Evidence for Correlated Evolution of Sexual Selection and Pseudogenization in Primate Fertilization

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Abstract

The mouse sperm protein ZP3R interacts with proteins in the egg coat and mediates sperm–egg adhesion in a species-specific manner. Understanding the function and evolution of such genes has important implications for studies of reproductive success, infertility, and speciation. A recent analysis showed that (1) the human pseudogene *C4BPAP1* is the ortholog of *Zp3r*, (2) *ZP3R* pseudogenization evolved independently in eight additional primate lineages, and (3) functional *ZP3R* genes evolve under positive selection among 20 other primate species for which the gene structure is known. The causes of this pseudogenization of *ZP3R* are unknown. To explore one plausible cause (relaxation of sexual selection on males), we compared sexual size dimorphism and mating system among primate lineages with and without a functional *ZP3R*. We found that traits associated with more intense sexual selection on males (greater sexual size dimorphism, polygyny) are associated with functional *ZP3R* expression, and suggest that a relaxation of sexual selection may be associated with *ZP3R* pseudogenization. This proposed causal relationship implies an evolutionary cost to maintaining redundancy in the suite of primate fertilization genes.

Introduction

Successful fertilization is critical for all sexually reproducing organisms. The process of sperm–egg recognition is mediated in part by a suite of proteins that are expressed on the surfaces of gametes and mediate several types of cellular interactions at the time of fertilization, ranging from sperm chemoattraction to gamete fusion (Hirohashi et al., 2008). The genes that encode these gamete surface proteins are often under selection (Metz & Palumbi, 1996; Vacquier, 1998; Swanson & Vacquier, 2002). Understanding how selection shapes the evolution of gamete interaction can potentially illuminate the phenotypic and genetic causes of fertility variation and reproductive compatibility within species, and the evolution of reproductive isolation between populations and species. Understanding the molecular basis for sperm–egg interactions in mammals is of

particular interest for the development of both contraceptives (Meeusen et al., 2007) and infertility treatments (Huang et al., 2014).

Though many important molecules have been described for their role in mammalian fertilization (e.g., sperm–egg plasma membrane fusion; Inoue et al., 2013; Allingham & Floriano, 2021; Brukman et al., 2022), a complete catalogue of the genes and proteins that mediate fertilization is not known for any single species, and the identification of all genes involved in any single step in fertilization can be incomplete even in the most well-studied species (Carlisle & Swanson, 2021). The catalog of fertilization genes is known to evolve in part by whole-gene duplication, the addition of new paralogs into related fertilization functions, and the loss of functional members of such gene families (recently reviewed by Carlisle et al., 2022; Rivera & Swanson, 2022). These effects have often been revealed from knockout experiments that identify single genes as functionally important but non-essential (with normal fertility in null-null homozygotes) due to the redundant function of other genes and proteins (Ikawa et al., 2010; Avella et al., 2013; Lu et al., 2019).

A prominent example of this mode of redundancy involves sperm adhesion to the inner layer of the egg coat called the vitelline envelope or zona pellucida in mouse fertilization (Wasserman, 2009). The mouse egg coat proteins ZP2 and ZP3 interact with sperm in a species-specific manner (Wasserman, 2009), and mouse *Zp3r* (or *Zona pellucida 3 receptor*, formerly known as *Sp56*) expressed in the sperm acrosome binds ZP3 (Buffone et al., 2008). However, male *Zp3r*- / *Zp3r*- null homozygotes are fertile (Muro et al., 2012). One interpretation of those knockout results is that they indicate potential redundancy of male-expressed genes and proteins that mediate sperm binding to the egg coat. Functional redundancies in fertilization may evolve as part of the arms race between males and females driven by sex differences in optimum values for reproductive traits including the specificity or strength of sperm–egg binding interactions (Gavrilets, 2000). Thus, the non-essential function of ZP3R in fertilization could be the result of selection to recruit multiple loci into sperm–egg binding functions.

Like other aspects of research using mice as model organisms, the ZP3/ZP3R system has been useful both for understanding the evolutionary ecology of mice in nature (e.g., mechanisms of reproductive isolation; Turner et al., 2008) and as a potential model for the compatibility of human sperm- and egg-surface proteins mediating human fertility. The human ortholog of mouse *Zp3* is well known (Buffone et al., 2008), but until recently the identity of human *ZP3R* was not. One significant obstacle to identifying *ZP3R* is the history of gene duplications and pseudogenization events that are known to have occurred in both the mouse and human lineages. The RCA gene cluster on mouse chromosome 1 (chr1) includes *Zp3r* and several other members of the C4b-binding protein family of genes and pseudogenes that each encode a series of CCP (or sushi) domains (McLure et al., 2004). Mouse C4bp and human C4BPA are orthologs that can form oligomers when expressed in blood plasma and moderate the inflammatory response to infection as one of the regulators of complement activation in the innate immune system (Arenzana et al., 1995). Mouse *Zp3r* is descended from mouse *C4bp* by an ancient gene duplication event, and the two genes are adjacent to each other on

chr1, but *Zp3r* has acquired a novel expression pattern (in the testis and acrosome) and a novel function (in sperm binding to the egg coat).

By contrast, the specific region of the human RCA cluster that is syntenic with mouse *Zp3r* and *C4bp* includes only one functional gene (*C4BPA*) and a non-functional pseudogene (*C4BPAP1*) previously annotated as a paralog of *C4BPA*. One of those two loci is probably the ortholog of mouse *Zp3r*. Several previous population genetic studies focused on human *C4BPA*. Rohlfs et al. (2010) showed that polymorphisms in *C4BPA* (which they called *ZP3R*) were in linkage disequilibrium (LD) with polymorphisms in human *ZP3* (on chr7). They argued that functional interactions between those polymorphisms when expressed in the sperm acrosome and in the egg coat might respond to sexual selection for pairs of advantageous alleles (and lead to the maintenance of LD between those physically unlinked loci). Hart et al. (2018) pursued that discovery by documenting evidence for selection on three balanced polymorphisms in the coding sequences of *C4BPA*, *ZP3*, and *ZP2*; LD between the two balanced polymorphisms in *C4BPA* and *ZP2* (like the LD between *C4BPA* and *ZP3* found by Rohlfs et al., 2010); and effects of maternal *ZP2* genotype and paternal *C4BPA* genotype on two measures of fertility (Kosova et al., 2012). Hart et al. (2018) and Morgan and Hart (2019) argued that *C4BPA* is the human ortholog of mouse *Zp3r* and ignored the human pseudogene *C4BPAP1*.

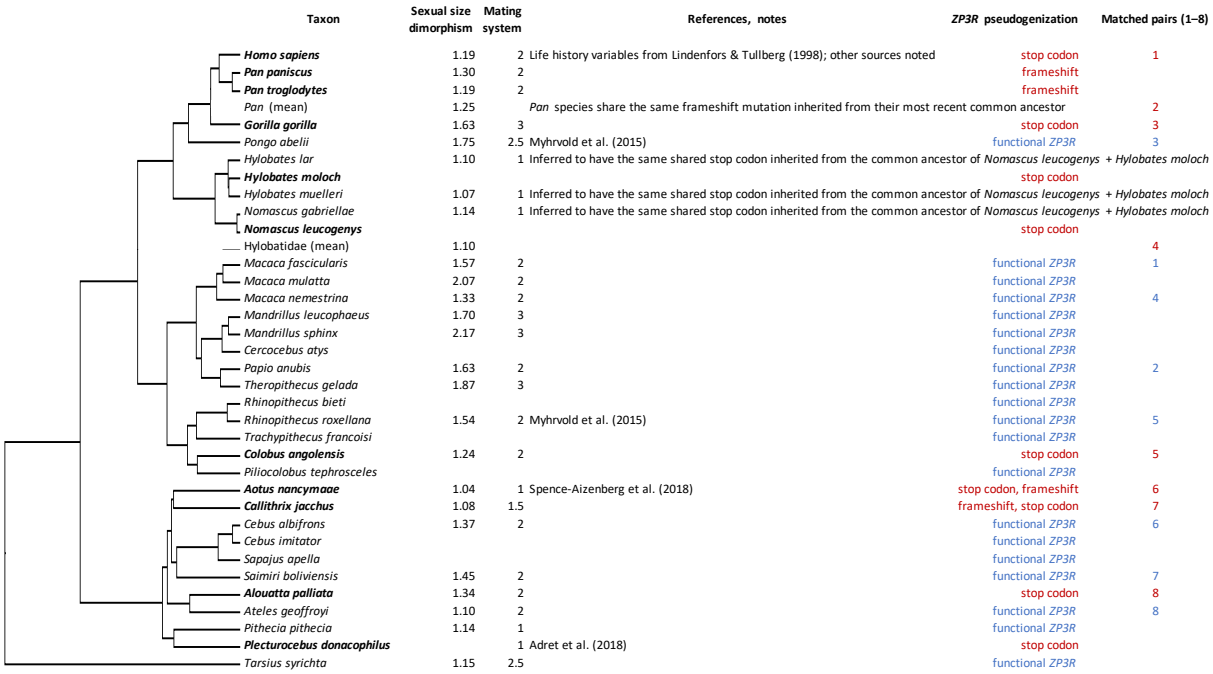
A more recent and more careful analysis by Carlisle et al. (2021) of synteny between genes in the mouse and human RCA clusters, and of phylogenetic relationships among the members of the C4b binding protein gene family, showed that *C4BPAP1* is the human ortholog of *Zp3r*. Carlisle et al. (2021) noted that *C4BPAP1* also shows highest RNA expression in the testis (Carithers & Moore, 2015), in contrast to the lack of testis expression for human *C4BPA*. Those expression data (which were also not accounted for in previous studies; Hart et al., 2018; Morgan & Hart, 2019) are consistent with a former function for human C4BPAP1 like the function of mouse ZP3R in fertilization. Together those results show unambiguously that human *ZP3R* (annotated in GRCh38/hg38 as the pseudogene *C4BPAP1*) is the functional homolog (and the genetic ortholog) of mouse *Zp3r*. Those results argue strongly against a function for C4BPA in human fertilization.

In addition to the human *ZP3R* pseudogene, Carlisle et al. (2021) identified *ZP3R* pseudogenization events that occurred independently across some but not all other primate lineages. The parallel loss of *ZP3R* function was unambiguously inferred from differences in the genomic locations and types of frameshift mutations and premature stop codons among *ZP3R* coding sequences from eight other lineages. Given the evidence for positive selection acting on divergence among functional primate *ZP3R* genes (Carlisle et al., 2021), and the general tendency for sexual selection to cause rapid divergence of genes that mediate reproductive success (Gavrilets, 2000; Uy et al., 2000; Van Doorn et al., 2001; Swanson & Vacquier, 2002; Patiño et al., 2016), Carlisle et al. (2021) concluded that “recurrent, lineage-specific gene loss events [are] suggestive of strong selection for gene loss [but] it is still unclear what is driving the loss of *ZP3r* in primates.”

Here we explore evidence for one possible mechanism driving the evolutionary loss of *ZP3R* expression: relaxation of sexual selection on males that permits or favours the loss of gene function and reduced gene redundancy in sperm adhesion to the egg coat. We use previously published life-history data to characterize variation among primate lineages in the overall intensity of sexual selection, and we identify a specific association between decreased sexual size dimorphism and *ZP3R* pseudogenization events. One interpretation of that pattern across primate lineages is that phenotypic markers of the intensity of sexual selection predict the conditions for loss (and perhaps for gain) of gene function in fertilization.

Methods

Our analysis was motivated by the results of Carlisle et al. (2021). We used data for primate *ZP3R* pseudogenization from Carlisle et al. (2021), including their discoveries of functional or non-functional *ZP3R* gene structure in 30 non-human primate species (Figure 1). Species with eight translated CCP domains and a translated transmembrane domain in the *ZP3R* coding sequence were considered to have a functional version of the gene. By contrast, species with any untranslated CCP or transmembrane domains resulting from premature stop codons or frameshift mutations were considered to have a non-functional *ZP3R* pseudogene. We analyzed 8 primate lineages with *ZP3R* pseudogenes and 14 others with functional *ZP3R* genes (Figure 1).



Taxon	Sexual size dimorphism	Mating system	References, notes	*ZP3R* pseudogenization	Matched pairs (1–8)
Homo sapiens	1.19	2	Life history variables from Lindenfors & Tullberg (1998); other sources noted	stop codon	1
Pan paniscus	1.30	2		frameshift	
Pan troglodytes	1.19	2		frameshift	
Pan (mean)	1.25		*Pan* species share the same frameshift mutation inherited from their most recent common ancestor		2
Gorilla gorilla	1.63	3		stop codon	3
Pongo abelii	1.75	2.5	Myhrvold et al. (2015)	functional *ZP3R*	3
Hylobates lar	1.10	1	Inferred to have the same shared stop codon inherited from the common ancestor of *Nomascus leucogenys* + *Hylobates moloch*		
Hylobates moloch				stop codon	
Hylobates muelleri	1.07	1	Inferred to have the same shared stop codon inherited from the common ancestor of *Nomascus leucogenys* + *Hylobates moloch*		
Nomascus gabriellae	1.14	1	Inferred to have the same shared stop codon inherited from the common ancestor of *Nomascus leucogenys* + *Hylobates moloch*		
Nomascus leucogenys				stop codon	
Hylobatidae (mean)	1.10				4
Macaca fascicularis	1.57	2		functional *ZP3R*	1
Macaca mulatta	2.07	2		functional *ZP3R*	
Macaca nemestrina	1.33	2		functional *ZP3R*	4
Mandrillus leucophaeus	1.70	3		functional *ZP3R*	
Mandrillus sphinx	2.17	3		functional *ZP3R*	
Cercocebus atys				functional *ZP3R*	
Papio anubis	1.63	2		functional *ZP3R*	2
Theropithecus gelada	1.87	3		functional *ZP3R*	
Rhinopithecus bieti				functional *ZP3R*	
Rhinopithecus roxellana	1.54	2	Myhrvold et al. (2015)	functional *ZP3R*	5
Trachypithecus francoisi				functional *ZP3R*	
Colobus angolensis	1.24	2		stop codon	5
Piliocolobus tephrosceles				functional *ZP3R*	
Aotus nancymaae	1.04	1	Spence-Aizenberg et al. (2018)	stop codon, frameshift	6
Callithrix jacchus	1.08	1.5		frameshift, stop codon	7
Cebus albifrons	1.37	2		functional *ZP3R*	6
Cebus imitator				functional *ZP3R*	
Sapajus apella				functional *ZP3R*	
Saimiri boliviensis	1.45	2		functional *ZP3R*	7
Alouatta palliata	1.34	2		stop codon	8
Ateles geoffroyi	1.10	2		functional *ZP3R*	8
Pithecia pithecia	1.14	1		functional *ZP3R*	
Plecturocebus donacophilus		1	Adret et al. (2018)	stop codon	
Tarsius syrichta	1.15	2.5		functional *ZP3R*	

Figure 1. Life history variables and *ZP3R* gene structure (from Carlisle et al., 2021) for 36 primate species or higher taxa. Sexual size dimorphism (SSD) is relative male size (male mass/female mass); higher values of the mating system index indicate greater polygyny. Derived pseudogenes are shown in red; ancestral functional genes are shown in blue. Eight matched pairs of species are indicated by red or blue numbers. Phylogenetic relationships are shown at the left. Bold species names in the phylogeny indicate pseudogenes.

We used previously published data for sexual size dimorphism (SSD) and an index of the mating system (Figure 1). Both traits are expected to covary with (and reflect past selection arising from) the intensity of male-male competition for mates or for fertilizations. We used estimates of sexual size dimorphism (male body mass/female body mass) from Lindenfors & Tullberg (1998) and Myhrvold et al. (2015). We used the index of mating system from the analysis and review by Cassini (2020), which varies from a value of 1 (social groups consisting of monogamous male-female pairs and their offspring) to 3 (social groups consisting of a dominant male and a harem group of females plus offspring), with larger values representing greater polygyny and larger among-male variance in reproductive success (see Lindenfors & Tullberg, 1998). We supplemented the data from Cassini (2020) with mating system observations from studies of other species (Adret et al., 2018; Spence-Aizenberg et al., 2018) that were not reviewed by Cassini (2020) but for which the structure of *ZP3R* was known (Carlisle et al., 2021). We used those two traits to characterize the past response to sexual selection on each species. Cassini (2020) reviewed several other life history traits that are also expected to covary with the intensity of sexual selection and noted correlations among some of those traits (including a positive association between SSD and the mating system index; Figure 2). We used these two traits (SSD, mating system) because values for them were available for the largest number of species for which *ZP3R* pseudogenization was observed by Carlisle et al. (2021).

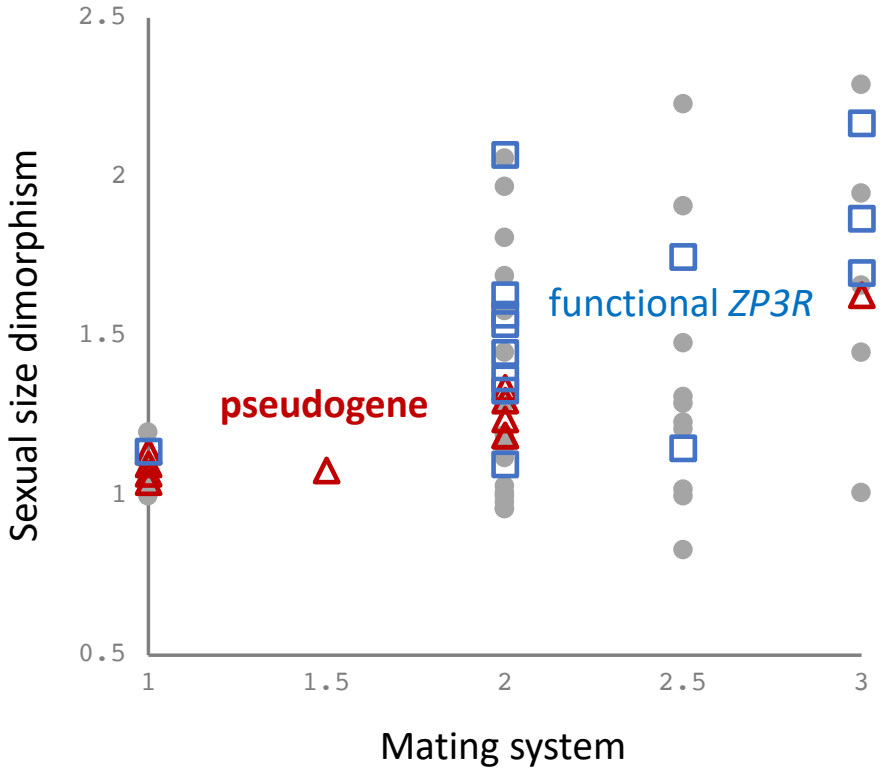


Figure 2. Sexual size dimorphism (SSD, male mass/female mass) and mating system (higher values indicate greater polygyny) for 11 primate species with a *ZP3R* pseudogene (red triangles), 14 species with a functional *ZP3R* (blue squares), and 36 other species for which the gene structure is not known (grey circles).

Because the mating system index is not a continuous trait and its value is somewhat subjective (see Lindenfors & Tullberg, 1998; Cassini, 2020), and because mating system and SSD covary among primate species, our quantitative analysis focused on variation in SSD. We calculated differences between mean SSD for 11 species (including humans) with a pseudogene and mean SSD for 14 other species with a functional *ZP3R* gene.

We used a matched pairs analysis to account for phylogenetic non-independence of changes in SSD values and *ZP3R* function. In order to focus on independent pseudogenization events, we used the mean SSD value for the two species of *Pan* that inherited the same *ZP3R* frameshift mutation (in the fifth sushi domain) from their most recent common ancestor (see Carlisle et al., 2021). Similarly, the gibbons *Hylobates moloch* and *Nomascus leucogenys* analyzed by Carlisle et al. (2021) share two of the same premature stop codons in *ZP3R* (in the second and fourth sushi domains) and were assumed to have inherited that pair of mutations from a recent common ancestor of those two genera. Consequently, we calculated the mean SSD value for three other gibbons (*H. lar, H. muelleri, N. gabriellae*) for which those data were available, and used that mean to characterize the SSD value associated with a single *ZP3R* pseudogenization event in the Hylobatidae. We downloaded the phylogenetic tree topology for 22 lineages (eight species or higher taxa in which a single independent pseudogenization event occurred, plus 14 other species that retained the ancestral functional *ZP3R*) from the 10kTrees database (Arnold et al., 2010; Carlisle et al. 2021). We used that phylogeny to match each lineage that has a *ZP3R* pseudogene to the most closely related lineage that has a functional *ZP3R*. In cases where there were several equally closely related pairs of lineages that could be matched, we chose pairs that resulted in the smallest difference in SSD within each pair. For example, among the great apes we matched the single species (*Pongo abelii*) that has a functional *ZP3R* to the species (*Gorilla gorilla*) that has the least different SSD value; we then matched each of the other three great ape lineages that have a pseudogene in order of increasing SSD (Hylobatidae, *Homo sapiens, Pan*) to the Old World monkey with the least different SSD value (*Macaca nemestrina, M. fascicularis, Papio anubis*, respectively). This approach gives a conservative estimate of the difference in SSD between pairs of lineages with and without a functional *ZP3R*.

Results

In the two-dimensional space defined by the mating system index and SSD value, species with relatively small males or a less polygynous mating system were more likely to have evolved a *ZP3R* pseudogene in comparison to species with relatively large males or a more polygynous mating system (Figure 2). This difference was evident both among species in the quadrant of that space characterized by weaker sexual selection (lower left of Figure 2) and among species in the quadrant characterized by stronger sexual selection (upper right), as well as among the large number of species with intermediate values of both traits. The distribution of both mating system scores and SSD values was not obviously different between species for which the *ZP3R* gene structure is known (from Carlisle et al., 2021) and other species for which only the life

history traits are known (Appendix 1). That similarity suggests that the sample of genomes analyzed by Carlisle et al. (2021) was probably not strongly biased with respect to primate life history variation.

Among the 11 species in which *ZP3R* pseudogenes were documented by Carlisle et al. (2021) (Figure 1), mean SSD (1.21) was much less than mean SSD among 14 other species with a functional *ZP3R* (1.56; Figure 3). Carlisle et al. (2021) noted that pseudogenization events were more common among great apes (four) relative to the number of species for which the gene structure is known (seven), but pseudogenization also occurred among Old World monkeys (one) and New World monkeys (four). The qualitative association between low SSD and pseudogenization was consistent across all three clades (e.g., functional *ZP3R* in the orangutan with the highest SSD among great apes; pseudogenes in the colobus and in the night monkey and the marmoset with lowest SSD among Old World and New World monkeys in the sample of species analyzed by Carlisle et al., 2021; Figure 3).

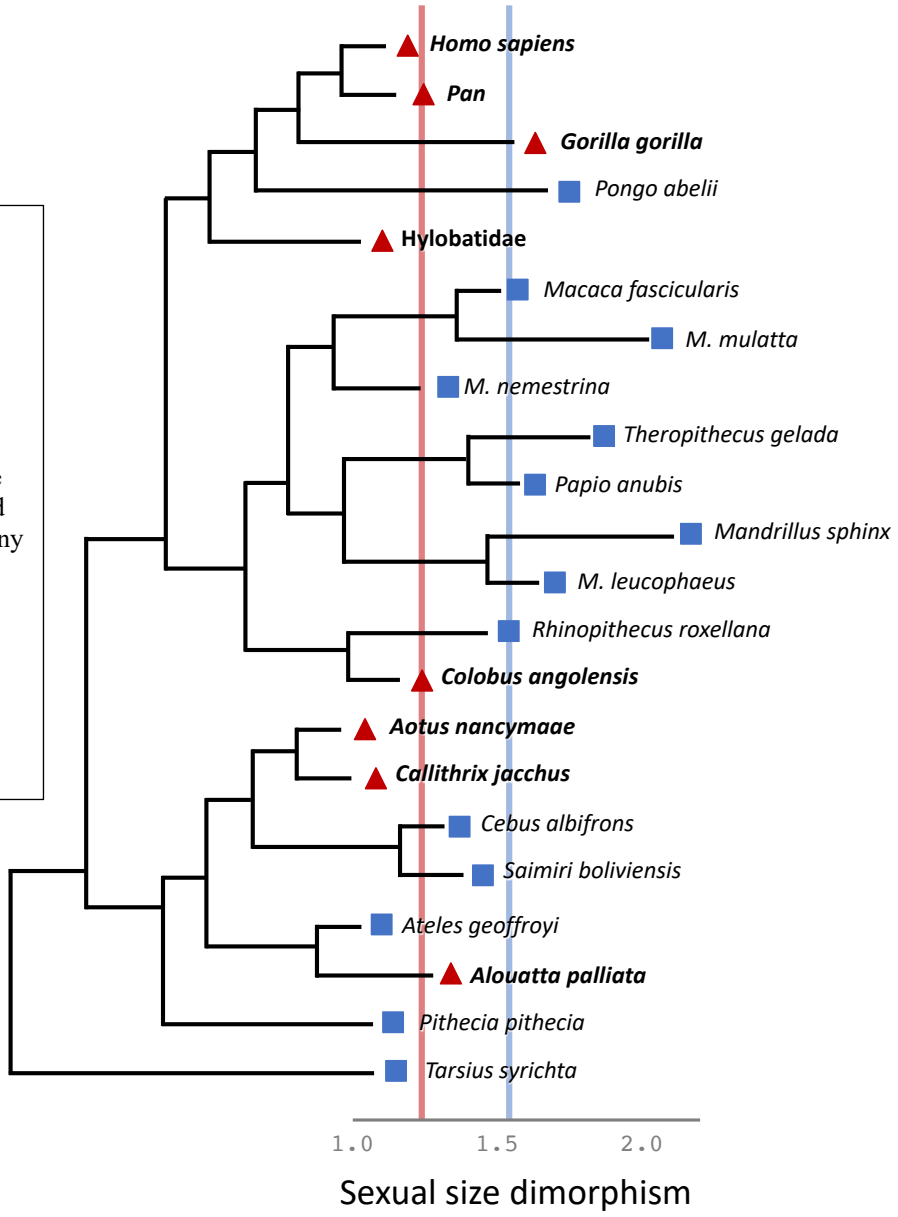


Figure 3. Sexual size dimorphism (SSD, male mass/female mass) for eight species or higher taxa with a *ZP3R* pseudogene, and 14 species with a functional *ZP3R*. Derived pseudogenes are shown in red (triangles); ancestral functional genes are shown in blue (squares). Bold species names in the phylogeny indicate pseudogenes. The phylogeny shows topological relationships only. Red and blue bars show mean SSD among all species for which the trait value is known (see Fig. 1).

Among eight matched pairs, we found seven in which the lineage with a functional *ZP3R* had greater SSD than the lineage with a *ZP3R* pseudogene (Figure 4). That pattern was consistent across pairs of lineages with overall high SSD (orangutan and gorilla) and low SSD (most New World monkeys). The direction of that difference was not significant in a two-sided sign test for matched pairs (p=0.070), but the magnitude of that difference was significant in a paired-sample t test (t=3.1, p=.017). The only exception was greater SSD in the howler monkey *Alouatta palliata* with a pseudogene in comparison to the spider monkey *Ateles geoffroyi* with a functional *ZP3R*.

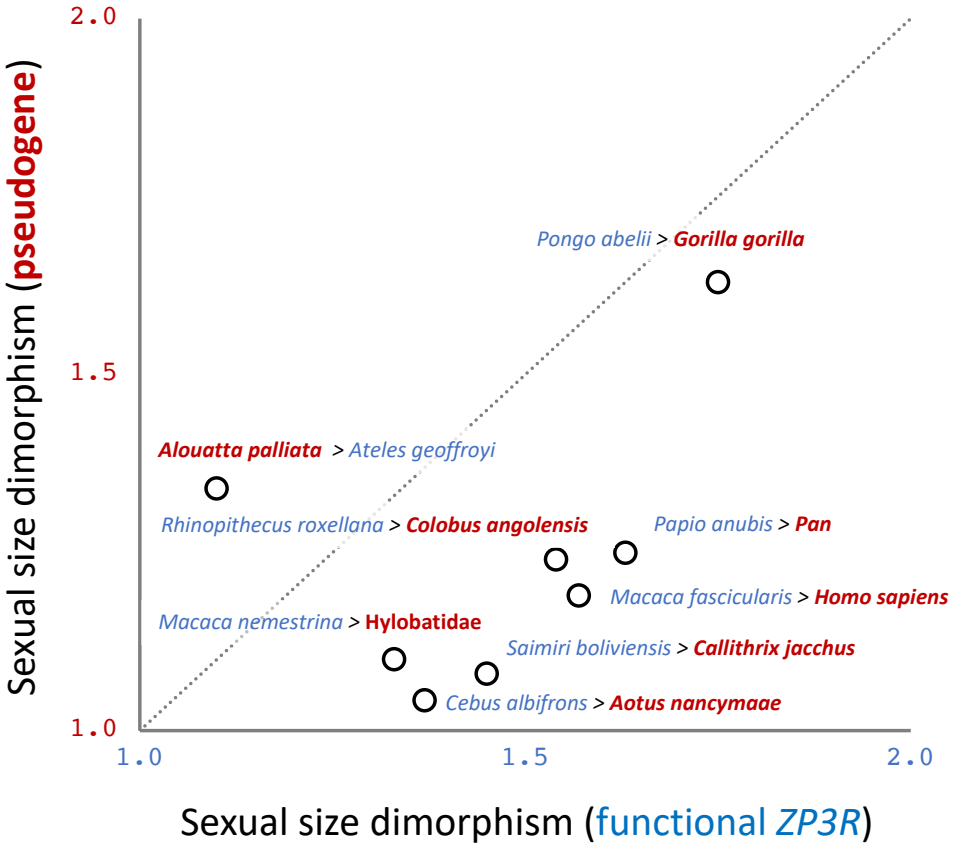


Figure 4. Sexual size dimorphism (SSD, male mass/female mass) for eight matched pairs of lineages that differ in gene structure. Derived pseudogenes are shown in red; ancestral functional genes are shown in blue. Bold species names indicate pseudogenes. The dashed line shows the 1:1 relationship; in most pairs, the lineage with reduced SSD has evolved a *ZP3R* pseudogene.

Discussion

Carlisle et al. (2021) asked "what is driving the loss of *ZP3r* in primates?" Here we develop one plausible explanation for recurrent independent *ZP3R* pseudogenization associated with variation in two traits that reflect the intensity of sexual selection. A reasonable interpretation of this pattern is that a relaxation of sexual selection on males permits or favours the loss of gene function in systems with sufficient redundancy such as sperm adhesion to the egg coat. Carlisle et al. (2021) showed that *ZP3R* evolves

under positive selection among primates with a functional version of the gene. Our findings are consistent with that result and suggest that sexual selection shapes both the divergence of functional *ZP3R* genes (among species that are experiencing strong sexual selection on males or sexual conflict between males and females; Gavrilets et al., 2000) and the loss of *ZP3R* function (among species that are experiencing weaker sexual selection or have resolved sexual conflicts).

That conclusion may depend in part on our choice of life history traits to characterize each species; other traits such as testis size (Kappeler, 1997) that might be more closely and specifically related to competition among males for fertilizations might also be better predictors of the selective conditions that favour loss of *ZP3R* function in fertilization. New studies should assay *ZP3R* structure and function in species for which testis size and other relevant traits vary. Those studies will be facilitated by the rapid accumulation of new assemblies for whole genomes from many primates, including many species for which some life history traits are already known (Lindenfors & Tullberg, 1998). Future studies could also extend the comparison of *ZP3R* structure and life history variation to the lemurs and lorises, in which complex patterns of variation in life history and mating system traits (Kappeler et al., 2017) are expected to parallel those found among apes, monkeys, and tarsiers and predict the evolution of *ZP3R* structure and function.

Carlisle et al. (2021) showed conclusively that *ZP3R* originated from a gene duplication of *C4BPA* in a common ancestor that was older than the divergence between Glires and Primates. Genes with other functions can be recruited to function in gamete binding, possibly as evolutionary solutions to complement or overcome adaptations in the other sex in the context of sexual conflicts of interest (Gavrilets, 2000) over the frequency or intensity of mating or reproductive allocation (Carlisle et al., 2022; Rivera & Swanson, 2022). How old was this gene duplication event? In the case of *ZP3R*, we do not know when this gene duplication event occurred or the evolutionary context of that duplication, or when the duplicated gene was recruited to function in gamete binding. Extending the analyses of Carlisle et al. (2021) to the other major lineages of mammals could identify the lineage in which the duplication occurred, and help to answer this question about the causes of increased redundancy in gene function at fertilization.

These future studies could also help to understand the causes of decreased redundancy in gene function when *ZP3R* (and other fertilization genes) is lost by pseudogenization. Our analysis suggests that a relaxation of sexual selection may be associated with this phenomenon. That proposed relationship implies an evolutionary cost to maintaining redundancy in the suite of primate fertilization genes under conditions of relaxed sexual selection on males or resolution of sexual conflict between mates. The nature of this cost is not known but might include high risk of fatal polyspermy relative to the reduced benefits of high-fidelity sperm binding in species with low sperm competition among males. Under this hypothesis, males in species that have resolved sexual conflict or reduced male competition under less polygynous mating systems may experience selection for lower sperm binding affinity to the egg coat as a way to lower the likelihood that two sperm from the same male fertilize the same egg

(resulting in the deaths of all three genome copies). Removing some male-expressed loci from the egg-binding repertoire of sperm could be one response to such selection, similar to changes in allele frequencies for single loci in response to changes in the male optimum for rates of sperm–egg contact (Levitan, 2012). That hypothesis also predicts that, in species that have not evolved this kind of resolution of sexual conflict, females may experience selection for lowering sperm binding affinity to the egg coat by adding new female-expressed genes to the egg coat repertoire in order to fend off supernumerary sperm and avoid polyspermy. Aagaard et al. (2013) developed extensive evidence in abalone that supports one hypothetical source of such additions via duplication of existing genes that encode sperm-binding molecules and deploying the paralogous gene products as sperm decoys that mimic the ligand for sperm molecules but do not facilitate fertilization.

The causes of decreased redundancy in human fertilization (through the evolution of the human *ZP3R* pseudogene) are of particular interest. The timing of the human pseudogenization event could point to its evolutionary cause. Because the premature stop codon in the second CCP domain of human *ZP3R* is not a polymorphism, and because both the divergence and loss of *ZP3R* among primates appears to be driven by selection (Carlisle et al., 2021), the origin and fixation of the human pseudogene is expected to be associated with a change in selection acting on the early human lineage. A careful comparison between the *ZP3R* pseudogene in modern humans and the syntenic region of chr1 in ancient human genomes (Neanderthals, Denisovans), contrasted with the functional gene in orangutans, would contribute to understanding the timing and possible circumstances of human *ZP3R* pseudogenization.

The discovery by Carlisle et al. (2021) that human *C4BPA* is not the ortholog of mouse *Zp3r* and is not a human fertilization gene greatly clarifies our understanding of the evolution of this part of human chr1. It also resolves important misunderstandings in some previous work (Hart et al., 2018; Morgan & Hart, 2019) that documented evidence of selection on human *C4BPA* and incorrectly linked that evidence to a function of C4BPA in human fertilization. Although the identity of the human ortholog of mouse *Zp3r* is now settled, the knowledge that the pseudogenization of *ZP3R* is associated with a relaxation of sexual selection among primates does point to potential effects of variation in that region of human chr1 and the RCA cluster on human reproduction. The evidence for linkage disequilibrium between polymorphisms in *C4BPA* and polymorphisms in human *ZP3* (Rohlfs et al., 2010) and *ZP2* (Hart et al., 2018), and associations between those balanced polymorphisms and variation in human fertility (Hart et al., 2018), all point toward a functional effect of human RCA variation on fertilization. However, the specific function of human RCA genes in fertilization is unknown: human *C4BPA* is not expressed in testes or sperm, and human *ZP3R* is expressed in testes but encodes a nonfunctional gene product. It is not clear how to account for this combination of evidence. One explanation is based on possible splice variants of *ZP3R* that excise the single known premature stop codon (in the second sushi domain) and encode a shorter functional gene product (of just seven sushi domains). Such a mechanism could account for the evolutionary persistence of testis-specific *ZP3R* mRNA expression in humans (Carithers & Moore, 2015). Expression data

for functional *ZP3R* genes and for pseudogenes in other primates would provide useful context for understanding the significance of the expression of the human pseudogene. We speculate that selection on *ZP3R* function, plus physical linkage between *ZP3R* and *C4BPA*, could account for evidence linking *C4BPA* polymorphisms to fertility and to the evolution of genes expressed in the egg coat. A plausible alternative explanation is that previous evidence for coevolution between human *C4BPA*, egg coat genes, and fertility (Hart et al., 2018) are false positives. We look forward to new analyses and evidence of RCA gene function in human fertilization.

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