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SEQUENCE EVOLUTION OF THE SPERM LIGAND ZONADHESIN CORRELATES NEGATIVELY WITH BODY WEIGHT DIMORPHISM IN PRIMATES

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Sexual selection has repeatedly been shown to be the probable driving force behind the positive Darwinian evolution of genes affecting male reproductive success. Here we compare the sequence evolution of the sperm ligand zonadhesin with body mass dimorphism in primates. In contrast to previous related studies, the present approach takes into account not only catarrhine primates, but also platyrrhines and lemurs. In detail, we analyze the sequence evolution of concatenated zonadhesin fragments (555 bp) of four Lemuroidea, five Platyrrhini, and seven Catarrhini, using the rate ratio of nonsynonymous to synonymous substitutions ($d_n/d_s = \omega$). Unexpectedly, subsequent regression analyzes between ω estimates for the terminal branches of a primate phylogeny and residual male body mass reveal that sequence evolution of zonadhesin decreases with increasing sexual dimorphism in body weight. Mapping published mating system classifications onto these results illustrates that unimale breeding species show a tendency for rather slow sequence evolution of zonadhesin and comparably pronounced sexual dimorphism in body weight. Female choice and sperm competition can be assumed to drive the evolution of zonadhesin. We speculate that the level of sperm competition is lower in more sexually dimorphic primates because males of these species monopolize access to fertile females more successfully. Thus, variation in sperm competition may be driving the observed negative correlation of sequence evolution and sexual dimorphism in body weight.

KEY WORDS: **Breeding system, female choice, mating system, sexual dimorphism, sperm competition, sperm–egg interaction, zonadhesin.**

With the increasing amounts of comparative sequence data, evidence is growing that positive selection plays a crucial role in the evolution of genes involved in mating behavior, fertilization, spermatogenesis, and sex determination (reviewed in Swanson and Vacquier 2002). As it affects the evolution of physical traits such as testis size, sperm morphology, and baculum length (Harcourt et al. 1981; Anderson and Dixson 2002; for review see Dixson and Anderson 2004), sexual selection may also drive the evolution of genes that influence the reproductive success of males. Increased levels of sexual selection should thus accelerate sequence evolution of genes affecting male reproductive success. The first suggestion of such a relationship came from comparative analysis of the protamine gene cluster in primates (Wyckoff et al. 2000; see also Rooney and Zhang 1999). Subsequently, Dorus et al. (2004) reported a positive correlation of sequence evolution of semenogelin 2, a main component of copulatory plugs, and the

level of sperm competition, a form of sexual selection. However, the estimates of sperm competition used by Dorus et al. (2004), that is, the number of peri-ovulatory partners and residual testis size are not available for most primate species. Moreover, except for one platyrrhine monkey serving as outgroup (Dorus et al. 2004), previous studies were confined to Hominoidea (Wyckoff et al. 2000) and Catarrhini (Dorus et al. 2004). Yet seasonality of breeding (Rowe 1996) suggest high levels of sexual selection in Lemuroidea. Finally, sexual selection may be primarily behavioral in many species, and successful males may prevent access to receptive females. Such behavior could thus reduce the opportunity for interindividual sperm competition.

ZONADHESIN

Based on a broad primate sampling, including Platyrrhini and Lemuroidea, we investigate possible correlations between the sequence evolution of the sperm ligand zonadhesin and sexual selection of males. Pseudogenization of zonadhesin might occur sporadically (see Hillier et al. 2003). However, we found no evidence for such a process in previous studies (Herlyn and Zischler 2005a,b) and, thus, consider zonadhesin a suitable subject for evolutionary studies. The ~62 kb of the human zonadhesin locus stretch over 48 exons coding for the 2811 amino acids of precursor zonadhesin (see ENSEMBL Gene: ENSG00000146839). In primates, *Sus scrofa*, and *Oryctolagus cuniculus*, precursor zonadhesin encodes for two MAM (meprin/A5 antigen/mu receptor tyrosine phosphatase) domains, a mucin-like tandem repeat, and five so-called D domains, homologous to von Willebrand factor D (Hardy and Garbers 1995; Gao and Garbers 1998; Lea et al. 2001; Herlyn and Zischler 2005a,b). The mucin-like domain of zonadhesin consists of tandemly arranged seven amino acid motifs that evolve in a concerted fashion. Contrary to this, zonadhesin MAM and D domains evolve divergently (Herlyn and Zischler 2006), apparently driven by positive selection at the level of both single codon sites (Swanson et al. 2003) and posttranslational modifications (Herlyn and Zischler 2005a,b). The processing of precursor zonadhesin leads to the formation of four subunits that partly bind to the egg's zona pellucida (see Hardy and Garbers 1995; Gao and Garbers 1998; Lea et al. 2001; Bi et al. 2003).

BODY MASS DIMORPHISM

In most primate species, long life spans, low reproductive rates, and practical difficulties in the field render it impossible to directly measure sexual selection on males (Plavcan 2004). Moreover, as mentioned above, surrogate measures of sperm competition such as residual testis size and number of peri-ovulatory partners are not available for most primate species. For both reasons, it is desirable to employ more available estimates when analyzing sexual selection of males on the basis of more comprehensive samplings. Data on body mass dimorphism fulfil the criterion of easy availability (for a compilation of body mass data in primates see, e.g., Kappeler 1991, Lindenfors 2002). Initially, a combination of haplorhine and strepsirhine data in one statistical approach might appear problematical because body mass dimorphism is much less common in strepsirhines than in haplorhines (Kappeler 1991, 1997; Lindenfors and Tullberg 1998; Plavcan 2001; Lindenfors 2002). However, in the context of the present study the compared species do not have to be sexually dimorphic. Instead, the sexes may be of similar or equal size, and females may even be larger than males. The only prerequisite is that the sample covers variance of dimorphism within primates. Another concern could be that body mass is under the influence of different selective pressures and constraints. Though this is certainly the case, recent studies have shown a strong correlation between body mass dimorphism and other indirect measures of sperm competition, such as operational sex ratio, mating systems, and male–male competition levels (Mitani et al. 1996; Plavcan 2001, 2004; Lindenfors 2002; Lindenfors et al. 2004). Therefore, we consider sexual dimorphism in body mass as another surrogate measure of sexual selection.

Materials and Methods

PCR, SEQUENCING, AND ALIGNING

As it is very difficult to obtain zonadhesin expressing tissue such as testis, new sequences were generated on the basis of genomic DNA rather than cDNA. Given the extensive intronic information covered by the zonadhesin locus (~53 kb, see ENSEMBL Gene: ENSG00000146839), we confined the present approach to the exonic information upstream and downstream of three exceptionally short introns, that is, intron 9–10 (108 bp, taking the human ortholog as reference), intron 20–21 (110 bp), and intron 32–33 (93 bp). Exons 9 and 10 encode for fragments of MAM domains 1 and 2. Exons 20 and 21 and exons 32 and 33 are located within domains D1 and D3, respectively.

Genomic DNA was extracted from different tissues of the following primate representatives: Lemuroidea (*Eulemur fulvus*, *Lemur catta*, *Microcebus murinus*, *Varecia variegata*), Platyrrhini (*Aotus azarai*, *Ateles belzebuth*, *Callicebus cupreus*, *Saguinus fuscicollis*, *Saimiri sciureus*), and Cercopithecoidea (*Cercopithecus mitis*, *Erythrocebus patas*, *Macaca mulatta*, *Mandrillus sphinx*, *Papio hamadryas*, *Pygathrix nemaeus*). Primers specific for Cercopithecoidea, Platyrrhini, and Lemuroidea (Table 1) were designed using published data from GenBank (U40024, AF244982, AF332975, AY428845-58). Fragments of 310–385 bp length comprising introns 9–10, 20–21, and 32–33, and part of the adjacent exons were amplified by wax-mediated hotstart PCR using the Taq PCR Core Kit (Qiagen). Each reaction (30 μL) contained 0.5 U Taq polymerase (AmpliTaq, Perkin Elmer), 15 pmol dNTPs, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton

Table 1. Primers used for amplification of the three analyzed zonadhesin fragments.

Location[1]	Taxon		Primer-sequence (5′-3′)	T_m (°C)
Exons 9,	cercopithecoidea-1	for	CGTGGT(AG)GGCGTTTTTG	54.0
Intron 9-10,		rev	TGCATTAGGGAAACCTCC	53.7
Exon 10	platyrrhini-1	for	TCGTGGTGGG(CT)(AG)TTTTTG	53.7
		rev	CTG(CT)(CA)CT(AG)GGGAAATCTC	54.8
	strepsirhini-1	for1	CGTGGTGGG(CT)(AG)T(ATG)TTTG	53.6
		rev1	CTCAAGGTAGATATAGTG	49.1
		for2	GGAC(AG)GATACAGTT(CT)ACCGTG	59.8
		rev2	TGGAGGGGTACATTT(CT)TATGTC	57.5
Exon 20,	cercopithecoidea-2	for	TGTCAGAAG(ATC)ACCAGGTG	54.4
Intron 20-21,		rev	CTCCAGGGCTTCACAGC	57.6
Exon 21	platyrrhini-2	for	TGTCAGAAG(ATC)ACCAGGTG	54.4
		rev	AGGGCTTCACGGCGTAG	57.6
	strepsirhini-2	for	GTGCCTGGAGAACCTGG	57.6
		rev	AGAACTGGGGCTCCCTC	57.6
Exon 32,	cercopithecoidea-3	for	TACGTGAGCTTTGATGGTAG	55.3
Intron 32-33,		rev	ATGGTGTAGATGCTGCTGG	56.7
Exon 33	platyrrhini-3	for	TATGTGAGCTTTGATGGCAG	55.3
		rev	ATCGTGTAGATGCTGCTGG	56.7
	strepsirhini-3	for	CTTCGATGGCAGTGAACAC	56.7
		rev	CTTGACAATGGTGTGGATG	54.5
Vector primers		for	GTTTTCCCAGTCACGAC	52.8
		rev	GGATAACAATTTCACACAGG	53.2

[1]Human zonadhesin as reference; T_m melting temperature.

X-100, 1.2 mg/ml bovine serum albumin, and 10 pmol each of forward and reverse primer. Depending on the primers used and the fragment length expected, PCR-conditions were as follows: 2 min, 94°C; 30–40 × (1 min, 94°C; 40 sec, 45–53°C; 20–25 sec, 72°C); 5 min, 72°C. Weak amplicons were re-amplified with lowered annealing temperature. Target amplicons were purified with gel purification spin columns (Millipore), and bi-directionally sequenced on an ABI PRISM 377 DNA Sequencer, using a Big Dye Terminator Cycle Sequencing Kit 3.1. If sequence quality turned out to be insufficient, the fragments were ligated into pGEM T-vector (Promega) and electroporated into *Escherichia coli* (TOP 10, Invitrogen) before sequencing with vector specific primers (Table 1). Conditions of vector PCR were as follows: 5 min, 94°C; 25 × (1 min, 94°C; 40 sec, 50°C; 35–40 sec, 72°C); 5 min, 72°C. Based on forward and reverse sequences, we generated consensus sequences that were aligned to the orthologs of *S. scrofa*, *O. cuniculus*, and *Homo sapiens* (see Table 2 for accession numbers), using the ClustalW algorithm implemented in BioEdit (Hall 1999). Subsequently, we concatenated the three separate alignments and excised primer binding sites and introns. The final dataset had a length of 555 bp and comprised orthologs of 16 primate species, *S. scrofa*, and *O. cuniculus*. Previously published orthologs of *Callithrix jacchus* and *Saguinus oedipus* (Herlyn and Zischler 2005a,b) have been excluded from present analysis because they represent compound sequences made of smaller consensus sequences derived from a probably polymorphic locus.

SEQUENCE ANALYSIS

We checked the final alignment for saturation using the method of Xia et al. implemented in DAMBE (Xia and Xie 2001; Xia et al. 2003). The test compares an index of substitution saturation to a critical value, beyond which the sequences fail to recover the true tree.

To assess the evolutionary regime acting on the 555 bp alignment, we employed the ratio of non-synonymous (amino acid altering) to synonymous (silent) nucleotide substitution rates ($=d_n/d_s = \omega$). Theory predicts that a sequence is under positive selection ($\omega > 1$) if many non-synonymous substitutions increase the fitness of an individual. This is a very conservative signature of positive selection. In turn, negative selection occurs and ω is <1 when mutations cause a reduction in fitness. Lastly, a sequence is expected to evolve neutrally ($\omega = 1$) when nonsynonymous substitutions have no average effect on fitness relative to synonymous substitutions. First, site-specific analysis of sequence evolution was performed using the maximum likelihood approach implemented in PAML version 3.14 (Nielsen and Yang 1998; Yang et al. 2000). The intree represented the widely accepted phylogeny within the primate sampling (see, e.g., Smith and Cheverud 2002)

Table 2. Accession numbers of the analyzed zonadhesin fragments.

Taxon	Species	Exon 9–10	Exon 20–21	Exon 32–33
Lemuroidea	*E. fulvus*	DQ910886	DQ910872	DQ904409
	L. catta	DQ910887	DQ910873	DQ904410
	M. murinus	DQ910889	AY428849	DQ904412
	V. variegata	DQ910888	DQ910874	DQ904411
Platyrrhini	*A. azarai*	DQ910894	DQ910879	DQ904417
	A. belzebuth	DQ910892	DQ910877	DQ904415
	C. cupreus	DQ910893	DQ910878	DQ904416
	S. fuscicollis	DQ910891	DQ910876	DQ904414
	S. sciureus	DQ910890	DQ910875	DQ904413
Catarrhini	*C. mitis*	DQ910896	DQ910881	DQ904419
	E. patas	DQ910897	DQ910882	DQ904420
	H. sapiens	AF332975	AF332975	AF332975
	M. mulatta	DQ910898	DQ910883	DQ904421
	M. sphinx	DQ910900	DQ910885	DQ904423
	P. hamadryas	DQ910899	DQ910884	DQ904422
	P. nemaeus	DQ910895	DQ910880	DQ904418
Outgroup	*O. cuniculus*	AF244982	AF244982	AF244982
	S. scrofa	U40024	U40024	U40024

and used orthologs of *O. cuniculus* and *S. scrofa* as outgroup sequences (Fig. 1). The codon frequency was estimated from a F3×4 matrix. Gap positions were not removed (cleandata = 0). To test for positive Darwinian evolution across sites, a likelihood ratio test (LRT) was carried out comparing models M7 and M8. To avoid local optima, we ran M8 twice with different initial ω values (0.6 and 1.6). Both models describe the ω distribution as a beta function. However, while the beta null model M7 confines ω to (0,1), the alternative beta&ω model M8 allows for a positively selected extra site class. In detail, M7 includes two freely estimated parameters, that is, p and q from the beta distribution. M8, in turn, comprises four free parameters, that is p and q from the beta distributed sites with ω (0,1), the proportion estimate of the non-positively selected site class, and ω of the positively selected site class. For LRT, twice the log likelihood difference ($2\Delta l$) between models M7 and M8 was compared to critical values from a chi-square distribution with degrees of freedom (df) equal to the difference in the number of free parameters between both models, that is, $\mathrm{df} = 4 - 2 = 2$.

In a second step, we inferred ω for the terminal branches of the phylogeny shown in Figure 1. Therefore, we ran the free-ratio model implemented in PAML 3.14 (Nielsen and Yang 1998; Yang et al. 2000) and the lineage dual model implemented in HyPhy 0.99 beta (Kosakowsky Pond and Frost 2004; Kosakowsky Pond et al. 2004). While PAML is generally based on a version of the codon-substitution model of Goldman and Yang (1994), we specified a combination of the Muse and Gaut (1994) and the Hasegawa et al.

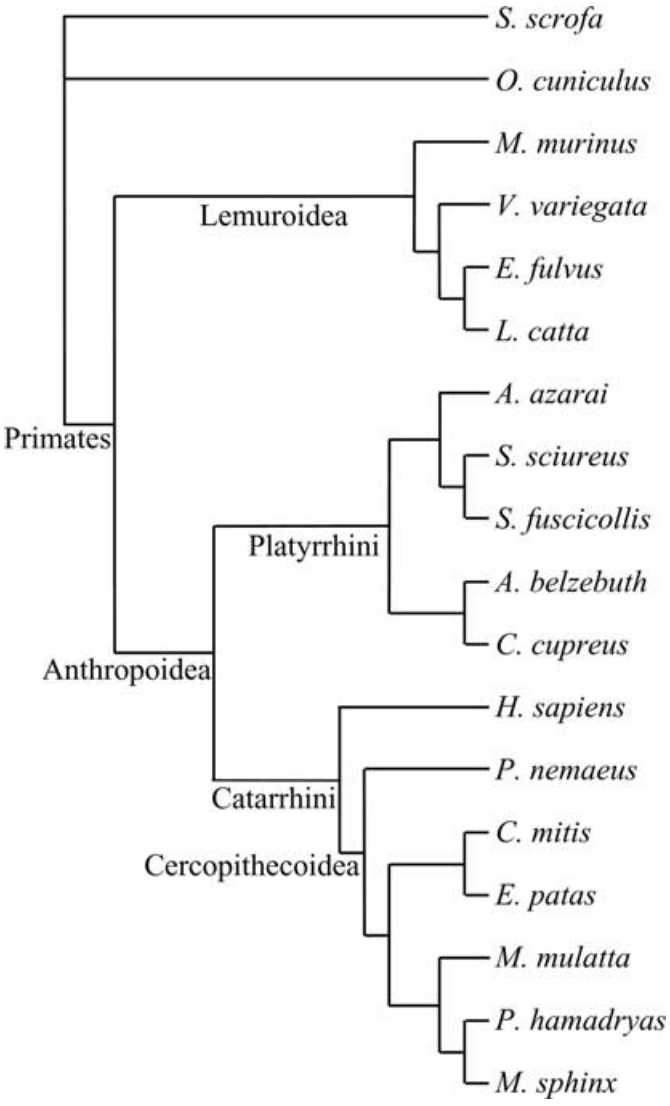

Figure 1. Probable phylogeny within the present sample (after Smith and Cheverud 2002). Superspecific taxon names (primates, anthropoidea, etc.) are written below the respective branches. For the complete Latin names see the Materials and Methods section.

(1985) model in the HyPhy menu. Moreover, we specified three synonymous and nonsynonymous site classes for HyPhy analysis. Gap positions were not excluded, neither in PAML nor in HyPhy analysis. Other parameters were set as default.

REGRESSION ANALYSIS

Linear regression analyses were conducted to test for the possible predictability of sequence evolution by sexual dimorphism of body weight, using the software implemented in the SPSS 11.0 package. Following the approach of Ranta et al. (1994), we first performed regression of male and female body weight data to assess whether ratios of male to female body weight (in case of isometry) or residual male body weights (in case of allometry) are more appropriate for further analyses. Body weight

data were taken from the literature (Kappeler 1991; Lindenfors 2002). Subsequently, we analyzed which residuals better met the requirements of linear regression, those inferred from regression of non-transformed male and female body weights or those inferred from regression of log10-transformed values. In detail, we ran a Kolmogorov–Smirnov test to determine which population of residuals better fits a normal distribution. Final regression analyses were carried out between terminal ω estimates (PAML and HyPhy) and residual male body weights to answer the initial question of a possible correlation of sequence evolution and sexual dimorphism of body weight. To assess the possible influence of phylogenetic non-independence on the results, regression analyses were repeated under exclusion of the cercopithecoid subsample. Subsequently, we mapped published mating system classifications (Rowe 1996; Garber and Leigh 1997; Lindenfors 2002) onto the graphs. In analogy to this procedure, we re-analyzed the PAML estimates of ω that Dorus et al. (2004) calculated for the terminal branches of a hominoid phylogeny (*Colobus guereza*, *Gorilla gorilla*, *Hylobates lar*, *H. sapiens*, *M. mulatta*, *Macaca nemestrina*, *Pan paniscus*, *Pongo pygmaeus*), using semenogelin II as an example. The respective body mass data were again taken from literature (Lindenfors 2002). We performed a *t*-test for the equality of slopes of the regression lines (Sachs 1992) between zonadhesin-based PAML estimates of ω and residual male body weight on one hand, and semenogelin-based PAML estimates of ω and residual male body weight on the other hand.

Results

SEQUENCE EVOLUTION AND SEXUAL DIMORPHISM

DAMBE indicates little to no saturation for the present 555 bp dataset encoding concatenated zonadhesin fragments of four Lemuroidea, five Platyrrhini, seven Catarrhini, *O. cuniculus*, and *S. scofa* ($P = 0.000$). Moreover, we did not find a single frame-shift or nonsense mutation and, thus, no evidence for pseudogenization. Instead, the 10 cysteine sites covered by the present dataset turned out to be widely conserved. The only exception is the *A. azarai* sequence that displays an arginine at amino acid position 77, instead of a cysteine. This conservation pattern speaks for the involvement of the cysteines in the formation of disulfide bridges and, thus, for their relevance in the folding of the functional protein. Given these findings, we do not expect a confounding bias of subsequent evolutionary analyses from saturation or unrecognized pseudogenes.

Site-specific PAML analysis provides strong evidence for the presence of positively selected codon sites. The alternative beta&ω model M8 calculates nearly identical parameter estimates, irrespective of the initial ω (= 0.6 and 1.6). Differences in the results of both runs are restricted to the fourth and fifth decimal places and can thus be ignored. When ω is inferred over the entire length of the sequences, model M8 indicates negative selection (ω = 0.514). Irrespective of this, about 2% of the codon sites are under strong positive selection (mean ω = 5.521). The log likelihood of beta null model M7 (= −2743.740) is clearly lower than the log likelihood of the alternative beta&ω model M8 (=−2735.746; $2\Delta l = 15.988$). Consequently, the null hypothesis (no positive selected codon sites) is rejected with high significance ($P < 0.001$; critical value from chi-square distribution with 2 df = 13.816).

Having shown by site-specific analysis that the evolution of single codons is governed by positive selection, we carried out branch-specific analyses. Repeated runs of the free-ratio model implemented in PAML calculated identical estimates for the terminal branches shown in Figure 1. The length of the dataset can thus be regarded as sufficiently long to yield stable ω estimates for each branch. Subsequently, we compared sexual dimorphism in body weight and branch-specific sequence evolution of the concatenated zonadhesin fragments. Following the approach of Ranta et al. (1994), we first assessed whether ratios or residuals represent the better measure of sexual dimorphism. The regression line between male and female body weights taken from the literature (Kappeler 1991; Lindenfors 2002) does not go through the origin and has a slope < 1 ($y = 0.785x - 0.716$, $r^2 = 0.921$, $P = 0.000$). Due to the allometric relationship of male and female body mass, we opted for residuals as the measure of sexual dimorphism, instead of ratios. According to the Kolmogorov–Smirnov test statistics the population of log10-transformed residuals better fits a normal distribution ($P = 0.989$, two-sided test) than the population of non-transformed residuals ($P = 0.192$, two-sided test). Therefore, log10-transformed residuals represent the more appropriate measure of sexual dimorphism, compared to non-transformed residuals.

Regression analyses between ω estimates for the terminal branches and residual male body mass reveal that the rate of sequence evolution of zonadhesin decreases with increasing sexual dimorphism (Fig. 2). The extremes might be best represented by *E. patas* on one hand, and *M. murinus* on the other: While ω approximates zero in the sexually dimorphic cercopithecoid *E. patas*, it reaches a maximum value of 1.421 (PAML) in *M. murinus*, a species characterized by a low residual male body mass (Fig. 2A). The HyPhy estimates (lineage dual) for the branch to *M. murinus* is even higher (= 2.562; Fig. 2B). Taking the PAML estimates of ω, the regression line has a slope of −1.326 and cuts the ordinate axis at 0.672 (Fig. 2A). About 44% of the variance is explained by the regression line ($r^2 = 0.436$) and support for the hypothesis that ω depends on the residual male body mass is highly significant ($P = 0.005$). Ignoring an unrealistic ω estimate for the *C. mitis* branch (6487), regression analysis between HyPhy estimates of ω (lineage dual model) and residual male body mass results in an even steeper negative slope of −2.262 (Fig. 2B). Stability index ($r^2 = 0.452$) and support ($P = 0.006$)

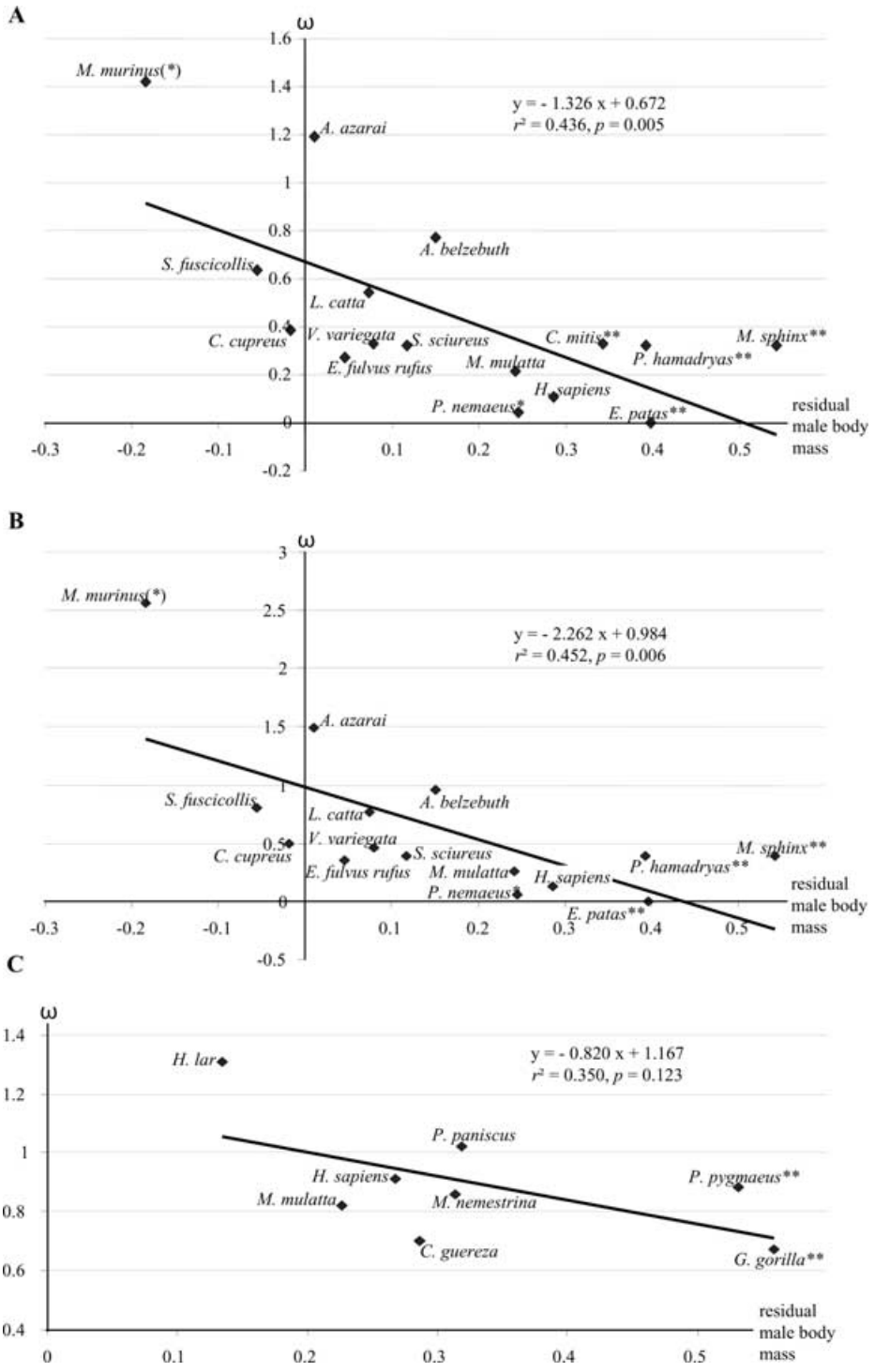

Figure 2. Regression between ω estimates for the terminal branches of a phylogeny and residual male body weight. (A) (B). The ω values have been inferred on the basis of concatenated zonadhesin fragments of 555 bp. Taking either free-ratio (PAML) (A) or lineage dual (HyPhy) estimates of ω (B) produces a regression line that reveals a negative correlation between sequence evolution and sexual dimorphism ($P = 0.005$ and $P = 0.006$, respectively). Unimale breeding species (highlighted by **) cluster to the lower right in the graphs. *Pygathrix nemaeus* is specified by a single asterisk because the species is unimale to multimale–multifemale breeding. Parentheses indicate that the mating system of *Microcebus murinus* is nominally unimale, but is actually multimale–multifemale (see Discussion). (C) The ω values are taken from a previous free-ratio analysis of semenogelin II (Dorus et al. 2004). Comparable to the results of the zonadhesin based regression analyses, there is a negative trend between sequence evolution and sexual dimorphism. Moreover, unimale breeding species (highlighted by **) again cluster to the lower right in the graph (for mating systems see Kappeler 1991; Garber and Leigh 1997; Lindenfors 2002).

are again similar to the values inferred from regression between PAML estimates of ω and residual body mass. The differences in detail can be partly ascribed to the different substitution models assumed under the free-ratio model (PAML) and the lineage dual model (HyPhy). While PAML is generally based on a version of the codon-substitution model of Goldman and Yang (1994), we ran the lineage dual model under a combination of the Muse and Gaut (1994) and the Hasegawa et al. (1985) model. Amongst others, this difference may explain why the lineage dual yields generally higher ω estimates, compared to PAML. This increase is higher when ω is high and vice versa and, therefore, the slope is steeper when the lineage dual estimates are regressed on residual male body mass, compared to the alternative use of the free-ratio estimates.

As can be seen in Figure 2, representatives of the Cercopithecoidea generally cluster in the lower right area of the graphs. To assess the possible influence of phylogenetic non-independence on the results, we performed analog regression analyses under the exclusion of the cercopithecoid monkeys. In doing so, we still found a negative correlation of sequence evolution and sexual dimorphism in body weight, irrespective of whether taking PAML estimates of ω ($y = -2.292x + 0.714$; $r^2 = 0.471$) or HyPhy estimates of ω ($y = -4.160x + 1.051$; $r^2 = 0.540$) as a measure of sequence evolution. The slope is even steeper when excluding Cercopithecoidea from analyses. The support for the hypothesis that ω depends on the residual male body mass is lower, but still significant ($P = 0.028$ and $P = 0.016$, respectively), when Cercopithecoidea are excluded from analyses. The cercopithecoid subsample thus influenced the support, but did not affect the general trend of regression analyses. Therefore, we rule out that phylogenetic non-independence is the reason for the observed negative correlation of ω and residual male body mass as shown in Figures 2A and B. But even if we presume a certain level of confounding background noise, its effect is apparently not strong enough to camouflage a negative correlation between ω and residual male body mass. We take the consistency of the results of the present regression analyses (lineage dual/free-ratio; with/without Cercopithecoidea) as evidence for the robustness of the finding that the rate of sequence evolution of the analyzed zonadhesin fragments decreases with increasing sexual dimorphism.

SEQUENCE EVOLUTION, SEXUAL DIMORPHISM, AND MATING SYSTEM

Subsequently, we combined the results from the regression analyses with published data on the mating systems in primates (Rowe 1996; Garber and Leigh 1997; Lindenfors 2002). In doing so, we found evidence for a trend between ω and residual male body mass on one hand, and mating system classification on the other hand. In detail, unimale mating species such as *C. mitis*, *E. patas*, *M. sphinx*, and *P. hamadryas* turned out to have small ω values and high residual male body masses (see species names marked with ** in Figs. 2A and B). Similarly, we calculated a very low ω value and a rather high residual male body mass for *P. nemaeus*, a species showing multimale–multifemale and unimale breeding (see *P. nemaeus** in Figs. 2A and B). In contrast, multimale–multifemale breeding and monogamous species such as *A. belzebuth* and *S. fuscicollis* show a tendency for less sexual dimorphism and higher ω values. The only exception from this general trend is the nominally unimale breeding lemur *M. murinus* that shows a high ω value and low residual male body mass (see *M. murinus*(*) in Figs. 2A and B). However, as we will outline in Discussion this seemingly contradictory observation can be easily explained by the semantic fuzziness of the term "unimale breeding." Actually, *M. murinus* is promiscuous and the breeding system might be better described as multimale–multifemale (Fietz 1999; see also Kappeler 1997).

SEMENOGELIN II

In a final step, we compared branch-specific sequence evolution of semenogelin II, a main component of so-called copulatory plugs, and sexual dimorphism in body weight. In detail, we regressed the ω values (PAML) that Dorus et al. (2004) computed for the terminal branches of a catarrhine phylogeny on residual male body mass (Fig. 2C). In accordance with the above results, the regression line has a negative slope ($y = -0.820x + 1.167$). However, stability index ($r^2 = 0.350$) and support ($P = 0.123$) are lower, compared to the results of the regression analyses shown in Figures 2A and B. Irrespective of this, *t*-test statistic shows that there is no significant difference in the slopes of the PAML-based regression lines shown in Figures 2A and C. In detail, the calculated *t*-value (=0.034) is much smaller than the critical *t*-value (two-sided) for $\alpha = 5\%$ and 20 df (= 2.09). Moreover, unimale breeding species such as *G. gorilla* and *P. pygmaeus* again cluster to the lower right of the graph (see *G. gorilla*** and *P. pygmaeus*** in Fig. 2C). Thus, the semenogelin II data corroborate that the rate of sequence evolution of genes affecting male reproductive success is lower in unimale breeding species with pronounced sexual dimorphism. On the other hand, phylogenetic non-independence might account for the smaller slope and the worse fit of the regression line between ω and residual male body mass in Hominoidea (Fig. 2C; see also Dorus et al. 2004), compared to the regression line shown in Figures 2A and B. Moreover, it can be assumed that the chosen gene (zonadhesin versus semenogelin II) influences the results of regression analyses. Finally, we cannot rule out that the relative importance of zonadhesin and semenogelin II varies between taxa such as lemurs and Anthropoidea.

Discussion

SEQUENCE EVOLUTION, SEXUAL DIMORPHISM, AND MATING SYSTEM

Polygynous haplorhines show a tendency for pronounced sexual dimorphism of body weight (Lindenfors and Tullberg 1998). Moreover, genes affecting male reproductive success have previously been shown to evolve faster in multimale–multifemale breeding catarrhines, such as *P. paniscus*, and slower in unimale breeding catarrhines, such as *G. gorilla* (Wyckoff et al. 2000; Dorus et al. 2004). Combining published information on breeding systems (Kappeler 1991; Garber and Leigh 1997; Lindenfors 2002) with a regression of sequence evolution of zonadhesin and semenogelin II on sexual dimorphism confirms both trends for a broader taxonomic sampling (present study). However, while previous related studies were confined to catarrhines (Wyckoff et al. 2000; Dorus et al. 2004), the present analysis also takes into account platyrrhines and lemurs (Fig. 2).

As foreshadowed in Results, the low residual male body weight and high ω value assigned to *M. murinus* do not disprove the general trend of slow sequence evolution and strong sexual dimorphism in unimale breeding primates (Fig. 2). Actually, *M. murinus* exhibits unimale breeding only in the sense that the territory of one male covers several female territories. Beyond this, male ranges overlap with those of many other rivals (Eberle and Kappeler 2002), and mating is promiscuous (Fietz 1999; Andres et al. 2003). In contrast to *M. murinus*, unimale mating cercopithecoids such as *P. hamadryas* and *M. sphinx* are group-living, and dominant males defend exclusive access to receptive females (Dixson et al. 1993; Colmenares et al. 2006). Given these differences, the mating system of *M. murinus* might be better categorized as multimale–multifemale (Fietz 1999; see also Kappeler 1997).

Similar to the situation in *M. murinus*, males and females of *P. pygmaeus* do not live in stable groups. Therefore, one could expect that as with *M. murinus*, *P. pygmaeus* is also characterized by a comparably high ω value and a rather low residual male body weight. A closer look at the social and mating system of *P. pygmaeus* reveals why the opposite is the case (Fig. 2). Females of *P. pygmaeus* prefer to copulate with territorial males that are very intolerant of one another whereas matings with non-territorial males occur only sporadically (Singleton and van Schaik 2002; Atmoko and van Hooff 2004; Goossens et al. 2006). Male–male competition on the postcopulatory level can thus be assumed to be lower in *P. pygmaeus* than in the promiscuous lemur *M. murinus*. These differences could explain why a maximum ω value is assigned to the *M. murinus* branch whereas a comparably low value has been calculated for the branch to *P. pygmaeus* (Fig. 2; Dorus et al. 2004). Finally, the ω value assigned to *H. lar* might appear rather high for a monogamous species (Fig. 2; Lindenfors 2002; Dorus et al. 2004). However, evidence is growing that extra-pair matings occur more often in gibbons than is commonly assumed (Fuentes 2000; Sommer and Reichert 2000) and sexual selection on males might thus be stronger in *H. lar* than suggested by the monogamy category. These examples illustrate that even seemingly contradictory findings can be brought in line with a general trend for lower rates of sequence evolution of zonadhesin and semenogelin II and stronger sexual weight dimorphism in unimale breeding primates, compared to multimale–multifemale breeding and monogamous primates (present study).

SEQUENCE EVOLUTION AND SEXUAL DIMORPHISM

The main finding of the present study is that sequence evolution of zonadhesin slows down in primates with more pronounced sexual dimorphism in body weight (Fig. 2). Though a causation cannot be conclusively inferred from the observed negative correlation, we assume that sexual selection represents the driving force behind the sequence evolution of zonadhesin. As suggested by the name, zonadhesin contributes to the adhesion of the spermatid to the zona pellucida (Hardy and Garbers 1995; Lea et al. 2001; Bi et al. 2003). Cryptic female choice exerted by a constantly changing zona pellucida receptor might thus drive single codon substitutions. On the other hand, the present data reveal a negative correlation of sequence evolution and sexual weight dimorphism, a parameter that, in turn, correlates with several measures of sperm competition, such as operational sex ratio, mating systems, and male–male competition levels (Mitani et al. 1996; Plavcan 2001, 2004; Lindenfors 2002; Lindenfors et al. 2004). Furthermore, the sequence evolution of zonadhesin as well as that of semenogelin II is slower in primates with stronger sexual weight dimorphism. Yet the probable driving force behind the sequence evolution of the copulatory plug component semenogelin II is sperm competition (Dorus et al. 2004). Therefore, we assume that a combination of cryptic female choice and sperm competition accounts for the sequence evolution of the analyzed zonadhesin fragments.

In a variety of taxa, including insects (Wicklund and Forsberg 1991; Wedell 1997) and fishes (Parker 1992), sexual weight dimorphism co-varies with sperm competition across species. There is ample evidence from both interspecific and intraspecific comparisons that sperm competition increases not only male body size, but also male physical traits such as testes size. Hosken (1997), for instance, found a positive relation between testes mass and group size across bat species (Hosken 1997). Similarly, Pitcher et al. (2005) reported larger testes in colony-breeding birds, compared to solitary breeding species. However, the principle is not confined to vertebrates, as the example of flatworms of the genus *Macrostomum* may illustrate (Schärer 2004). Contrary to a positive relationship of sperm competition and dimorphism in a broad range of taxa we observe a negative correlation between sperm

competition mirrored by an increased rate of sequence evolution at the zonadhesin locus and sexual weight dimorphism in primates. This negative relationship suggests two extremes of male primates counteracting male–male competition. Either they compete on the postcopulatory level of sperm competition, or they compete precopulatory on the behavioral level, thus lowering the level of sperm competition. In the first scenario, males and females are of similar size, fertile females mate with multiple partners and the rate of sequence evolution of zonadhesin and semenogelin II is increased. In the latter scenario, males are the larger sex and defend exclusive access to fertile females. We speculate that the intensity of sperm competition is lower in more sexually dimorphic species because males more successfully fend off male competitors. Thus, the negative correlation of weight dimorphism and pace of sequence evolution at the two presented loci in primates can be speculated to result from different sexual selection regimes on the behavioral and sperm competition level.

CONCLUSIONS

The present study provides initial evidence for a negative correlation of sequence evolution of the sperm–ligand zonadhesin and sexual dimorphism in body weight. The findings support a general trend of lower rates of sequence evolution and stronger body weight dimorphism in unimale breeding primates, compared to monogamous and multimale–multifemale breeding species. However, contrary to previous related studies the present approach is based on a sampling comprising not only catarrhines, but also platyrrhines and lemurs. Probably, a combination of female choice and sperm competition drives the evolution of zonadhesin. We speculate that sperm competition is lower in more sexually dimorphic primates because males manage more successfully to defend exclusive access to receptive females. As a consequence, the present findings suggest two principally different ways for male primates to counteract male–male competition. Either they compete on the postcopulatory level of sperm competition, or they compete precopulatory on the behavioral level by monopolizing oestrous females, thus lowering the level of sperm competition. Future analyses will show whether the rate of sequence evolution of genes affecting male reproductive success is generally lower in primates and in other mammal species with more pronounced sexual dimorphism in body weight. Such a negative correlation would open up new perspectives for the comparison of sperm competition and sequence evolution because data on body mass are more available than data on, for instance, testis size.

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