. gularis* and *A. tesselata* both harbor 46 chromosomes (Wright and Lowe, 1967), a direct comparison of genome sizes was needed to inform our analysis. Taking advantage of the fact that reptilian erythrocytes are nucleated, we subjected blood samples to flow cytometry analysis and found that the nuclear DNA content in somatic cells differed by less than 1% between the two species (Fig. 1d). For comparison, samples from sexual diploid *A. tigris* and parthenogenetic triploid *A. exsanguis*[*] were also analyzed, with the latter showing an approximately 50% increase in DNA content as expected (Fig. 1d). Independent confirmation for a doubling in chromosome number was obtained by examining GVs in late prophase. At this stage chromosomes are highly condensed and consistent with a doubling in chromosome number, and we were able to distinguish 46 bivalents in *A. tesselata* GVs (Fig. 2).

## Maintenance of recombination

Entering meiosis with an 8n chromosome complement would allow parthenogenetic animals to utilize the two normal meiotic divisions to generate diploid gametes. However, the long-term maintenance of heterozygosity across the genome is only ensured if cross-overs between homologs are suppressed. Two-photon imaging of diplotene chromosomes from *A. tesselata* and another parthenogenetic species, *A. neomexicana*, revealed no differences compared to sexual controls besides the increased DNA content. Notably,

[*] *A. exsanguis* is the product of two consecutive hybridization events involving the sexual species *A. inornata*, *A. burti* and *A. gularis*[13].

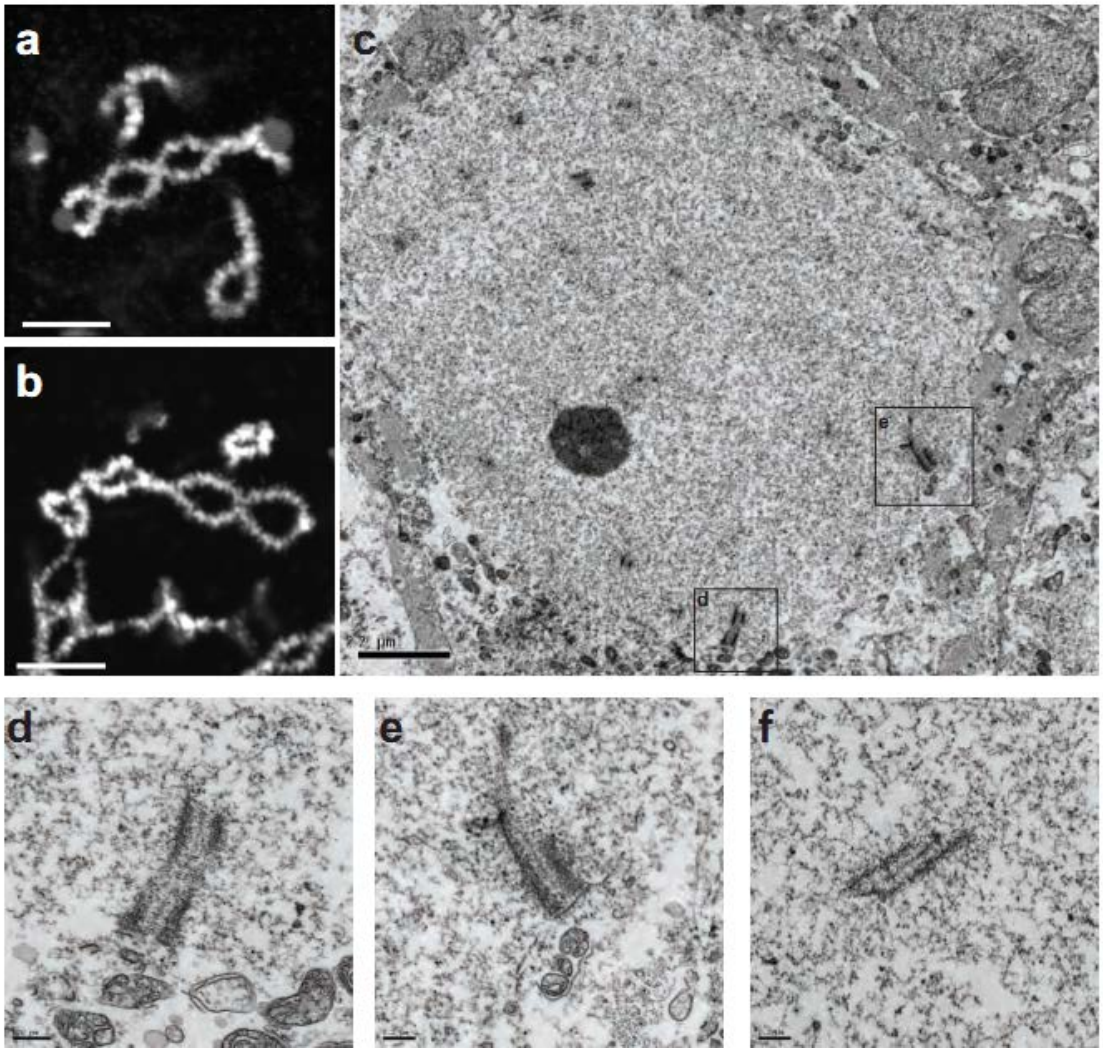



**Figure 3. Visualization of chiasmata and synaptonemal complexes in parthenogenetic *A. tesselata* and sexual *A. tigris*.** Projection of bivalents from *A. tigris* **(a)** and *A. tesselata* **(b)** GVs in diplotene. Scale bars correspond to 10 µm. **(c)** EM image of *A. tesselata* GV in pachytene. Several sections of synaptonemal complexes are visible. Scale bar corresponds to 2 µm. Close-ups for two areas are shown in **(d)** and **(e)**. A close-up of a SC from *A. tigris* is shown in **(f)**. Scale bars correspond to 200 nm.

bivalents appeared to be connected by chiasmata in all samples, indicating that crossing-over is not abandoned (Fig. 3a, b).

To further examine chromosome pairing, thin sections of ovaries from *A. tesselata*, *A. tigris*, and *A. neomexicana* were examined by electron microscopy. Synaptonemal complexes (SCs), characterized by well-defined lateral and central elements, were observed in all species examined providing further support that a typical meiotic program is underway (Fig. 3c to f, Fig. 4). Based on the presence of SCs in pachytene and chiasmata in diplotene, we surmise that meiotic chromosome pairing and recombination are not bypassed in parthenogenetic *Aspidoscelis* species.

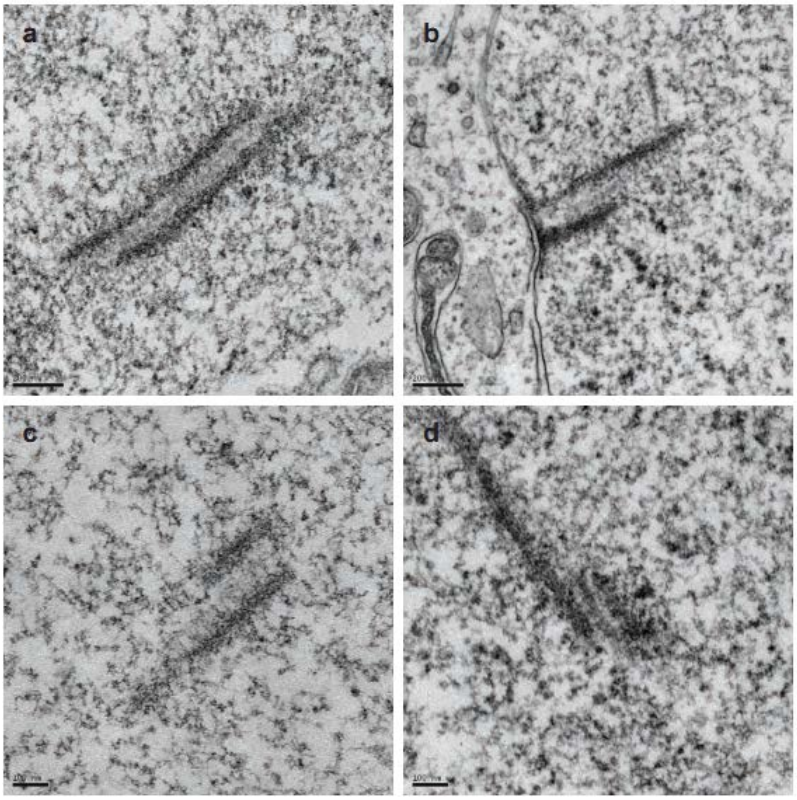


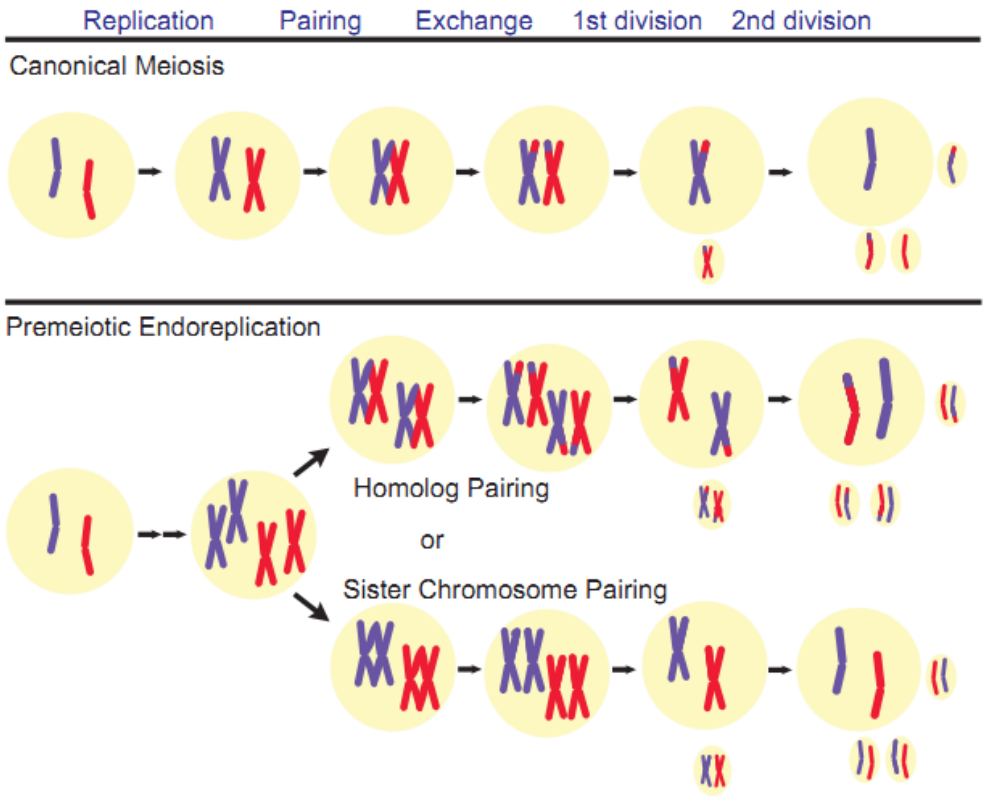

**Figure 4. Synaptonemal complexes visualized by electron microscopic examination of thin sections from *A. neomexicana* oocytes.** Scale bars correspond to 200 nm in **(a)** and **(b)** and to 100 nm in **(c)** and **(d)**.

## Possible scenarios for chromosome pairing

The premeiotic doubling of chromosomes allows for bivalent formation to occur either between homologs as in normal meiosis or between sister chromosomes (Fig. 5).

To distinguish between these possibilities we sought to identify probes that selectively recognize one particular chromosome in a pair of homologs. We discovered that 26 of the 46 *A. tigris* chromosomes, including all 22 macrochromosomes and 4 microchromosomes, harbor large tracks of internal telomeric repeats in addition to the signal at chromosome ends (Fig. 6a). In contrast, staining metaphases of *A. inornata* chromosomes with a telomeric protein-nucleic acid probe only revealed signal at the chromosome termini (Fig. 6b). Consistent with its hybrid origin from these two sexual species(Lowe and Wright, 1966; Neaves, 1969b; Neaves and Gerald, 1968b), *A. neomexicana* chromosomes contained large internal repeats on 13 chromosomes, allowing us to unambiguously identify 13 chromosomes inherited from *A. tigris* in the original F1 hybrid (Fig. 6c). In the context of a bivalent, hybridization signals on both sides indicates sister chromosome pairing, whereas hybridization on only one side supports homolog pairing.

**Figure 5.** Meiosis in sexual and parthenogenetic *Aspidoscelis* species. In normal meiosis a single round of DNA replication is followed by two consecutive divisions that result in a haploid gamete and three polar bodies. Homologs are shown in red and blue. Recombination generates chimaeric chromosomes. Premeiotic doubling of chromosomes allows for pairing of homologous or sister chromosomes. Homolog pairing and recombination result in some loss of heterozygosity in the mature oocyte. Recombination between pairs of sister chromosomes maintains heterozygosity at all loci.

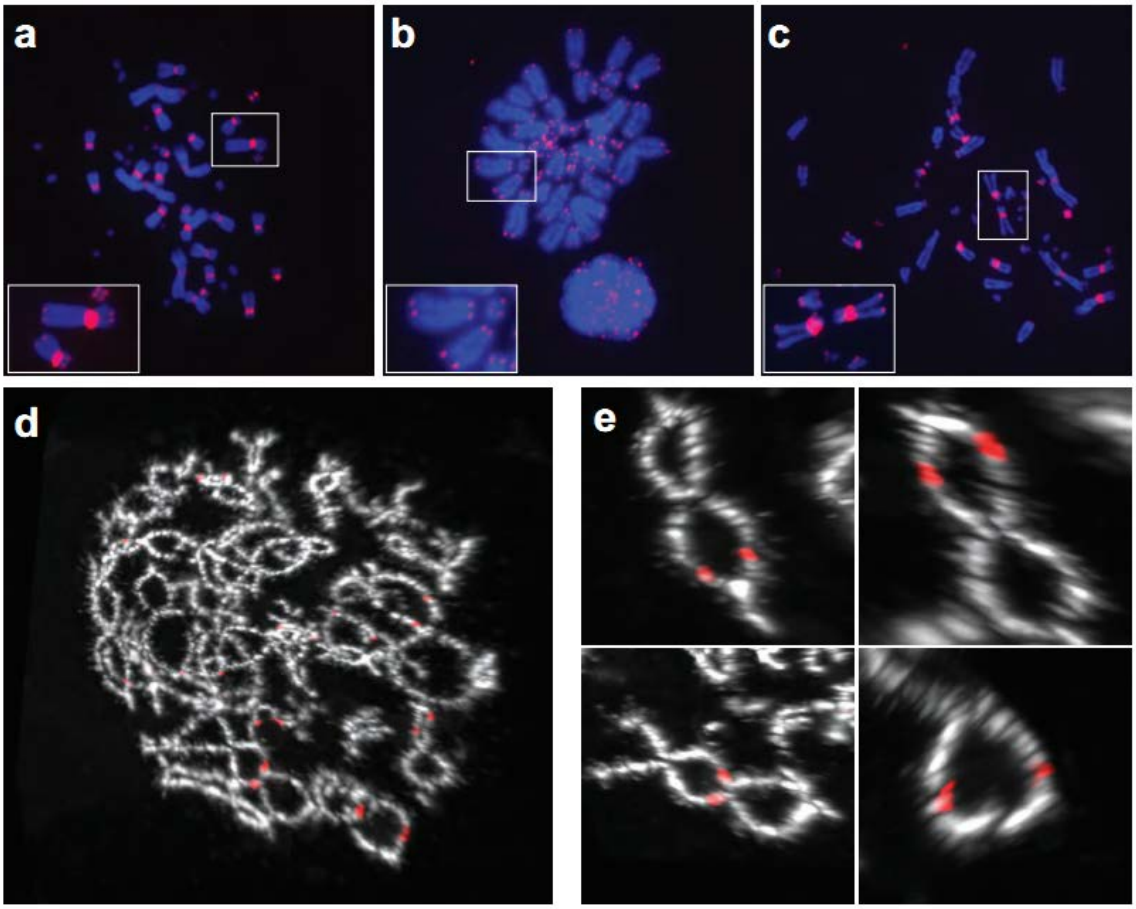



Pairing occurs between sister chromosomes rather than homologs

To preserve the three-dimensional arrangements of chromosomes in GVs and to provide better spatial resolution than commonly obtained in chromosome spreads, we adapted the FISH procedure to perform hybridization on intact GVs. At each site where chromosome internal hybridization was detected, a signal was observed on both sides of the bivalent (Fig. 6d, e). It is important to note that sister chromatids resulting from the most recent round of replication appear as one cytologically, as they are closely associated with each other along their length during this stage of meiosis. The exclusive

presence of paired hybridization signals therefore strongly suggests that bivalents are composed of sister chromosomes, not homologs. Based on this experiment, we concluded

**Figure 6. Internal telomeric repeats distinguish homologs in *A. neomexicana* and demonstrate sister chromosome pairing. (a)** Fluorescence *in situ* hybridization with a $CCCTAA_{(3)}$ peptide nucleic acid probe (red) identifies chromosome termini as well as large internal telomere repeat regions on metaphase spreads of *A. tigris* chromosomes prepared from fibroblast cultures. DAPI stained chromosomes are shown in blue. **(b)** Internal telomeric repeats are absent from *A. inornata* chromosomes, whereas chromosome termini signals are readily detected. **(c)** Chromosomes inherited from *A. tigris* but not from *A. inornata* are identified by internal telomeric repeats in *A. neomexicana*. **(d)** Projection of a subset of images from an *A. neomexicana* GV visualized by confocal microscopy. DAPI-stained chromosomes are shown in white and the telomeric probe in red. **(e)** Close-up of four representative areas visualizing paired fluorescence signals. The differences in resolution stem from differences in projection angles.



that for the 13 chromosomes for which the telomeric hybridization probe distinguishes sisters and homologs in *A. neomexicana*, sister chromosome pairing is the rule.

Screening of several tri-, tetra-, and hexanucleotide repeat probes identified $(CCAAGG)_n$ as an additional marker for at least nine chromosomes in *A. neomexicana* that are of *A. tigris* origin (Fig. 7a to c). When hybridized to diplotene chromosomes in acrylamide-embedded GVs, only paired signals were observed (Fig. 7d and e). In summary, two independent probes enabled us to distinguish sister chromosomes from homologs, and for over 20 bivalents examined, pairing occurred exclusively between sister chromosomes.

## Discussion

Entering meiosis with twice the usual number of chromosomes allows parthenogenetic species to produce oocytes carrying the complete somatic chromosome complement while preserving the established meiotic program. There are two principal pathways by which a diploid species' premeiotic oocytes may acquire eight rather than four sets of chromosomes. One is the process in which chromosome duplication occurs without cytokinesis; this has been termed endomitosis or endoreplication (Edgar and Orr-Weaver, 2001). Alternatively, 8n germ cells may arise by fusion of two cells either before or after the final premeiotic doubling of chromosomes. There is ample precedent for either mode of genome amplification in plants and animals, but the regulatory mechanisms are largely unclear.

In sexual species, homologous chromosomes form bivalents, and meiotic

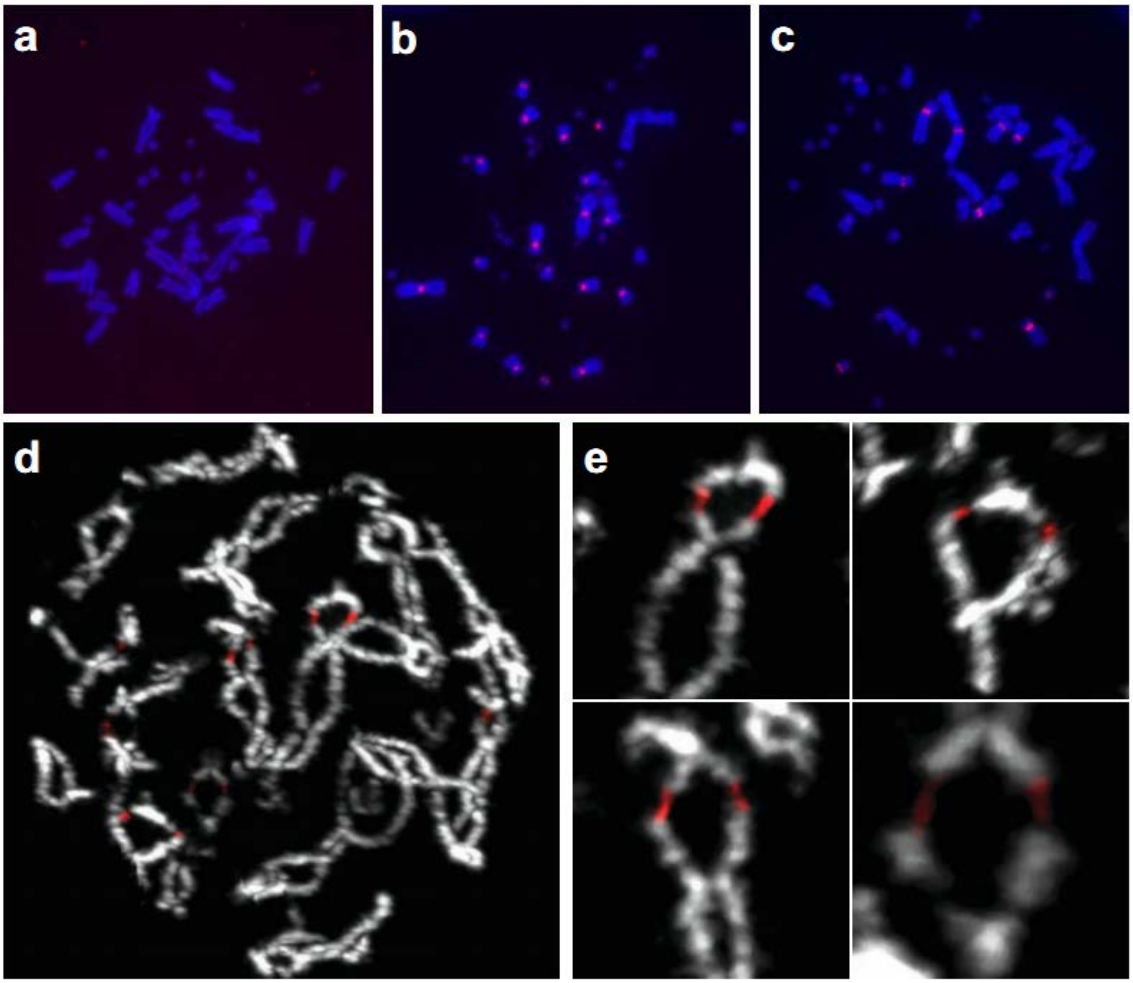



**Fig. 7. Assessment of pairing partners using a fluorescent $(CCAAGG)_2CC$ hybridization probe on an *A. neomexicana* GV. (a)** A $(CCAAGG)_2CC$ locked nucleic acid probe (red) does not hybridize strongly to *A. inornata* chromosomes. **(b)** 18 chromosomes from *A. tigris* are labeled by the same probe. **(c)** Chromosomes inherited from *A. tigris* but not from *A. inornata* are identified by the fluorescent probe in *A. neomexicana.* The inheritance of one set of chromosomes from *A. tigris* and the other from *A. inornata* leads to the expectation of nine foci in *A. neomexicana* cells. In reality ten brightly stained loci were observed. The tenth signal could be the result of homolog pairing and cross-over resulting in homozygozity at that locus. Alternatively, a different chromosome may have acquired the repeat region either before or after the hybridization event that gave rise to *A. neomexicana*. **(d)** Projection of a subset of images from an *A. neomexicana* GV visualized by confocal microscopy. DAPI-stained chromosomes are shown in white and the CCAAGG probe in red. **(e)** Close-up of four representative areas visualizing paired fluorescence signals.

recombination promotes genetic diversity while ensuring orderly segregation of chromosomes during the first meiotic division. The same mechanism would result in loss of heterozygosity in parthenogenetic species, whereas formation of bivalents from genetically identical sister chromosomes preserves heterozygosity. Interestingly, this same variation of the meiotic program appears to enable parthenogenetic reproduction in widely diverged species. Premeiotic doubling of chromosomes has been documented in triploid *Ambystomid* salamanders (Macgregor and Uzzell, 1964) as well as a parthenogenetic grasshopper (*Warramaba virgo*) (White et al., 1963). In both cases, sister chromosome pairing was suggested based on bivalent morphology. Although the lack of molecular markers in these studies precludes definitive conclusions, the striking parallels with whiptail lizards strongly indicate that a conserved mechanism enables parthenogenetic reproduction in diverse groups of animals. It seems likely that a relatively simple deviation from the established program of oogenesis is sufficient to permit parthenogenesis. However, loss of heterozygosity, paternal inheritance of centrosomes, and a requirement for fertilization in triggering completion of female meiosis are seemingly unconnected obstacles to parthenogenetic reproduction. A better understanding of the changes that permit a small but diverse group of animals to reproduce without males is clearly needed and may well shed light on the overwhelming success of sexuality.

## Acknowledgements

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## Materials and methods

### Animals

Laboratory colonies of *A. tigris*, *A. inornata*, *A. neomexicana*, *A. exsanguis* and *A. tesselata*, were established from animals collected in Socorro, Sierra and Otero Counties, New Mexico under a permit from the New Mexico Department of Game and Fish (permit # 3199 and 3395). *A. gularis* were collected in Dickens County, Texas. Animals were propagated in our Reptile and Aquatics Facility under conditions similar to a previously published description of captive lizard husbandry (Townsend, 1979), details to be published elsewhere.

### GV isolation and quantification of chromosome volume

Ovaries from adult and sub-adult lizards were placed in PBS and GVs were isolated using jeweler's forceps. GVs were transferred with a pipet to glass-bottom dishes (MatTek) containing 40 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS, and

allowed to incubate at 4 °C overnight. The following day the GVs were imaged using a LSM 510 META (Carl Zeiss Jena GmbH, Germany) system in two-photon excitation mode (Denk et al., 1990) equipped with a C-Apochromat 40x, N.A. 1.2 water immersion lens (Carl Zeiss Jena GmbH, Germany) optimized for ultraviolet and infrared transmission. Two-photon excitation at 735 nm was used to avoid out-of-focus bleaching and all fluorescence emission below 650 nm was collected. Images were cropped in Adobe Photoshop to digitally remove the nuclear envelope.

To obtain an unbiased measurement of chromosome volumes, we used the nonparametric and unsupervised, automatic threshold selection developed by Otsu (Otsu, 1979). In this method the gray-level histogram of an image suffices to find a gray level threshold, which yields the optimal separation of the chromosomes from the background without other *a priori* input. The Otsu method treats the normalized histogram as a probability distribution of possible pixel values. Pixels are dichotomized into two classes $C_0$ and $C_1$ (background and objects, in our case chromosomes) by a threshold at level k. $C_0$ denotes the class of pixels with values [0, …, k], and $C_1$ denotes the class of pixels with values [k+1, …, L], 0 being the smallest and L the largest pixel value in the image. The goal is to determine the threshold value k corresponding to the best class separation. Otsu showed, that k can be found by maximizing the between-class variance $\sigma_B^2(k)$ for all k between 0 and L. $\sigma_B^2(k)$ is defined as:

$$\sigma_B^2(k) = P_0(k)(m_0(k) - m_G)^2 + P_1(k)(m_1(k) - m_G)^2$$

$P_{0,1}(k)$ is the probability that a pixel k is assigned to class $C_0$ or $C_1$, respectively.

$m_{0,1}(k)$ represents the mean intensity of all the pixels assigned to class $C_0$ or $C_1$ (Gonzalez and Woods, 2008).

$m_G$ is the average intensity of the whole image.

To calculate the threshold we used the implementation of Otsu's method by Christopher Mei, Anthony Joshua, and Tony Collins as plug-in for the open-source image processing package ImageJ (Rasband, 1997-2009). The volume was determined with the commercial 3D image processing software IMARIS 6.3 (Bitplane AG, Switzerland). This software bases its volume calculation on an iso-surface rendered at an intensity value k, using the threshold determined as described above. This protocol ensures an objective, reproducible, and unbiased comparison of chromosome volumes measured for independent samples.

### Embryonic fibroblast cell culture

Cell cultures were modified from (Moore et al., 1997). Briefly, *Aspidoscelis* eggs were incubated at 29 °C for 30-40 days, sterilized in 90% ethanol, 120 mM potassium iodide and 39 mM iodine and opened under sterile conditions in a laminar flow hood. Embryos were immediately decapitated and cut into 1 cm or smaller pieces, rinsed with cold PBS and incubated with trypsin-EDTA solution (T4049, Sigma) on a stir plate for 5 min at room temperature. The supernatant was discarded and replaced with fresh PBS/trypsin, and incubation was continued for 15 min. The supernatant was decanted

through sterile cheesecloth into a 50-ml Falcon tube containing 2 ml ice-cold M199 cell culture media (Sigma) supplemented with 20% fetal bovine serum, 50 µg/ml gentamycin (Sigma), 1X glutamax (Invitrogen), 1X MEM non-essential amino acids (Invitrogen), 1X MEM vitamin solution (Invitrogen), 56 U/ml nystatin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Cell suspensions were kept on ice while the remaining tissue was trypsin treated for another 15 min and the supernatants were combined. Cells were then pelleted, washed in M199 media plus supplements and finally resuspended in 2-6 ml M199 media plus supplements and seeded in 6-well dishes (Falcon, cat.# 353046). Cells were cultured at 30 °C, 5% $O_2$ and 2% $CO_2$, and split once they had reached 85-100% confluency.

### Fluorescent *in situ* hybridization (FISH)

Embryonic fibroblasts were treated with 0.5 µg/ml Karyomax colcemid (Invitrogen) at 50-70% confluency and incubated for 3 hr at 30 °C, 5% $O_2$ and 2% $CO_2$. The cells were harvested by trypsin treatment and subjected to hypotonic swelling in 0.075 M KCl at 37 °C for 10 min. The cells were then pelleted, washed twice and finally resuspended in methanol / acetic acid fixative (3:1). Coverslips were cleaned with a 1:1 ethanol: ethyl ether solution and cells were dropped onto the coverslips and immediately washed liberally with fixative. Cover slips were then incubated on a heat block at 75 °C for 1 min. FISH was performed on dried coverslips as previously described (Sarthy et al., 2009) using either an AlexaFluor 543-labeled peptide-nucleic acid probe comprised of 5'-$(CCCTAA)_3$-3'or an AlexaFluor 488-labeled locked-nucleic acid (LNA) probe comprised of 5'-$(CCAAGG)_2$CC -3'. Washes were modified for the LNA probe as

follows: three 2 min washes in 2X SSC, 0.05% Tween 20 at 37 °C, two 6 min washes with 0.1X SSC at 55 °C, three 2 min washes with 2X SSC, 0.05% Tween 20 at room temperature, followed by two 2 min washes with PBS. Samples were imaged on a fluorescence microscope with a 100X, 1.4 NA Plan-APOCHROMAT objective and images were analyzed with AxioVision software (Carl Zeiss Jena GmbH).

Ovaries were isolated from *A. neomexicana* and placed in Buffer A (15 mM PIPES, pH 6.8, 20 mM NaCl, 60 mM KCl, 0.5 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 1 mM DTT). GVs were then embedded in an acrylamide mixture consisting of Buffer A, 5% acrylamide, 1 mM DTT, 15 mM sodium sulfite, and 11.5 mM sodium persulfate. Embedding chambers consisted of 22 x 55 coverslips that had been siliconed to stainless steel washers. After the GV was added to the washer, half of a 22 x 22 coverslip was dropped onto the washer to seal the top and promote polymerization of the acrylamide. After 30 to 60 min, the coverslip was carefully lifted and the gel was washed 4 times for 20 min with Buffer A on a shaking platform. Prehybridization was carried out in 50% deionized formamide, 2X SSC for 80 min with three changes of the prehybridization solution. Hybridization mixtures were as described for somatic cells and samples were incubated in a sealed chamber to minimize evaporation. Slides/washers were placed on a PCR machine with the following program: non-heated lid, 1 hr at room temp, 30 min at 40 °C, 6 min at 94 °C, and overnight at 37 °C. The following day, samples were washed four times in PBS with 0.1% Tween 20, and three times in PBS and stained with DAPI (40 ng/ml) for at least 1 hr at room temperature. Samples were imaged on an inverted confocal microscope with a C-apochromat 40x/1.20 W objective (Carl Zeiss Jena GmbH). The Alexa Fluor 543-labeled peptide-nucleic acid probe [5'-$(CCCTAA)_3$-3']

was excited at 561 nm and fluorescence emission was collected above 575 nm. The Alexa Fluor 488-labeled locked-nucleic acid probe [5'-$(CCAAGG)_2$CC-3'] was excited at 488 nm and fluorescence emission from 505-550 nm was collected. DAPI stained chromosomes were visualized by excitation at 405 nm excitation and collection of fluorescence emission between 420 nm and 480 nm. To avoid emission cross-talk we switched the excitation between the different laser lines and collected data in the appropriate detection channel only (multi-tracking).

## Transmission electron microscopy

Ovaries were isolated from *A. tigris* and *A. neomexicana* in PBS then transferred to a tube containing 70% hexane, 0.75% paraformaldehyde (Electron Microscopy Sciences), and 0.125% Nonidet (US Biological) in PBS. The tube was gently inverted 10 times then placed in a 20 °C waterbath for 2.5 hr, gently inverting the tube five times every 20 min. The ovaries were washed three times with 0.2% Tween 20 in PBS and three times with PBS (20 min each), followed by overnight fixation in 2.5% glutaraldehyde (Electron Microscopy Sciences) in PBS at 4 °C. Fixed tissues were washed three times in PBS and water, then post-fixed in aqueous 1% $OsO_4$, 1% $K_3Fe(CN)_6$ for 10 min at room temperature. Following 3 PBS washes, the tissue was dehydrated through a graded series of 30-100% ethanol, 100% propylene oxide then infiltrated in 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 hr. After two to three changes of 100% resin over 24 hr, tissues were embedded in molds, cured at 37 $^{o}$C overnight, followed by additional hardening at 65 $^{o}$C for two more days. Ultrathin (60

nm) sections were collected on copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were photographed using a FEI transmission electron microscope at 80 kV.

# **Chapter 3:** Laboratory synthesis of an independently reproducing vertebrate species

## Summary

Speciation in animals commonly involves an extrinsic barrier to genetic exchange followed by the accumulation of sufficient genetic variation to impede subsequent productive interbreeding. All-female species of whiptail lizards, which originated by interspecific hybridization between sexual progenitors, are an exception to this rule. Here the arising species instantaneously acquires a novel genotype combining distinctive alleles from two different species, and reproduction by parthenogenesis (*virgin birth*) constitutes an effective intrinsic barrier to genetic exchange (Cole, 1985). Fertilization of diploid parthenogenetic females by males of sexual species has produced several triploid species, but these instantaneous speciation events have neither been observed in nature nor have they been reconstituted in the laboratory. Here we report the generation of four self-sustaining clonal lineages of a new tetraploid species resulting from the superimposition of haploid sperm from *Aspidoscelis inornata* onto triploid oocytes from a parthenogenetic *A. exsanguis*. Molecular and cytological analysis confirmed the genetic identity of the hybrids and revealed that the females retain the capability of parthenogenetic reproduction characteristic of their triploid mothers. The tetraploid females have established self-perpetuating clonal lineages which are now in the third postformational generation. Our results describe the first reproductively independent vertebrate species to be generated in a laboratory and validate that ploidy increase can drive instantaneous speciation in reptiles when favorable combinations of parental genomes are assembled. We anticipate that these animals will be a critical tool in understanding the mechanisms underlying hybrid incompatibility and speciation in parthenogenetic vertebrates.

## Introduction

New species ordinarily arise over many generations through the gradual accumulation of incremental differences that eventually result in self-sustaining populations phenotypically distinctive and reproductively isolated from their progenitors (Coyne and Orr, 2004; Mayr, 1963). With few exceptions (e.g. (White et al., 1977)), interspecific hybridization has been viewed as detrimental to the process of speciation in animals rather than a driving force for it. However, the recent application of molecular tools in *Heliconiu*s butterflies (Mavarez et al., 2006), tephritid fruit flies (Schwarz et al., 2005) and several other taxa has led to the realization that hybrid speciation may be more common in animals than previously thought (Mallet, 2007b). At the extreme of instant speciation, hybridization combined with parthenogenesis has given rise to almost all unisexual lizards (Neaves and Baumann, 2011b). The incidence of such speciation events varies widely among families and is unusually high in Caucasian rock lizards (genus *Darevskia*) and North American whiptail lizards (*Aspidoscelis*, (Kearney et al., 2009)). For example, of the 12 *Aspidoscelis* species found in New Mexico, seven are parthenogenetic and five of these are triploid (Degenhardt et al., 1996; Reeder et al., 2002b).

Karyotypic and molecular evidence revealed that diploid parthenogenetic species arose from hybridization events between sexual progenitors (Dessauer and Cole, 1986; Lowe and Wright, 1966; Neaves and Gerald, 1968b, 1969). Subsequent hybridization between diploid parthenogenetic females and males of sympatric sexual species produced triploid unisexuals. How the unisexual mode of reproduction is induced in diploid hybrids and maintained in triploids remains unknown. Several lines of evidence suggest

that productive hybridization events are exceedingly rare. Firstly, histocompatibility studies support that single hybridization events have given rise to each of several parthenogenetic species (Cordes and Walker, 2003; Cuellar, 1976, 1977; Maslin, 1967; Taylor et al., 2003). Secondly, *de novo* hybridization events between sexual species have only been reported for closely related species or subspecies where they result in offspring that reproduce sexually (Dessauer et al., 2000). In contrast, quite a few first generation hybrids between parthenogenetic *Aspidoscelis* species and males of sexual species have been observed in field studies over the past 40 years. When hybridization occurs between a diploid parthenogenetic female and a sexual male, the hybrid offspring are triploid (e.g. (Cuellar and McKinney, 1976)) whereas hybridization events involving triploid parthenogenetic females produces tetraploid hybrids (Cole, 1979; Lowe et al., 1970; Neaves, 1971; Walker et al., 1990). Notably, in no case has successful reproduction of a hybrid been documented; and with one exception (Neaves, 1971) the animals were clearly infertile where examined (e.g. (Hardy and Cole, 1998)). In addition, a 29-year study aimed at creating a hybrid species in the laboratory involving 74 males and 156 females of nine species produced five confirmed hybrids which were all sterile (Cole et al., 2010). In summary, these findings indicate that in most cases ploidy elevation coincides with a loss of the ability to reproduce parthenogenetically in the offspring.

## Results

### Generation of tetraploid hybrids

To gain more insight into the relationship between hybridization and infertility, we paired males of the diploid sexual species *A. inornata* with females of the triploid

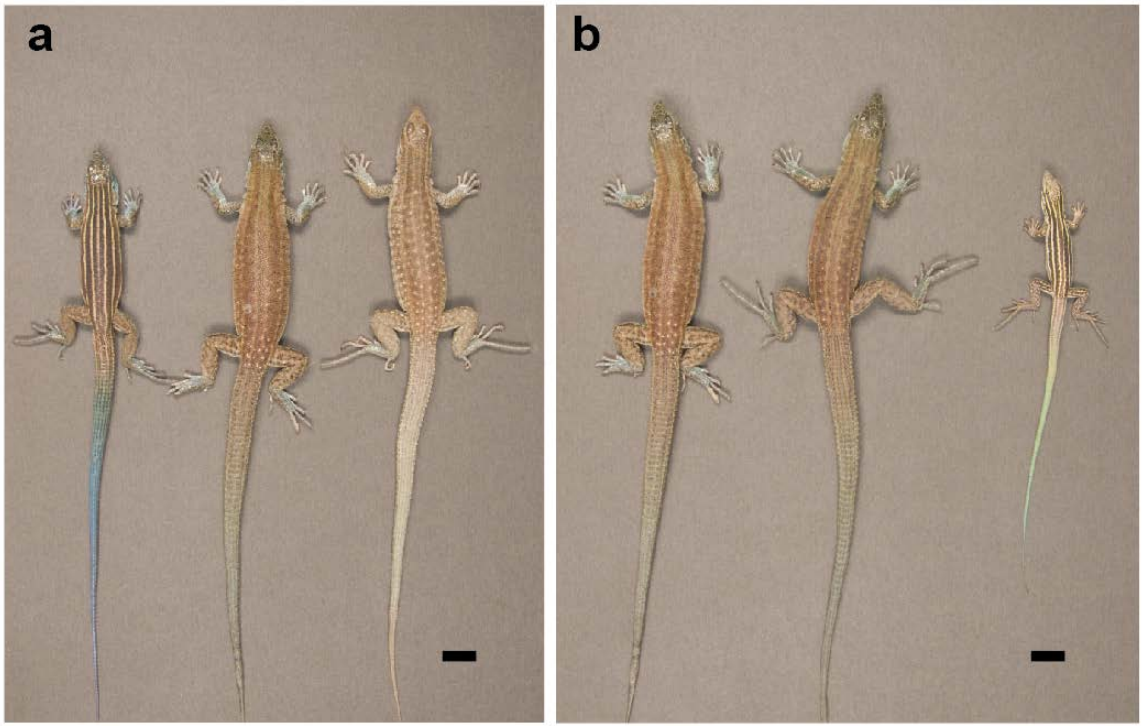



**Figure 1. Morphology of parental species and tetraploid hybrid animals.** (**A**) Dorsal view of *A. inornata* (left), *A. exsanguis (right)*, and the *A. exsanguis/A. inornata* hybrid (center). The scale bar corresponds to 10 mm. (**B**) Individuals representing the first (H1, left), second (H2, middle) and third (H3, right) hybrid generation of the tetraploid species. The H1 and H2 individuals are adults photographed on day 1168 and 645 post hatching, respectively. The H3 individual is shown at an age of 44 days and displays the color and pattern typical for juveniles.

parthenogenetic species *A. exsanguis*. This choice was inspired by the description of an apparent hybrid between *A. inornata* and *A. exsanguis* that was captured in August 1967. While in captivity this animal laid two fully-yolked eggs, but desiccation made it impossible to determine if the eggs could have produced viable offspring (Neaves, 1971). In our present study, the *A. inornata* male was observed mating with *A. exsanguis* females on several occasions. Three clutches totaling six eggs were recovered from the enclosure and incubated at 28 degree Celsius. Hatching occurred after 63 to 67 days and the six offspring appeared morphologically similar to *A. exsanguis* with the exception of

subtle blue pigmentation visible especially on the tail and indicative of a hybrid origin (Fig. 1).

The ploidy of the animals was determined by quantifying the DNA content in nucleated erythrocytes by flow cytometry. Blood samples from *A. inornata* and *A. exsanguis* served as diploid and triploid controls, respectively. The analysis revealed a 4C DNA content in somatic cells of the hybrid lizards (Fig. 2A). Tetraploidy was further confirmed by karyotyping cultured fibroblasts isolated from the heart of a hybrid female that died at 20 months of age. The somatic cell karyotype comprised 90-92 chromosomes

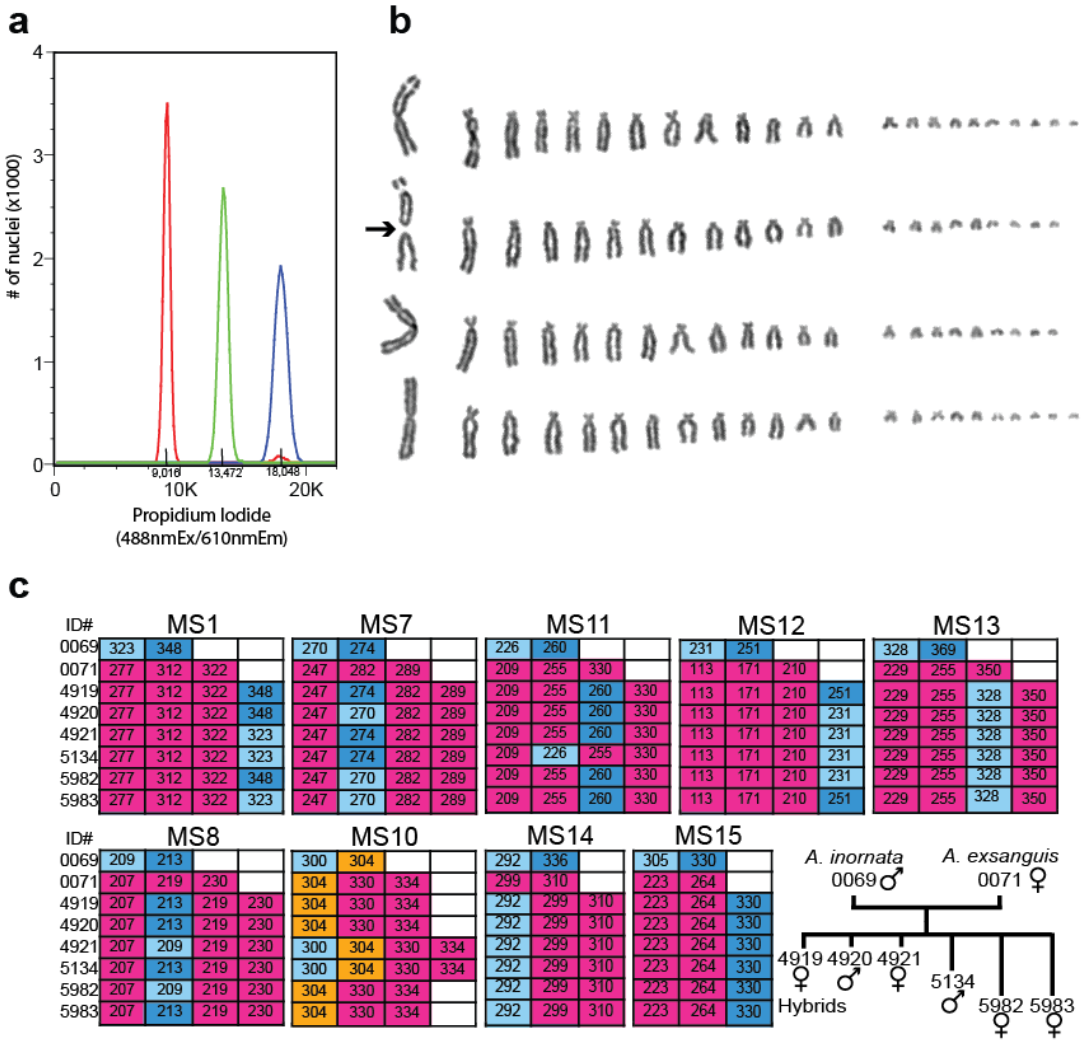


**Figure 2: DNA and chromosome analysis.** (**A**) Determination of DNA content of whole blood nuclei by propidium-iodide PI staining followed by FACS analysis. A comparison between diploid *A. inornata* (red), triploid *A. exsanguis* (green) and a putative hybrid (blue) indicates a tetraploid DNA content for the hybrid. (**B**) The karyotype of the *A. exsanguis/A. inornata* hybrid was determined from metaphase chromosomes of cultured cells. Each row shows a haploid chromosome set, with chromosomes arranged by decreasing size. The centric fission of one of the three large chromosomes is indicated with an arrow. (**C**) Microsatellite analysis at 9 loci in the *A. exsanguis* and *A. inornata* parents and the six hybrid progeny. The tree depicts the relationships between the 8 animals. Unique alleles of *A. exsanguis* and *A. inornata* are highlighted in red and blue, respectively. In some experiments an additional peak at 261 was observed for MS15, but was not reproducible in repeat runs and is considered a technical artifact.

(Fig. 2B), consistent with a combination of the haploid (n=23) chromosome complement of an *A. inornata* sperm with the unreduced triploid (3n ≈ 69) chromosome complement of parthenogenetic *A. exsanguis* (Fig. 3).

Microsatellite analysis

We next developed a panel of microsatellite markers to examine the parentage and genetic fingerprint of each hybrid lizard. Microsatellite analysis for nine highly polymorphic loci consistently reflected the parentage of the six animals and identified the parent individuals (Fig. 2C). The *A. exsanguis* mother had three different alleles at each of the MS1, 7, 8 and 10-13 loci but only two at loci MS14 and 15, the latter presumably reflecting the presence of the same allele on two of the three homeologous chromosomes. The male *A. inornata* was heterozygous at each of the 9 loci, but at locus MS10 one of the two alleles was the same as one of the three alleles in *A. exsanguis*. All alleles present at the 9 loci in *A. exsanguis* were detected in the 6 hybrid offspring, a finding consistent with the mother ovulating eggs carrying the unreduced somatic chromosome complement as previously observed in other *Aspidoscelis* species (Cuellar, 1971; Lutes et


**Figure 3: Karyotypes of *A. inornata* and *A. exsanguis*.** The karyotype of the *A. exsanguis* (**a**) and *A. inornata* (**b**) were determined from metaphase chromosomes of cultured cells. Each row shows a haploid chromosome set, with chromosomes arranged by decreasing size. *A. exsanguis* collected at Alamogordo, NM in 2003-2005 show centric fission of one of the three large metacentric chromosomes in the triploid karyotype (indicated with an arrow). This chromosomal change from the ancestral karyotype has previously been reported for *A. exsanguis* from Hidalgo county.

al., 2010). Importantly, all six hybrids had received *A. inornata* alleles from the fertilizing sperm. Two of the animals had enlarged femoral pores and more intense blue pigmentation characteristic of *A. inornata* males. Based on these criteria as well as the presence of hemipenal bulges at the base of the tail we surmise that these two individuals are males, whereas the other four hybrids are females.

## Preservation of meiotic mechanism

At 20 months of age, one of the hybrid females died of unknown cause. A large egg was removed from the oviduct during necropsy (Fig. 4A) and incubated unsuccessfully. However, unlike previous hybrids examined by others, the ovaries of this lizard appeared normal and contained numerous developing follicles (Fig. 4B). Germinal vesicles (GVs) were isolated, stained with 4’, 6-diamidino-2-phenylindole (DAPI), and examined by confocal microscopy to compare the chromosome content with GVs from diploid sexual and parthenogenetic species.

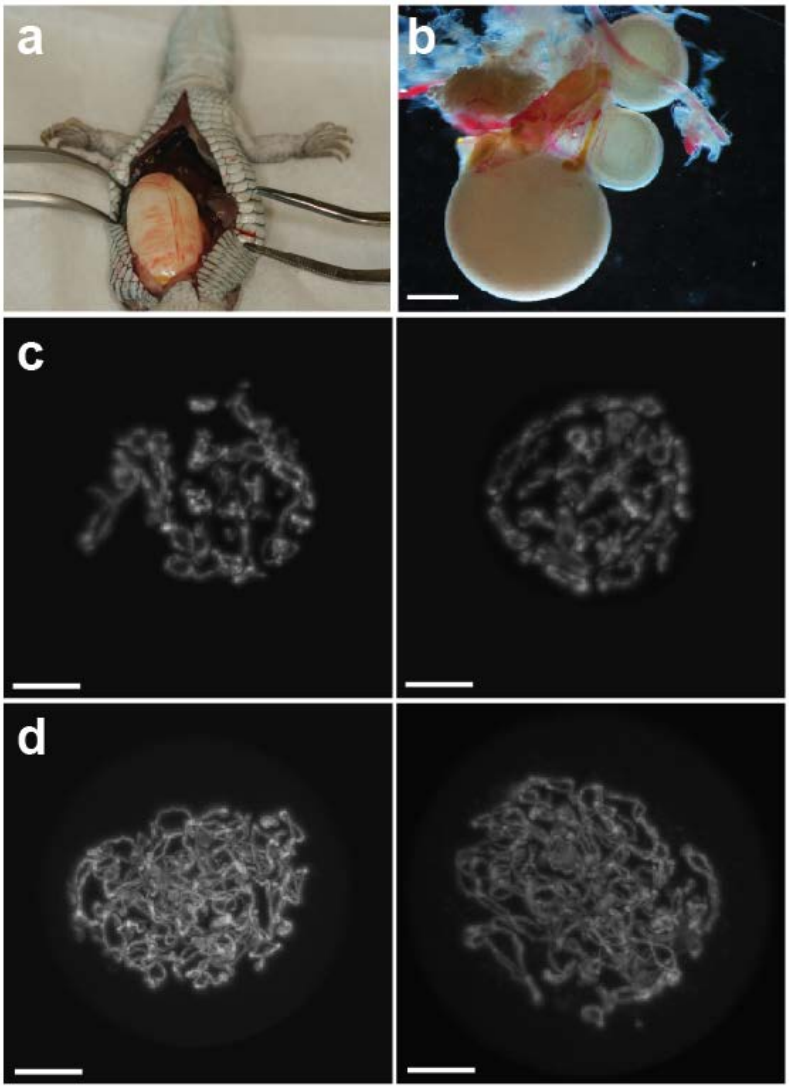



**Figure 4: Ovaries and mechanism of oogenesis.** (**A**) A large yolked and shelled egg was found in the body cavity of a deceased tetraploid hybrid. (**B**) One of two fully developed ovaries from the same lizard shown in (A). Scale bar corresponds to approximately 0.5 mm. (**C**) Three-dimensional projections of DAPI-stained germinal vesicles (GV) in prophase I of meiosis from a diploid parthenogenetic *A. tesselata*. Scale bar is 20 µm. (**D**) GVs from the tetraploid hybrid.

In diploid parthenogenetic *Aspidoscelis* species, meiosis proceeds with twice the number of chromosomes found in sexual species so that diploid oocytes are produced following the two meiotic divisions (Lutes et al., 2010). If sexual reproduction occurred in the tetraploid hybrids, the premeiotic nuclei would contain twice the amount of DNA found in prophase I of meiosis in a sexually reproducing diploid species or the same amount as in a GV from a diploid parthenogenetic species (Fig. 4C). In contrast, if the tetraploid hybrids were capable of parthenogenetic reproduction, their GVs should

contain twice the amount of DNA found in GVs of *A. tesselata*, an outcome that is consistent with our experimental findings (Fig. 4D). These observations indicate that the deceased tetraploid hybrid had been capable of producing eggs with a tetraploid chromosome content by employing the same mechanism previously characterized for diploid parthenogenetic species in this genus (Lutes et al., 2010). Indeed, genotyping later revealed that the deceased lizard had previously laid an egg from which a viable offspring had emerged.

### Establishment of four tetraploid lineages

Between April 2009 and October 2010 twenty-five offspring were produced in aggregate by the four tetraploid females. The microsatellite analysis was first extended to these second generation (H2) animals and later to 22 third generation (H3) animals that hatched between April and December 2010. With one exception (see below), all alleles present at the nine loci were identical between the first generation hybrids (H1) and their respective daughters and granddaughters, providing evidence for four independent parthenogenetic lineages. The example shown in Figure 5 includes one H1 hybrid, two of its daughters and four granddaughters. The single exception to clonal inheritance occurred at the MS14 locus where the H3 animal 9706 deviates from its siblings and progenitors by the appearance of a new allele 302 not otherwise found in the lineage. This allele appears to be the result of a repeat expansion confined to a single individual.

It should be noted that the two H2 animals represented in figure 5 were produced by the H1 female while she was housed with two H1 males (4920 and 5134), which are distinguishable at two and four loci from the H1 female, respectively (Fig 2C).

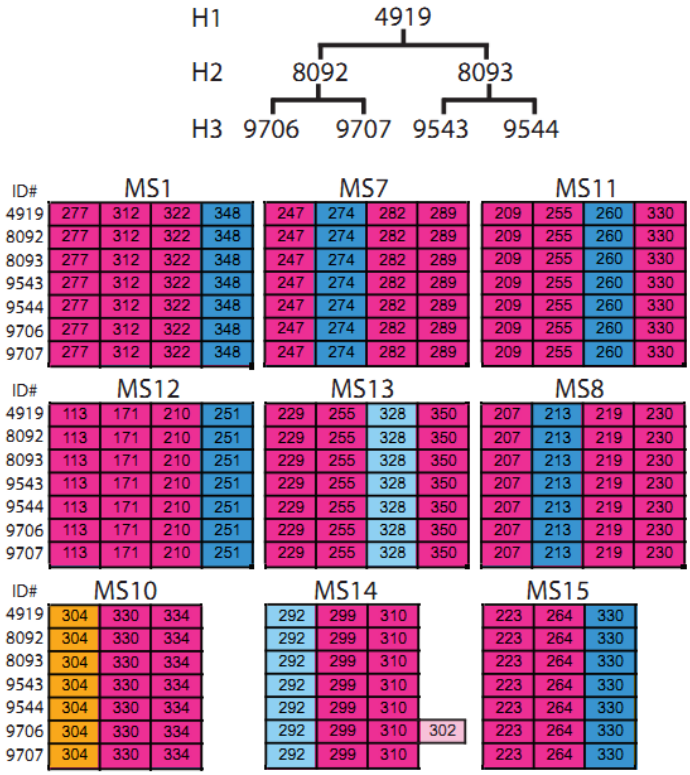



**Figure 5: Maintenance of heterozygosity over three generations.** Microsatellite analysis at 9 loci for a first generation hybrid (H1), two of its daughters (H2) and 4 granddaughters (H3). Alleles originally inherited from *A. inornata* are highlighted in blue, those from *A. exsanguis* in red. A single novel allele of MS14 is highlighted in pink.

Nevertheless, only maternal alleles were detected in the daughters providing further evidence for unisexual reproduction. The other members of the H2 generation thus far were daughters of H1 females housed separately from males, and microsatellite analysis revealed each to be genetically identical to its mother. Together with the increased DNA content in meiotic prophase and the fact that all H2 and H3 animals are female, the genotyping results therefore strongly support a parthenogenetic mode of reproduction.

## Discussion

The breeding experiment described here has produced over 50 tetraploid animals representing three generations with more forthcoming as eggs hatch and additional eggs are laid. The maintenance of reproductive competence following ploidy elevation was highly unexpected as other *Aspidoscelis* hybrids (both field-collected and laboratory-

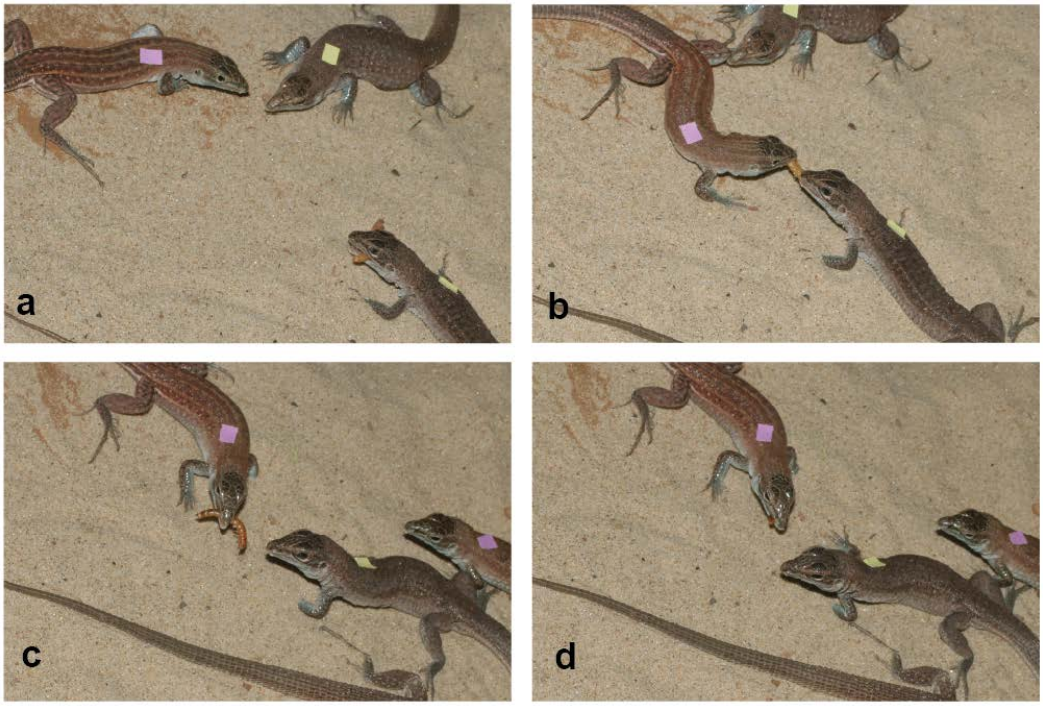
generated) have failed to reproduce. It has even been suggested that the decline of parthenogenetic *A. dixoni* in Antelope Pass, New Mexico is a result of fertilization of already diploid *dixoni* eggs by sexual *A. tigris* males resulting in sterile triploid hybrids (Cole et al., 2007). We have now described the first case where ploidy elevation in a reptile has not resulted in embryonic lethality or infertility, providing the proof of principle for how triploid parthenogenetic species are likely to have arisen in nature.

While evidence from field and laboratory studies (Cole et al., 2010; Taylor et al., 2005) indicates that speciation by ploidy elevation is exceedingly rare in reptiles, unisexual lineages of some fish and amphibians are polyclonal and in a few cases are readily reconstituted. For example, laboratory hybridization of *Poeciliopsis monacha* females and *P. lucida* males reconstituted a hybridogenetic species of fish, *Poeciliopsis monacha-lucida,* found in northwestern Mexico (Schultz, 1973b; Wetherington et al., 1987b). Similarly, the hemiclonal frog *Rana esculenta* was recreated in the laboratory by crossing the two parental species *Rana ridibunda* and *R. lessonae* (Hotz et al., 1985; Hotz et al., 1999). Inherent to the hemiclonal mechanism of hybridogenetic reproduction, eggs only contain one of the parental genomes and diploidy must be restored via fertilization by sperm from sympatric males each generation. Even in clonally reproducing (i.e. gynogenetic) unisexual salamanders and fish where the sperm genome is not incorporated into the offspring, sperm from a related species is required to trigger embryogenesis in eggs carrying the full somatic chromosome complement (Bogart et al., 2007; Lamatsch and Stöck, 2009) This absolute requirement for males from related sexual species is shared by all unisexual anamniotes and prevents establishment of reproductively independent unisexual species in these taxa.

The four lineages described here constitute the first example of a laboratory-generated vertebrate species that can reproduce independently of its progenitors. In addition it is the first tetraploid species of lizard. The absence of gene flow both within and between unisexual taxa has fueled debate about the taxonomic treatment of parthenogenetic animals (Cole, 1985; Frost and Wright, 1988; Walker, 1986). Although the lack of interbreeding prevents application of the biological species concept, parthenogenetic lizards exist as phenotypically and genetically discrete, self-reproducing entities, and they have been recognized as valid taxonomic units with the same status as sexually reproducing species (Reeder et al., 2002b).

The origin of the first reproducing line of tetraploid whiptail lizards in the laboratory raises the question whether this new species could survive in nature in

**Figure 6: Competition over food item between *A. exsanguis* and tetraploid hybrid.** (**a**) *A. exsanguis* (marked with yellow tape) on bottom right has captured *T. molitor* (**b**) Tetraploid hybrid (marked with pink tape) bites end of food item and (**c**) pulls it out of the mouth of the *A. exsanguis*. (**d**) Tetraploid hybrid consumes the food item.



competition with other *Aspidoscelis* species. Or is it comparable to domestic species that depend on husbandry under captive conditions to persist? It is premature to speculate on answers, but tetraploids of all three generations in the laboratory pursue and capture live crickets as effectively as their progenitors and exhibit no obvious competitive disadvantage when housed with individuals of sexual or parthenogenetic *Aspidoscelis* species. In experiments where a single food item (larvae of the darkling beetle *Tenebrio molitor*) was offered to a group comprised of four *A. exsanguis* and four tetraploids, the item was consumed as frequently by a tetraploid as by an *A. exsanguis*. On several occasions, we observed a tetraploid removing the food item from the mouth of an *A. exsanguis* (Fig. 6).

The laboratory synthesis of a new tetraploid *Aspidoscelis* species, coupled with the collection of a tetraploid hybrid between *A. inornata* and *A. exsanguis* in Alamogordo more than 40 years ago (Neaves, 1971), raises the question of why a tetraploid species derived from hybridization between these two species has not yet been found in nature. The apparent health and vigor of the tetraploids and their parthenogenetically produced offspring in captivity does not ensure their ability to succeed in nature, but it does suggest that sporadic mating of *A. inornata* with *A. exsanguis* could result in self-sustaining tetraploid lineages in locations where both species are sympatric. The new tetraploid species synthesized in captivity may be the prototype of a species that might eventually emerge in the deserts of the southwestern US or northern Mexico. Perhaps its existence in the laboratory, together with recognition of the subtle phenotypic differences that distinguish it from its triploid progenitor, will stimulate a productive search for its counterpart in nature.

## Materials and Methods

### Animals

Laboratory colonies of *A. exsanguis*, *A. inornata*, *A. tesselata*, and *A. exsanguis* x *A. inornata* hybrids were established from animals collected in Socorro, Sierra and Otero Counties, New Mexico, under a permit from the New Mexico Department of Game and Fish (permit numbers 3199 and 3395). Animals were propagated and maintained in the Reptile and Aquatics Facility under conditions similar to a previously published description of captive lizard husbandry (Townsend, 1979) and in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research.

### Flow Cytometry

Blood was isolated from tail-clips in acid citrate dextrose anticoagulant (45 mM sodium citrate, 22.8 mM citric acid, 81.5 mM dextrose). Cells were centrifuged at 500 g and resuspended in citrate buffer, pH 7.6 (0.25 M sucrose, S-0389 Sigma, 38.6 mM trisodium citrate, C-8532, Sigma and 5% DMSO) then pelleted again at 500 g. After decanting the supernatant, the cells were resuspended in citrate buffer at a density of 2.5 x $10^6$/ml. 100 µl of cell suspension was transferred into a 15 ml conical tube and incubated with 900 µl 30 mg/L trypsin, pH 7.6 (T-0134, Sigma), diluted in buffer S (3.4 mM trisodium citrate, 0.1% Triton X-100, T-6878, 1.5 mM spermine, S-1141 Sigma and 0.38 mM Tris-HCl, T-7149 Sigma) for 10 min with gentle rotation, followed by the addition of 750 µl trypsin inhibitor solution (0.5 mg/ml trypsin inhibitor, T9003 Sigma, 0.1 mg/ml RNase A, R-5500 Sigma prepared in buffer S) for an additional 10 min with

gentle rotation. An additional 750 µl of propidium iodide solution (propidium iodide 0.42 mg/ml P-4170 Sigma, 3.33 mM spermine in buffer S) was added and incubated with gentle agitation for 10 minutes while protected from light. Events were collected with an Influx instrument (BD Biosciences) by excitation at 488nm and collection at 610nm with a threshold set to exclude small debris. No gates were used.

### Cell Culture

Primary cell lines were established from embryos (*A. exsanguis and A. inornata*) or heart tissue (tetraploid hybrid) using a procedure modified from(Moore et al., 1997). Briefly, *A. exsanguis and A. inornata* eggs were incubated at 29 °C for 30–40 days, sterilized in an ethanol-iodine mixture (90% ethanol, 120 mM potassium iodide and 39 mM iodine), and embryos were removed under sterile conditions and immediately decapitated. Minced embryos or, in the case of the hybrid animal, heart tissue was rinsed with cold phosphate-buffered saline and agitated for 15 min at room temperature in the presence of trypsin-EDTA solution (T4049, Sigma). The suspension was passed through sterile cheesecloth for *A. inornata* and *A. exsanguis* samples into a 50 ml Falcon tube containing 2 ml ice-cold M199 cell culture medium (Sigma) supplemented with 20% fetal bovine serum, 50 µg/ml gentamycin (Sigma), glutamax (Invitrogen), MEM non-essential amino acids (Invitrogen), MEM vitamin solution (Invitrogen), 56 U/ml nystatin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). For the *A. exsanguis* x *A. inornata* hybrid sample larger tissue fragments were manually removed using forceps prior to the addition of 2 ml ice-cold M199 cell culture medium plus supplements as above. The filtered cell suspensions were kept on ice and the remaining tissue was

trypsinized for another 15 min. Larger tissue fragments were again removed and the cell suspensions were combined and centrifuged to pellet cells, then washed in M199 media plus supplements and finally resuspended in 2 to 6 ml M199 media plus supplements. Cells were seeded in 6-well dishes (Falcon, 353046) and cultured at 30 °C, 5% $O_2$ and 2% $CO_2$. When cells exceeded 85% confluency, the cultures were passaged at a 1 in 3 dilution.

### Karyotyping

At 50-70% confluency, cultured cells were treated with 0.5 µg/ml Karyomax colcemid (Invitrogen) and incubated for 3 h at 30 °C, 5% $O_2$ and 2% $CO_2$. The cells were harvested by trypsinization and subjected to hypotonic swelling in 0.075 M KCl at 37 °C for 10 min. The cells were then pelleted, washed twice in PBS and resuspended in methanol:acetic acid fixative (3:1). Coverslips were cleaned with a 1:1 ethanol:ethyl ether solution, air-dried and stored in water at 4 °C. Cells were dropped onto the coverslips and immediately washed with 1-3 ml fixative. Coverslips were then incubated on a heat block at 75 °C for 1 min. Coverslips were further processed by RNase treatment (0.5 mg/ml in PBS) for 30 min at 37 °C, washed twice briefly in PBS, then fixed for 2 min in 4% formaldehyde (Sigma F8775) in PBS. After rinsing briefly in PBS three times, the coverslips were incubated with 1 mg/ml Pepsin (Sigma P6887, 3,200-4,500 units/mg) for 10 min at 37 °C, rinsed twice in PBS, and fixed again in 4% formaldehyde. Following three washes in PBS, the samples were dehydrated in an ethanol series (70%, 90%, 100%) and air-dried. Giemsa staining was performed by mixing giemsa (VWR, 15204-144) and phosphate buffer, pH 6.8 (VWR, 34171-002) in a 1:12 ratio, then filtering the

mix through a 0.2 micron filter with an attached 18 gauge needle onto coverslips. The samples were incubated for 10-15 minutes, rinsed in water and air-dried in a dust-free environment prior to mounting. Samples were imaged on an Axiovert microscope equipped with an Axiocam HRm camera using a 100×, 1.3 NA Fluar objective. Images were analyzed with AxioVision software and karyotypes were assembled in Adobe Photoshop.

### Microsatellite Analysis

Microsatellites MS1, MS7, MS8 were isolated from a genomic library prepared from *A. tesselata* liver tissue. Genomic gDNA was isolated using QIAGEN Genomic-tips and the library was constructed using the Lambda FIX II library construction kit (Stratagene). Briefly, BAMH1-digested gDNA was ligated into the Lambda FIX II vector and packaged according to the Gigapack III manual (Stratagene). Plaque lifts were performed and membranes were hybridized with a $^{32}$P-labelled $CA_{(10)}$ probe. Positive plaques were isolated and phage DNA was purified using the Wizard Lambda Prep DNA Purification System (Promega) and sequenced by primer walking to identify microsatellites and flanking sequences. Microsatellites MS12 to 15 were isolated from genomic DNA libraries enriched for tetranucleotide repeats that were generated by Genetic Identification Services using genomic DNA isolated from liver of *A. exsanguis*. MS10 primers were modified from (Rowe, 2002) to amplify the Cvanμ7 microsatellite in *Aspidoscelis* species and MS11 (Ai5062) was as described (Crawford et al., 2008). For each primer set one primer was 6-carboxyfluorescein-labeled at the 5’ end. Primer sequences are listed in Table 1.

| Microsatellite | Oligo sequence (5' → 3') |
|---|---|
| MS1 | TGCATGATGGAGGAATCTTC<br>CTAGTGGTGATAGAAACATGG |
| MS7 | AACTAAGTGCTAAGTGTGAC<br>ACAGTCTTAGAGATCACAAG |
| MS8 | ACACCCAAAGTCCTCAACAG<br>CTAGTACATGTGTAAGGGTG |
| MS10 | GACCAATAATGTGGAAGCTG<br>ACATGGCTGAGTAATTGGTG |
| MS11 | GTGCATTCAATGATGTATT<br>TTTGCTCAGTGTAACTAG |
| MS12 | TACCCACCTGGAGATGTTTAG<br>AGGACGCCTTAAAATAGGAAG |
| MS13 | CCAGCAGTCTGTTCTCATAGG<br>TTTATTGTTTGCAGCCTTGAG |
| MS14 | TGGAGGCAGTCTTGGTATC<br>GAACATTGACCGCATCAC |
| MS15 | TTAAAGCAGAGGTCAGGTTATC<br>GATGGAAGAATAGGATGATGAA |

One millimeter lizard tail-clips were placed in 300 µl of a solution consisting of 50 mM Tris pH 8.8, 1 mM EDTA, 0.5% Tween-20, and 100 µg/ml proteinase K. Samples were incubated for 12-18 hrs at 55°C then placed in a 95°C heat block for 10 min followed by direct storage at -80°C until use. One microliter of each sample was used as template and PCR was performed with Biolase DNA polymerase (Bioline) or Taq DNA polymerase (New England Biolabs). PCR products were detected by capillary electrophoresis on a 3730 DNA Analyzer and analyzed with GeneMapper Version 4.0. Size ranges used to bin each allele are listed in Table 2.

## Confocal Microscopy

Germinal vesicles were isolated using jeweler's forceps, incubated with 40 ng/ml 4',6-diamidino-2-phenylindole (DAPI) and imaged using a LSM 510 META (Carl Zeiss Jena) system equipped with a C-Apochromat 40×, NA 1.2 water immersion lens. A 405 nm laser was used to excite the fluorescent dye and signal was collected using a long-

pass 420 nm filter. Images were cropped in Photoshop to digitally remove the nuclear envelope. Noise was removed by smoothing in Imaris with a 3x3x3 median filter.

| Microsatellite | Allele | size range |
|---|---|---|
| MS1 | 277 | 276.25 - 276.87 |
| | 312 | 312.09 - 312.57 |
| | 322 | 321.87 - 322.35 |
| | 323 | 322.73 - 323.45 |
| | 348 | 348.04 - 348.38 |
| MS7 | 247 | 246.68 - 247.06 |
| | 270 | 269.71 - 270.05 |
| | 274 | 273.55 - 273.80 |
| | 282 | 281.43 - 281.87 |
| | 289 | 289.08 - 289.57 |
| MS8 | 207 | 206.98 - 207.17 |
| | 209 | 208.66 - 208.74 |
| | 213 | 212.74 - 212.93 |
| | 219 | 218.92 - 219.13 |
| | 230 | 230.39 - 230.56 |
| MS10 | 300 | 299.83 - 299.92 |
| | 304 | 304.06 - 304.26 |
| | 330 | 329.82 - 329.98 |
| | 334 | 334.10 - 334.39 |
| MS11 | 209 | 208.94 - 209.07 |
| | 226 | 226.23 - 226.27 |
| | 255 | 255.09 - 255.69 |
| | 260 | 260.24 - 260.72 |
| | 330 | 330.09 – 330.20 |
| MS12 | 113 | 112.91 - 113.22 |
| | 171 | 170.52 - 170.89 |
| | 210 | 210.13 - 210.39 |
| | 231 | 231.05 - 231.29 |
| | 251 | 250.41 - 250.79 |
| MS13 | 229 | 228.72 – 228.95 |
| | 255 | 254.90 - 255.21 |
| | 328 | 327.44 - 327.74 |
| | 350 | 349.72 - 350.08 |
| | 369 | 369.25 |
| MS14 | 292 | 291.74 - 291.95 |
| | 299 | 298.92 - 299.14 |
| | 302 | 301.64 |
| | 310 | 309.88 - 310.18 |
| | 336 | 335.77 |
| MS15 | 223 | 223.14 - 223.38 |
| | 264 | 263.73 - 263.97 |
| | 305 | 305.32 |
| | 329 | 329.43 - 329.74 |

**Table 2.** Size ranges determined for each allele as determined by Genemapper.

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# Chapter 4: Conclusions and future prospects

## Summary

Through meiotic modifications that yield diploid gametes, some species within *Aspidoscelis* are able to overcome the sterility that is common to hybrids. In this work, we have identified cellular mechanisms, and their consequences, in these animals. First we determined that chromosomal content doubles (8C) in diploid parthenogenetic *Aspidoscelis* oocytes. This appears to be specific, as erythrocytes - a proxy for somatic cells - are 2C. Oocytes were identified in diplotene stage by the presence of bivalents which exhibited chiasmata. Next, we differentiated between homolog pairing and sister chromosome pairing by identifying homolog specific microsatellites that were suitable for FISH. These sequences were inherited from the maternal species, *A. tigris*. Bivalents with FISH signal were shown to be exclusively comprised of maternally inherited chromosomes; thus chromosome pairing occurs between sister chromosomes. To gain an understanding into the conservation of this meiotic mechanism and its relation to the prevalence of polyploid *Aspidoscelis* species, we generated tetraploid hybrids by crossing parthenogenetic triploid *A. exsanguis* and bisexual *A. inornata*. Unlike other reported tetraploid *Aspidoscelis*, these hybrids are fertile and reproduce through the chromosomal doubling mechanism described in *Aspidoscelis* diploids.

## *A. exsanguis/A. inornata* hybrids

### Absence of tetraploids in nature

Previous tetraploids have been documented as sterile or infertile. On the other hand, the *A. exsanguis/A. inornata* crosses are quite robust, with an ability to effectively

compete with diploid and triploid relatives in regards to both food competition and viable offspring. Chromosome pairing is not a problem with these animals. With 16C DNA content in meiosis, reproduction appears as efficient as in diploids, either unisexual or bisexual. Why then might tetraploid species be absent in nature? Lack of tetraploids may stem from incompatible interactions involving the *A. tigris* genome; these hybrids do not readily produce higher ploidy animals, the exception being *A. neotesselata* which contains one genome from *A. tigris* and two from the *A. sexlineata* group (*A. gularis* and *A. sexlineata viridis*, Fig. 1, Chapter 1). In contrast, the *A. exsanguis/A. inornata* cross is comprised of genomes exclusively from the *A. sexlineata* group, suggesting that these may be more amenable to chromosome doubling. Further we speculate that the meiotic mechanism may be dosage dependent, because as our lab has observed, fertile tetraploid lineages form more easily than triploid counterparts. Alternatively, tetraploids may be unable to form a unique environmental niche, rendering them inept to compete with *A. exsanguis*. Because the fourth genome is largely redundant with two of its predecessors, it may not add genetic variation to the animal, serving only an energy sink. Performing gene expression analyses may yield insights into the differences between a triploid and closely related tetraploid. Although we saw no disability in prey capture with the tetraploids, these animals may harbor unknown detriments that result in their demise. Testing this in a natural setting would eliminate many potential artifacts from gene expression studies. By monitoring competition between these two species in an enclosed area in their natural environment, one can extrapolate how the two would fare in nature. To test the idea of the Red Queen, for example, one could compare mite loads because, as previously discussed, unisexual species exhibit higher rates compared with bisexuals.

According to several evolutionary hypotheses, tetraploids should have an advantage over triploids. The Fisher-Muller hypothesis predicts a higher probability of favorable allele combinations with a ploidy increase. According to Muller's ratchet, the increase in gene redundancy will reduce the 'click' of the ratchet. And the increased genetic variability should reduce the parasitic susceptibility described in the Red Queen hypothesis. However, the variation in a polyploid does not appear to be simply additive, as 'genomic shock' – the variance in gene expression between parent and hybrid – appears to play a role in hybrid survival (McClintock, 1984). In *Arabidopsis*, most of these differences result from selective repression of a parental allele, possibly as a consequence of nucleolar dominance (Wang et al., 2006). Hence, although a tetraploid may contain 33% more DNA than its predecessor, it may not be expressed as such. If a fourth chromosomal complement is present, but largely silenced, then it would not serve as an advantage in regards to any of the evolutionary hypotheses.

## Genetic variation

Surprisingly, a microsatellite change was observed between the second and third generation *A. exsanguis/ A. inornata*, between mother 8092 and offspring 9706. Single generation variation has also been observed in parthenogenetic rock lizards of the genus *Darevskia*, in both the germline and somatic cells (Malysheva et al., 2007). This variation was detected using genomic fingerprinting methods; in comparison, by using our microsatellite tools we are able to measure genetic changes at single nucleotide resolution. As our tetraploid colony approaches its sixth generation, it would be informative to measure additional variation between present and forthcoming

generations. To gain insight into genetic stability of the tetraploid genome, it would be fruitful to compare variability between diploid, triploid, and tetraploid populations. Because microsatellite variation positively correlates with that of the genome in mammals (albeit at a difference variance) an observed increase in variation between tetraploids may indicate larger genome-wide fluctuations (Driscoll et al., 2002; Väli et al., 2008). Changes in the genome would undermine the idea that these animals reproduce clonally.

### Genetic differences between *Aspidoscelis* species

We demonstrated sister chromosome pairing by finding large microsatellite arrays in the *A. tigris* karyotype that were absent in that of *A. inornata*. This unexpected discrepancy predicts a larger diversity than first expected from superficial gene comparison (Lutes and Baumann). Ironically, genome sequencing would not have uncovered the breadth of diversity, as large repetitive regions are often unable to be sequenced to completion. It would be interesting to determine the extent of these differences. Of the three bisexual species we examined as well as one additional (Meyne et al., 1990), *A. inornata* appears to be the exception rather than the rule in *Aspidoscelis*. Internal telomeric repeats are common in both bisexual and parthenogenetic whiptails. How would such repeats arise and what are their functions, if any? As exemplified by chromosomal reduction in the evolution of great apes to humans, an internal telomere repeat may be a remnant of an ancestral chromosome fusion (IJdo et al., 1991). One hypothesis suggests that chromosome break healing may be mediated by the enzyme telomerase (Azzalin et al., 2001). However, these mechanisms would create insertions of short length and are

therefore unlikely to explain the karyotype of *Aspidoscelis* lizards. Additionally, these mechanisms do not provide an explanation for the prevalence of the other, non-telomeric, array we observed. Alternatively, the formation of large internal sites may be mediated through the insertion of a double-stranded repeat sequence into a chromosome break. Over time, it would be lengthened through faulty DNA replication (Lin and Yan, 2008). In addition to the formation of internal repeats, their function remains a mystery as well. As observed in dozens of organisms ranging from plants to mammals, such large arrays are observed near centromeres (Meyne et al., 1990), suggesting that they may play a related role (Tek and Jiang, 2004). Whether microsatellite regions serve a function at centromeres or are merely a consequence of nonlethal insertions remains to be known. Nevertheless, these repeats may consequently serve as a species barrier by preventing normal chromosome pairing and reproduction in hybrids.

### Chromosome pairing between sisters

A significant finding in our report of parthenogen meiosis was the observation of pairing between sister chromosomes, the genetically identical products of the chromosome doubling event. Pairing between identical chromosomes ensures that heterozygosity is maintained. We found that sisters not only pair, but they also undergo recombination and genetic exchange, as revealed by chiasmata on each of the bivalents. This implies that the sister chromosome is indistinguishable from, and treated as, the homolog. With effort expended to eliminate incorrect interactions and ensure homolog pairing, how are sister chromosomes able to pair in *Aspidoscelis*? Looking at similar situations may provide answers. For example, hexaploid wheat, an allopolyploid originating from three

progenitor species, contains a true set of homologs as well as three sets of genetically similar *homeologs*, which seemingly predisposes it to pairing difficulties (Yousafzai et al., 2010). However, despite this apparent obstacle, homolog pairing is robust and triploid gametes are reliably attained. The Ph1 locus, present on chromosome 5 of one of the homeologs, plays a significant role in strict homolog pairing. A Cdk2-like gene, Ph1 appears to interfere with the normal progression through meiosis through a dominant negative mechanism (Griffiths et al., 2006; Knight et al., 2010). In wild type cells, incorrect interactions can be remedied prior to zygotene completion whereas Ph1 deletion mutants demonstrate mild chromosome pairing defects, resulting in infertility after several generations (Yousafzai et al., 2010). Therefore, potentially detrimental interactions probably occur between homeologs, but with sufficient time, homologous interactions are enforced. As wheat does not appear to have a zygotene checkpoint, the Ph1 locus apparently affords them with sufficient time to correct homeologous interactions. *Aspidoscelis*, as with other vertebrates, appears to have a pairing checkpoint that would prevent progression into meiosis without proper pairing.

Mechanistically, it may not seem surprising that sister chromatids are differentiated from sister chromosomes. Homolog-specific recombination increases during meiosis; a reverse occurs in mitosis, in which intersister recombination is favored (Jackson and Fink, 1985; Kadyk and Hartwell, 1992). Elucidation of the involved factors and pathways is ongoing and has predominantly been demonstrated in *Saccharomyces cerevisiae* (for review see (Pradillo and Santos, 2011)). It is known that meiosis-specific genes, such as Dmc1, Hop1, and Red1 as well as the mitotic factors Rad51 and Rad 54 are involved in interhomolog bias (Carballo et al., 2008; Schwacha and Kleckner, 1997). Although many

mediating proteins have been found, the mechanism regulating interhomolog preference is still debatable. One hypothesis suggests that intersister cohesion attenuated by the axial components Red1/Mek1 plays a role (Kim et al., 2010). Another favored hypothesis suggests that DMC1 filaments are directed towards the homolog by RAD51 filaments (Sheridan and Bishop, 2006; Sheridan et al., 2008). Quite likely, there are multiple mechanisms utilized; and it appears that they are also relevant in sister chromosome pairing.

The recent breakthrough from the Hiraoka lab regarding non-coding RNA as a mediator in initial chromosome recognition suggests a widespread mechanism by which organisms initiate sequence-specific pairing (Ding et al., 2012). The same mechanism may be utilized in *Aspidoscelis*, and the huge microsatellite arrays present in the *A. tigris* genome may contribute. Whether or not these microsatellite regions are transcribed is yet to be determined, however transcription of such a vast region may have the potential to positively influence sister chromosome pairing. Observing the frequency of pairing in these microsatellite regions and their transcription will help shed light on pairing mechanisms among vertebrates. Possibly, these seemingly innocuous regions play a crucial role in homolog recognition and hybrid incompatibility.

## Chromosome doubling

### Effects and Consequences

Subsequent to finding that chromosome doubling provides the basic mechanism by which parthenogenetic lizards arise, we have investigated earlier events in oogenesis to

determine the cellular mechanism responsible. It seems reasonable that such a doubling would coincide with premeiotic S-phase and the onset of meiosis; however, studies from *Oryzias* hybrids have indicated that ploidy increase may occur earlier and may be inefficient (see Chapter 1). And although not fertile, either sexually or parthenogenetically, the reproductive biology of *Oryzias* may be applicable to other vertebrates. Indeed, we have found a similar mechanism in *Aspidoscelis* parthenogens (Fig. 1). The majority of oocytes have a DNA content of 4C or less and are present in zygotene, presumably due to a pairing disability. Therefore, it is likely that doubling occurs in the mitotic germ cells and that the polyploid cells progress through meiosis. Additionally, it is likely that a population of polyploid germ cells exists.

What would be the consequences of such polyploid germ cells? Prevalent in certain cell types, such as trophoblasts and megakaryocytes, polyploid cells are generally inhibited in most tissues. The unstable nature of polyploidy seems to predispose a cell to tumorigenesis in p53 compromised tissue (Fujiwara et al., 2005). The resulting cancer appears to be influenced in part by induced aneuploidy caused by supernumerary centrosomes. Support for this stems from the fact that cancers frequently contain aneuploid karyotypes (Storchova and Kuffer, 2008). In *Aspidoscelis* parthenogens, there have been no reported propensities towards tumor formation, indicating that either tetraploid cells are stable, or that checkpoint mechanisms prevent cell proliferation outside of meiosis. Because centrosome degradation occurs during meiosis (Manandhar et al., 2005), polyploidy may be stable only at this stage. In contrast, mitotically dividing cells would encounter difficulties in division, triggering checkpoint activation and apoptosis. Alternatively, polyploid cells may have few regulations, as has been suggested

for various human cell lines (Uetake and Sluder, 2004). Thus, identification of mitotically-dividing polyploid oogonia and their properties, such as abundance, stability, and centrosome number, will undoubtedly contribute to our understanding of checkpoints in the oogenesis of lower vertebrates.

The effects of polyploid cells may not be restricted to carcinogenesis. Gene

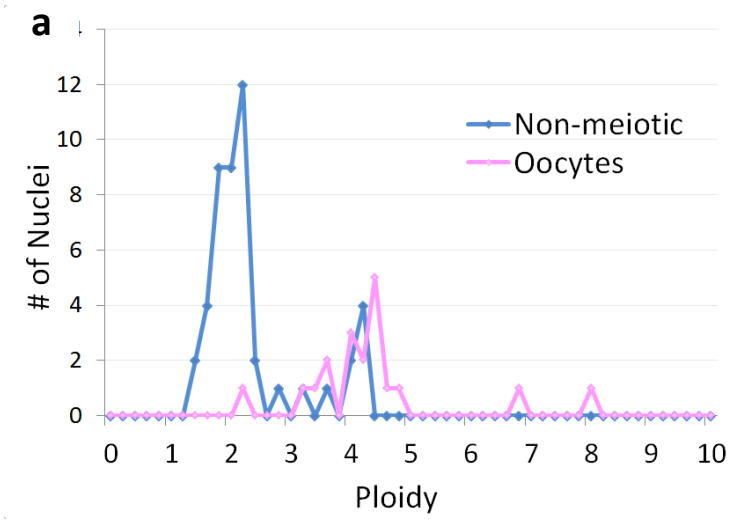
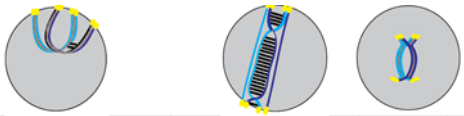



**Fig. 1. Analysis of ploidy and meiotic stage in *Aspidoscelis.* (A)** Ploidy analysis of the *A. neomexicana* germinal bed. The majority of oocytes are 4C or less. **(B)** Meiosis stage analysis of germinal beds in *A. tigris and A. neomexicana*. All identified cells in *A. tigris*, 2 months of age or older, were in diplotene. In contrast, the majority of cells were found in zygotene in the parthenogen, regardless of age.



**b**

| | Specimen | Age | Total number nuclei counted | Meiotic nuclei | % meiotic cells | Meiosis stage: tight cluster | tight to loose | loose cluster | loose to peripheral | peripheral | diplotene | ambiguous |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. tigris* (Sexual) | 1 | hatchling | 1031 | 31 | 3.0 | 0 | 0 | **28** | 0 | 0 | **3** | **4** |
| | 2 | 2 months | 500 | 4 | 0.8 | 0 | 0 | 0 | 0 | 0 | **4** | **1** |
| | 3 | 2 mo | 1040 | 6 | 0.6 | 0 | 0 | 0 | 0 | 0 | **6** | **2** |
| | 4 | 4 mo | 1136 | 11 | 1.0 | 0 | 0 | 0 | 0 | 0 | **11** | 0 |
| | 5 | 4 mo | 1003 | 10 | 1.0 | 0 | 0 | 0 | 0 | 0 | **10** | **1** |
| * → | 6 | 25 mo | 504 | 0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. neomexicana* (Partheno-genetic) * | 1 | hatching | 1024 | 6 | 0.6 | 0 | 0 | **5** | 0 | 0 | **1** | **1** |
| | 2 | 2 mo | 505 | 25 | 5.0 | **3** | **2** | **17** | **2** | **1** | 0 | **1** |
| | 3 | 12 mo | 1020 | 22 | 2.2 | 0 | 0 | **19** | **0** | **1** | **2** | **1** |
| | 4 | 12 mo | 1008 | 42 | 4.2 | **1** | **2** | **33** | **4** | **1** | **1** | 0 |
| | 5 | 12 mo | 999 | 57 | 5.7 | **3** | **5** | **31** | **3** | **11** | **4** | **3** |

***Sexual maturity**

expression may also be affected, and could have profound downstream effects in meiosis, such as in ribosomal DNA amplification, piRNA formation, and the regulation of oocyte-specific genes (Brown and Dawid, 1968; Khurana and Theurkauf, 2010; Zheng and Dean, 2007). However, if oogenesis is indeed disrupted from a ploidy increase in *Aspidoscelis*, it is certainly not detectable upon superficial comparison between unisexual and bisexual species; the fecundity of unisexual species rivals that of bisexual counterparts.

### DNA damage response and checkpoints in *Aspidoscelis* germline

While the consequences of polyploid cells may be intriguing, perhaps more interesting is *how* polyploid cells persist in *Aspidoscelis* parthenogens. As noted previously, polyploidy is tolerated in few tissues, with liver as one exception. Substantial progress has been made in understanding how these relics are able to survive. For example, tetraploid hepatocytes grown in culture do not activate the p53-mediated damage response, and divide with extra centrosomes that polarize in pairs (Guidotti et al., 2003). Functionally, tetraploid liver cells may have evolved to generate stress-induced adaptation from the resulting aneuploidy (Duncan et al., 2012). The relationship between stress and polyploidy is unclear, although there is certainly a correlation. Diseased cardiomyocytes, for example, contain higher numbers of polyploid cells (Lazzerini Denchi et al., 2006). Very little is known about DNA damage checkpoints in lizards, therefore we can merely speculate about the biology in *Aspidoscelis*. Perhaps parthenogens possess a compromised checkpoint in the ovary, allowing polyploid cells to

escape apoptosis. Alternatively, as with cardiomyocytes, the large number of failing oocytes may serve as a stressor which promotes dysfunction of dividing oogonia.

What checkpoint might be comprised in *Aspidoscelis*? Although p53 is critical for DNA damage response in tissues, including testes, whether or not it plays a significant role in oogonial apoptosis is still unclear. Irradiated ovaries from mouse p53 knockout mice undergo apoptosis normally, suggesting that the ubiquitous oncogene is not required for germ cell death (Guerquin et al., 2009). In mouse oocytes, p63 – a related family member of p53 – has been shown to be essential for the germline, albeit probably not in oogonia (Guerquin et al., 2009; Livera et al., 2008; Suh et al.). In contrast to mammals, p53 may indeed perform its important task in the germ cells of lower vertebrates. Zebrafish mutants of *fancl*, a DNA repair gene that results in massive germ cell death, are partially rescued by a p53 deletion (Rodriguez-Mari et al., 2010). Further studies are needed to ascertain the checkpoints required in the ovaries of lower and higher vertebrates. Nonetheless, a tissue-specific apoptosis factor may explain the concurrent prevalence of polyploid germ cells and negligible tumor formation in *Aspidoscelis*.

Alternatively, mutations in downstream targets of p53 can also yield an increase in cell survival. And the plethora of p53 downstream targets increases the options for tissue specificity (Slee et al., 2004; Wang et al., 2007). For example, the variable DNA damage responses downstream of the apoptosis regulator have been observed in intestinal cells (Fei et al., 2002). Dominant negative versions of many of these factors could explain the dosage-dependent phenotype observed in the tetraploids (discussed next under “cellular and genetic mechanisms”).

Another source of tetraploidy-inducing chromosome instability has been recently uncovered and involves telomere dysfunction. Resulting from shortened or unprotected chromosome ends, this type of chromosome instability was reported in liver and human cell lines (Davoli and de Lange, 2012; Lazzerini Denchi et al., 2006). Given the conservation of telomeres and their associated proteins, this detrimental mechanism may occur in other organisms. As we reported, in addition to containing true telomeres (located at the ends of chromosomes), huge telomeric sequences are also present internally in *Aspidoscelis*, with lengths on the order of megabases. How these loci affect normal function at terminal loci is unknown. Speculatively, if these internal sites interact with telomeric proteins, they may dilute protective factors from the ends of the chromosomes, thereby creating unprotected and dysfunctional telomeres.

## Cellular and genetic mechanisms

We can look to other polyploid cells to gain insight into the cellular mechanism

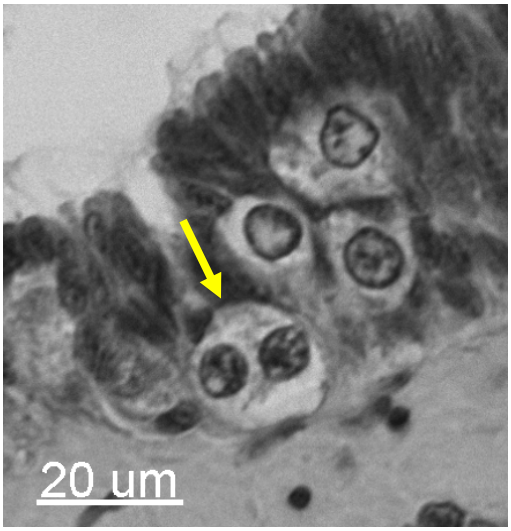



**Fig. 2. Germinal bed in the parthenogenetic species, *Aspidoscelis tesselata*.** Some oogonia and oocytes are binucleated (arrow) in both unisexual and bisexual *Aspidoscelis*. H&E stained section.

responsible in the *Aspidoscelis* germline. Binucleated cells, for example, are intermediates observed in liver, an organ in which cells frequently undergo failed cytokinesis or cell fusion. Subsequently, nuclear contents merge after nuclear envelope breakdown of the following mitosis. There is precedence for similar mechanisms in the ovary, as well. Failed cytokinesis was demonstrated in spontaneously immortalized ovarian epithelial cells in mouse. These were shown to generate near tetraploid daughter cells with carcinogenic properties (Lv et al., 2012). In *Aspidoscelis*, we have observed binucleated oogonia in the parthenogen ovary, suggesting a mechanism involving cytokinetic failure or cell fusion (Fig. 2). Interestingly, these cells are present in the bisexual species as well, implying that chromosomal doubling may not be restricted to parthenogenetic *Aspidoscelis*. More surprising is the observation of binucleated oocytes in the American alligator, *Alligator mississippiensis*, demonstrating a possible widespread phenomenon in reptilian oogonia (Moore et al., 2010). The biological significance of these cells remains to be found. They may simply be the result of rare cytokinetic errors that rapidly initiate cell death; or perhaps, they are the result of something more meaningful.

Oogonia and spermatids in both vertebrates and invertebrates inherently exhibit cytokinetic abnormalities, as demonstrated by the intercellular bridges which connect these cells. Arising from incomplete abscission of the midbody, a defect in this process could potentially yield oogonia-specific chromosome doubling. In somatic cells, tetraploidy can arise from deletion of midbody components (Carlton and Martin-Serrano, 2007; Singh and Westermark, 2011). In recent years, substantial progress has been made in understanding these enormous junctions in germ cells. TEX14, a germline specific

protein, was found to localize to intercellular bridges in testes and is essential for successful spermatogenesis (Greenbaum et al., 2006). It interacts with the ubiquitous abscission protein CEP55, preventing complete separation of germ cells. TEX14 also appears to be required for oogonial bridges and localizes to discrete foci within these regions. In contrast to males, female knockout mice are fertile, albeit with fewer ovarian follicles (Greenbaum et al., 2009). Normal abscission, as well as oogonial bridges, can be restored by overexpression of TEX14. In *Aspidoscelis*, abnormalities could arise in a variety of ways during this process. For example, a loss of abscission - and tetraploidy - could result from a gain-of-function mutation in TEX14 that mislocalized along the cleavage furrow.

An alternative hypothesis elaborates on the DNA damage response. Given the numerous zygotene-stage oocytes in the parthenogens, it is quite likely that these 4C oocytes are suspended in the telomere clustering stage for extended periods of time. Because double strand breaks are indicative of DNA damage, possibly there is a resulting chromosome instability that allows a small percentage of oocytes to increase in ploidy.

How can we interpret these possible mechanisms at the genetic level? Fortunately, in the herpetological field, several genetic analyses have been performed by crossing unisexual and bisexual species in the laboratory. While no fertile (i.e. parthenogenetic) diploids and triploids have been generated, reproductive tetraploids appear to form easily (at least within the *A. sexlineata* group), suggestive of a dosage dependent mechanism. Therefore, a gain-of-function defect as described for TEX14 seems unlikely as it would be dominant and yield fertile triploids. A dominant negative mutant, on the other hand, could explain this inheritance pattern. Consider a hypothetical scenario in which two

forms of a protein exist, WT and dominant negative (DN). To be fertile (i.e. parthenogenetic), a diploid would need a genotype of DN/WT (or the less likely double mutant), a triploid would necessitate DN/DN/WT (or DN/DN/DN), and a tetraploid could minimally be DN/DN/WT/WT. This would explain the ease with which tetraploids, and not triploids, are formed in the laboratory. A fertile tetraploid could contain two wildtype alleles provided that the other two were DN. Conflicting with this hypothesis is the discovery of the sterile tetraploid between *A. sonorae* and *A. tigris*; how can this be explained? One possibility is that genome expression levels differ in this hybrid compared with those in the *A. sexlineata* clade, thereby unbalancing the expression of the four alleles. Alternatively, the inclusion of the *tigris* genome may independently interfere with reproduction. It is difficult to draw conclusions based on one instance. In our experience with *A. sexlineata* species, tetraploids are created relatively easily. It is interesting that the doubling mechanism in *Oryzias* appears so robust, as diploid hybrids capable of diploid egg formation are generated readily in the laboratory. Perhaps this is the consequence of a physiological leniency, such as a checkpoint abnormality, in the Medaka ovary.

The dominant negative scenario could be applied to a checkpoint protein; in fact, dominant negative versions of p53 have been detected in cancers (Sun et al., 1993). Thus, a dominant negative mechanism appears plausible and could potentially explain a variety of possible mechanisms, including a cytokinesis mutant. Using the aforementioned example, a dominant negative version of CEP55 could occlude cytokinesis and yield binucleated cells. Certainly, more analysis at the cell and molecular level will be required to uncover the true mechanism.

## Future prospects

Once considered an obscure field of study, recent advancements – such as microscope technology and sequencing - have led to mechanistic insights into parthenogenesis. Now, with the accessibility of genome sequencing and RNA sequencing, the field appears to be at the onset of major discoveries, both in regards to the importance of sex as well as cellular and genetic mechanisms that enforce its persistence.

The extent to which genetic variability exists between parthenogen individuals has been cursorily studied using microsatellite analysis and fingerprinting. Although modest genetic variation among parthenogens has been demonstrated, how this relates to the remainder of the genome is unknown. Measuring changes throughout the genome can be performed relatively easily by whole genome sequencing methods and would serve as an accurate gauge of diversity. Additionally, the correlation between certain hybrid genotypes with parthenogenetic persistence can also be investigated to determine if particular traits benefit parthenogens. Further, the role of genomic shock in the formation of unisexual lineages within vertebrates is unknown. RNA expression analyses between parental and parthenogens would help elucidate the adaptability of these animals. Comparisons between sterile tetraploid hybrids (such as *A. sonorae*/ *A. tigris*) and their fertile relatives (*A. exsanguis*/ *A. inornata*) may reveal expression changes that affect reproduction.

Forthcoming prospects include descriptions of cellular mechanisms in various parthenogens. In *Aspidoscelis*, it will be interesting to determine how sister chromosomes pair and the dynamics involved in this process. Whether homologs initially try to pair is unknown. It may be that homologous interactions initiate but are disrupted and corrected

prior to diplotene. Additionally, the role of the homolog-specific microsatellite repeats is unknown; they may aid in pairing of sister chromosomes. The pairing of these loci can be determined from *in situ* hybridizations in various stages of zygotene. To further analyze the persistence of polyploid cells, studies into the DNA damage response and centrosome dynamics will be undertaken. Candidate genes can be sequenced and compared with bisexual species and sterile hybrids. Any positive candidates can be targeted for disruption or rescue, using RNAi or transfection, respectively. The conservation of this mechanism can be analyzed in other reptiles and lower vertebrates such as *Oryzias*. In regards to cellular mechanisms in the field as a whole, organisms presumed to reproduce mitotically will need to be clarified to determine to what extent they utilize meiotic proteins and how this influences their use of cryptic sex (Schurko and Logsdon, 2008).

In regards to vertebrate parthenogenesis, it will be important to uncover the variety of organisms which reproduce through facultative parthenogenesis. Because accounts are sparse and typically originate from controlled environments such as zoological parks, it is likely that the majority of facultative parthenogens remain to be discovered. By gaining information about the molecular mechanism and the genetic predispositions of such organisms, we may be able to predict an organism's reproductive plasticity without the confines of a zoo. The characteristics of facultative parthenogens, such as viability and fertility, can uncover the biological significance of this reproductive mode.

## Significance

Meiosis is an intricate process that is essential for the propagation of most eukaryotes. One of the most critical events occurs at its inception – faithful duplication of DNA during pre-meiotic S-phase. How this process is regulated without ploidy elevation remains unknown. Most studies utilize unicellular organisms *S. cerevisiae* and *S. pombe*, and although these have been instrumental in furthering our understanding of premeiotic replication, it is unclear how insights gained from yeast relate to higher organisms. *Aspidoscelis* may hold key information that links unicellular organisms and mammals. Although many organisms are not tolerant of genome duplications in the oocyte (Sun et al., 2008), such elevation appears to be relatively common in whiptail lizards, suggesting that these animals have less stringent cell cycle regulation. This relaxed stringency may allow us to study processes that otherwise cannot be observed in vertebrates without additional genetic manipulation. Finally, discovery of the mechanism that causes chromosome doubling will be a novel finding that will serve as an entry point for future studies to parthenogenesis.

The genomic loci responsible for speciation are largely unknown; however, it is likely that numerous and complex genetic changes explain the evolution between most extant species. In contrast, a single generation is sufficient to create distinct species in *Aspidoscelis* through the deregulation of oogenesis in parthenogens. Therefore, these studies will help elucidate the factors responsible for speciation in *Aspidoscelis* and other parthenogenetic animals.

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# Appendix A:

Glossary

**Allopolyploidy** – ploidy elevation in an interspecies hybrid

**Asexual** – an organism that does not reproduce by sexual reproduction

**Autopolyploidy** – ploidy elevation within one species

**Bisexual** – consisting of two sexes

**Endoreduplication** (endoreplication or endomitosis) – ploidy elevation without intervening cytokinesis

**Facultative parthenogenesis** – reproductive mode in which the organism typically reproduces by sexual reproduction, but sporadically or seasonally reproduces by asexual means

**Fisher-Muller hypothesis** – evolutionary hypothesis that attempts to explain the prevalence of sex. Sex allows for the merging of favorable genotypes from different individuals, whereas parthenogenesis would require the same mutations within a single lineage over a longer period of time

**Genomic shock** – first proposed by Barbara McClintock, it is the initial change in gene regulation that accompanies hybrid formation

**Gynogenic** – organism that requires sperm for egg activation, but does not incorporate paternal genetic material into the zygote

**Muller's ratchet** – evolutionary hypothesis that suggests that parthenogenetic species are unable to purge deleterious alleles, thus contributing to their demise in the long-term

**Obligate parthenogenesis** – reproductive mode in which the organism reproduces exclusively through parthenogenesis

**Parthenogen** – parthenogenetic organism

**Parthenogenetic** – organism that does not require sperm to reproduce. Gynogens, which require sperm for egg activation, but do not include paternal DNA, are sometimes included in this category

**Polar body fusion** – modification to meiosis in which the polar body (from either the first or second division) fuses with the oocyte to restore ploidy

**Recombinational load** – hypothesis that recombination may break up favorable genotypes

**Recombination nodule** – proteinaceous structure that is often indicative of a crossover, especially those categorized as late nodules; visible by electron microscopy

**Red Queen** – evolutionary hypothesis that suggests that co-evolution with parasites favors sexual reproduction, as sex produces novel genotypes that reduce parasitic affliction

**Synaptonemal complex** – proteinaceous structure that facilitates crossover formation during meiosis in most eukaryotes

**Unisexual** – consisting of one sex, usually female

# Appendix B:

Publications and meetings attended

## Peer-reviewed Publications:

Lutes, A.A., Neaves, W.B., Wiegraebe, W., Baumann, D.P., and Baumann, P. Sister Chromosome Pairing Maintains Heterozygosity in Parthenogenetic Lizards. *Nature* **464**, 283-286, 2010.

Lutes, A.A, Baumann, D. P., Neaves, W.B., and Baumann, P. Laboratory Synthesis of an Independently Reproducing Vertebrate Species. *Proc Natl Aca Sci U.S.A* **108**(24): 9910-9915, 2011.

Lutes, A.A., and Baumann, P. Visualizing Telomeres in Whole Mount Lizard Germinal Vesicles: Chromosome Dynamics in Meiosis. *Journal of Visual Experiments*, in preparation.

## Professional Presentations:

Lutes, A.A., Neaves, W.B., Baumann, D.P., and Baumann P. Oogenesis in *Aspidoscelis*: Comparative Analysis between Sexual and Parthenogenetic Species. Gordon Meiosis Conference, June 2012. Poster.

Lutes, A.A., Neaves, W.B., Wiegraebe, W., Baumann, D.P., and Baumann P. The Mechanism of Meiosis in the Parthenogenetic Lizard *A. neomexicana*. EMBO Conf Meiosis, Sept 2009. Oral.

Lutes, A.A., Neaves, W.B., Wiegraebe, W., Baumann D.P., and Baumann, P. The Mechanism of Meiosis in the Parthenogenetic Lizard *Aspidoscelis neomexicana.* American Society for Cell Biology Annual Meeting, Dec 2008. Poster.

Lutes, A.A., Baumann, D.P., and Baumann P. Microsatellite Analysis of *Aspidoscelis* Species. Society for Integrative and Comparative Biology Conference Annual Meeting, Jan 2008. Poster.