

Sexual and Temporal Dynamics of Molecular Evolution in *C. elegans* Development

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Dissection of the phenotypic and molecular details of development and differentiation is a centuries-old topic in evolutionary biology. However, an adequate understanding is missing for the molecular evolution of genes that are expressed differentially throughout development—across time, tissues, and the sexes. In this study, we investigate the dynamics of gene evolution across *Caenorhabditis elegans* ontogeny and among genes expressed differentially between each sex and gamete type. Using gene classes identified by genome-wide gene expression developmental time series and comparative sequence analysis with the congener *C. briggsae*, we demonstrate that genes expressed predominantly after reproductive maturity evolve more rapidly than genes expressed earlier in development and that genes expressed transiently during embryogenesis evolve faster than other embryonic transcripts. These results are indicative of relaxed selection on genes expressed after maturity, in accord with the mutation-accumulation model of aging. Furthermore, genes involved in spermatogenesis reveal more rapid evolution than other phenotypic classes of genes. Average rates of evolution among male soma-related genes indicates that selection acts to maintain males in these androdioecious species, despite their rarity, and the rapid evolution of sperm genes suggests that sexual selection acts on sperm development and function.

Introduction

Organismal development is the pattern and process of differentiation from zygote to adult. Morphological descriptions provide more than a century of results, yet molecular details have a more recent history and genome-scale perspectives on the molecular evolution of development are only now becoming feasible (Jones et al. 2001; Castillo-Davis and Hartl 2002; Arbeitman et al. 2002; Baugh et al. 2003; Reinke et al. 2004). Despite rapid growth in our knowledge of the cellular and genetic bases of development across ontogeny and between the sexes for the nematode *Caenorhabditis elegans* (Brenner 1974; Sulston and Horvitz 1977; Sulston et al. 1983; Wood 1988; Riddle et al. 1997), understanding of molecular evolutionary patterns in this organism has lagged behind. Here, we describe the relationships between rates of protein evolution and *C. elegans* development and infer how natural and sexual selection shape molecular evolution across the genome.

Pervasive throughout the development literature is the notion that early stages of ontogeny are more similar across species and, therefore, are most evolutionarily constrained (Arthur 1988; Raff 1996). This hypothesis follows from the idea that mutational changes to components of early development will generate a larger cascade of effects than changes acting later, because of pleiotropy among a large suite of genetic interactions (Raff 1996; Galis and Metz 2001). Consequently, mutations affecting early development are expected to be subject to stronger purifying selection because most nonneutral mutations are detrimental (Li 1997), and nucleotide substitution rates at amino acid changing sites will be lower among genes associated with earlier development (Arthur 1988). Empirical support for

this model of developmental molecular evolution is mixed, however. For example, rates of protein evolution show no difference among early-expressed versus late-expressed genes in *C. elegans* embryogenesis (Castillo-Davis and Hartl 2002), whereas some data in *Drosophila melanogaster* are consistent with this “pleiotropy model” (von Dassow et al. 2000; Galis, van Dooren, and Metz 2002; J. Davis, personal communication). An alternative explanation for the strong morphological conservation that is observed in early developmental stages across species is that it arises from modular genetic networks that are robust to mutation (Goodwin, Kauffman, and Murray 1993; von Dassow et al. 2000). If this were true, we should not expect to see a positive association between rates of protein evolution and timing of developmental expression. Across the longer developmental timescale separating life stages before and after the onset of reproduction, selection may be important in the context of senescence. The decreasing reproductive value of individuals after maturity relaxes selection and might allow late-expressed genes and phenotypes to evolve more rapidly because of the chance accumulation of mutations (Medawar 1952; Charlesworth 1994; Promislow and Tatar 1998; Partridge 2001). This argument led to the mutation-accumulation theory of aging and senescence (Medawar 1952).

Sexual selection theory proposes that traits expressed differentially in the development of each sex, and the genes underlying those traits, will often evolve rapidly. This follows from models of selection whereby mate preferences or intrasexual and intersexual conflicts can cause traits that are limited to either males or females (e.g., sperm and egg characteristics) to evolve in an arms-race fashion, potentially leading to reproductive isolation and speciation (Fisher 1958; Andersson 1994; Rice 1998; Parker and Partridge 1998; Palumbi 1998; Gavrillets 2000; Singh and Kulathinal 2000). Many examples of faster gender-related and gamete-related gene evolution have been described in outbreeding taxa, particularly for male and sperm genes (Singh and Kulathinal 2000; Swanson and Vacquier 2002). For species with alternative breeding

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systems, such as the androdioecious (hermaphrodite/male) *C. elegans*, it is less clear whether similar patterns of evolution might emerge. Sexual selection is a much weaker force on hermaphrodites in general (Greeff and Michiels 1999), and opportunities for male conflict in mating or sperm competition will be restricted in *C. elegans* because males are rare (Stewart and Phillips 2002; Chasnov and Chow 2002; Cutter, Avilés, and Ward 2003; Cutter and Payseur 2003b). Nonetheless, inseminated male sperm must compete with hermaphrodite self-sperm and substantial heritable variation in male sperm competitive ability is found among different strains of *C. elegans* (Lamunyon and Ward 1995; Hodgkin and Doniach 1997; Lamunyon and Ward 2002).

In this study, we characterize the dynamics of protein evolution across *C. elegans* ontogeny and among genes expressed differentially between each sex and gamete type. From microarray-based developmental categories of genes in *C. elegans* (Baugh et al. 2003; Reinke et al. 2004), we calculated rates of evolution for orthologs in the congener *C. briggsae* (Stein et al. 2003). We demonstrate that genes expressed predominantly after reproductive maturity evolve more rapidly than larval genes. Furthermore, sperm genes (expressed both in males and in hermaphrodites) evolve more rapidly and have fewer orthologs with *C. briggsae* than most genes in the worm genome. These patterns are consistent with theories of senescence that propose relaxed selection late in life and with the operation of sexual selection on sperm genes.

Methods

Molecular Evolution Analysis

We identified *C. elegans*–*C. briggsae* orthologs with a reciprocal best-hit Blast approach (Rivera et al. 1998; Cutter et al. 2003) from the Wormbase gene predictions in WS110 for *C. elegans* and the “hybrid” *C. briggsae* gene set (Stein et al. 2003), with paralogs defined as those genes with better within-genome than between-genome bitscore values. Genes were categorized as orphans when neither a paralog nor an ortholog was identified at $E < 10^{-6}$.

Ortholog conceptual amino acid translations were aligned using GCG Gap, from which rates of site substitution of the corresponding nucleotide sequences were computed with several methods: Li method in Diverge (Li, Wu, and Luo 1985; Pamilo and Bianchi 1993; Li 1993) and maximum-likelihood and Nei-Gojobori methods with codeml in PAML (Yang 1997). We report only analyses using the Li method (K_A), given the strong correlation among all three algorithms for estimating nonsynonymous site divergence (figure 4 in Supplementary Material online). Genes for which K_S could not be computed were excluded in analyses of site substitution, leaving 7,281 orthologous loci with data for both expression levels and site-substitution rates. Codon usage bias (F_{op}) of genes from both species were computed with CodonW (J. Peden; <http://www.molbiol.ox.ac.uk/cu/codonW.html>). *C. elegans* optimal codon designations were used for both species because no significant departures in optimal codons have been identified (Stein et al. 2003). Other sequence features (e.g., intron number) were extracted from the predicted

exon structures using Perl scripts. Synonymous substitution rates were adjusted for correlation with *C. elegans* F_{op} (Ikemura 1985; Stenico, Lloyd, and Sharp 1994; Sharp and Bradnam 1997) to yield the adjusted synonymous substitution rate estimator δ_S as in Cutter and Payseur (2003a). The principle trends were identical whether K_A or K_A/δ_S values were used (e.g., fig. 1C), so we focus on K_A because of the observed saturation of synonymous sites.

Phenotypic and Developmental Gene Categories

The expression data set of Reinke et al. (2004) was used to partition genes into phenotypic classes associated with sperm, oocytes, germline (“intrinsic”), soma, all tissues (“ubiquitous”), males, hermaphrodites, or both males and hermaphrodites (“shared”) according to the definitions in their “exclusive categories” designation. The experimental design yielded some genes that showed expression patterns that were elevated in “sperm” or “oocyte,” but could not be associated with either male or hermaphrodite germline; such genes are denoted “other sperm” or “other oocyte” (Reinke et al. 2004). For convenience, we use the term “sperm genes” to refer to genes expressed during spermatogenesis, recognizing that transcription does not occur in sperm per se (Ward, Argon, and Nelson 1981). Average log-transformed substitution rates for orthologs were then calculated for these gene categories, and 3×2 contingency table tests were used to identify nonrandom distributions of these gene categories among orthologs, paralogs, and gene orphans. Expected gene counts for each category were calculated from the global frequency of ortholog, paralog, and orphaned genes scaled to the total number of genes observed in a given category. The functional categorizations correspond to the Gene Ontology designations in Reinke et al. (2004).

To quantify the trends of molecular evolution and codon bias over 12 timepoints (T_i) from larvae (L3 and L4) to adult, we employed three approaches: (1) partitioning evolutionary rates in bins of peak expression level (L3: T_1 – T_3 , L4: T_4 – T_6 , and adult: T_7 – T_{12}), (2) partitioning evolutionary rates among gene expression clusters (A to C larval and D to F adult) defined by Reinke et al. (2004), and (3) correlation of expression level with substitution rates and codon bias (nonparametric Spearman’s ρ). For the binning method, timepoints T_3 and T_7 represent transitions between stages; for simplicity we arbitrarily assigned these timepoints to a single bin. For the correlation method, high expression ratios of a sample at time T_i relative to a standard mixed-stage population indicate elevated gene expression at that timepoint (Reinke et al. 2004). Consequently, more positive correlation coefficients signify higher evolutionary rates (or codon bias) in the given timepoint relative to genes expressed in the mixed-stage control population.

The embryonic expression data of Baugh et al. (2003) were analyzed in a similar way to infer relative rates of protein evolution over the course of embryogenesis. Embryonic gene expression levels were derived from 10 points in embryogenesis up to 165 hours after the four-cell stage from an Affymetrix array study (Baugh et al. 2003). We partitioned substitution rates among nine mutually

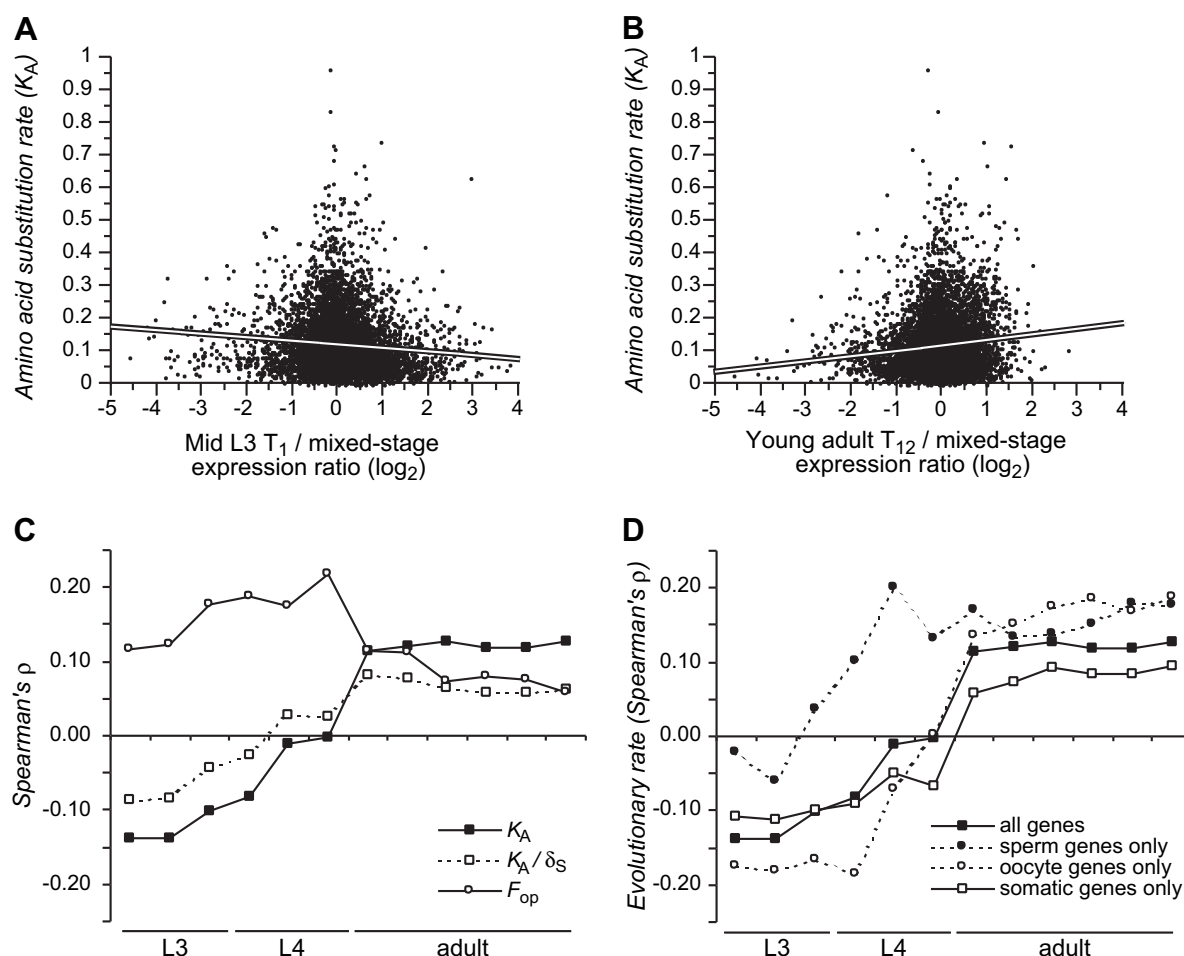


FIG. 1.—Evolutionary rates across larval and adult development. Higher correlation coefficients indicate faster rates of protein evolution for genes that are expressed predominantly in the given time sample. Larval gene expression levels correlate negatively with amino acid substitution rate (A), whereas gene expression correlates positively with amino acid substitution rate during adult timepoints (B). Lines in (A) and (B) indicate a linear regression line, the slope of which is analogous to the correlation coefficients in (C) and (D). (C) Molecular evolution across development as measured by the correlation (Spearman's ρ) between the nonsynonymous site substitution rate (adjusted, K_A/δ_S , or unadjusted, K_A , for synonymous site substitution rate) and expression level ratio. Correlation coefficients for codon bias (F_{op}) indicate the strength of selection for translational efficiency (higher values indicate stronger codon bias). (D) Molecular evolution across development separated into sperm genes, oocyte genes, and somatic genes (gamete and germline genes excluded) using the K_A -expression correlation coefficient. Spearman's ρ is significantly different from zero for all points ($P < 0.04$, except “all genes” and “oocyte genes only” T_5 and T_6 and “sperm genes only” $T_1 - T_4$).

exclusive expression classifications and among the expression pattern clusters in their supplementary material (Baugh et al. 2003). The nine categories depend on four patterns of expression to yield the groupings given in table 2: maternally derived (present M, absent m), embryonically derived (present E, absent e), transiently expressed (present T, absent t), degraded maternal transcript (present D, absent d), or no clear pattern. The set of genes classified as MDET were excluded from the grouping of other “transient” genes because of the highly divergent expression profile of this group compared with METd and ETmd genes (see figure 8b in Baugh et al. [2003]), such that MDET gene expression tends to be transiently reduced rather than increased. Wilcoxon rank-sum tests, G -tests, and ANOVA were used to test for differences among categories. Variables were $\log_{10}$ transformed as appropriate to improve fit to a normal distribution for parametric statistical analyses.

Results

Evolution Across Late Ontogeny

In groups of genes defined by the stage at which their expression is maximal (larval L3, L4, adult), adult-related genes evolve significantly faster than larvae-related genes ($F_{2,6874} = 99.7$, $P < 0.0001$ [table 1]). Synonymous substitution rates (δ_S) also are higher for adult-related genes ($F_{2,6874} = 92.1$, $P < 0.0001$ [table 1]), yet when K_A is adjusted for δ_S , this class of genes still shows an elevated rate of sequence evolution ($F_{2,6874} = 38.7$, $P < 0.0001$ [table 1]). The expression clusters defined by Reinke et al. (2004) show an identical pattern in that both K_A ($\chi^2 = 298.0$, $df = 1$, $P < 0.0001$) and K_A/δ_S ($\chi^2 = 87.3$, $df = 1$, $P < 0.0001$) are higher among genes in clusters D, E, and F associated with adult expression than among larval A, B, and C. This result holds when gamete and germline genes are excluded (K_A : $\chi^2 = 52.4$, $df = 1$, $P < 0.0001$; K_A/δ_S : $\chi^2 = 15.4$, $df = 1$, $P < 0.0001$).

Table 1
Site Substitution Rates for Genes with Larval and Adult Peak Expression

Peak Expression	n	Median K_A	Mean K_A (SE)	Median δ_S	Mean δ_S (SE)	Median K_A/δ_S	Mean K_A/δ_S (SE)
L3	2847	0.080	0.102 (0.0015) ^a	1.30	1.35 (0.0067) ^a	0.066	0.079 (0.0010) ^a
L4	1559	0.100	0.120 (0.0022) ^b	1.33	1.38 (0.0089) ^b	0.079	0.092 (0.0015) ^b
adult	2471	0.111	0.136 (0.0020) ^c	1.43	1.48 (0.0071) ^c	0.080	0.092 (0.0012) ^b

NOTE.—Different superscripted letters indicate Tukey honestly significant differences; significance for K_A and K_A/δ_S based on $\log_{10}$ transformed values.

We corroborated this result using the correlation coefficient measure of sequence evolution: gene expression in larval (L3 and early L4) worms correlates negatively with rates of protein evolution, indicating that genes tend to evolve more slowly when they have high expression during these larval stages relative to the mixed-stage control population (fig. 1A, C, and D). In contrast, the positive correlation in samples of adult-age worms demonstrates that genes overexpressed in adults tend to evolve more rapidly (fig. 1B, C, and D). The correlation coefficients differ significantly from zero at all timepoints (excepting the 5th and 6th timepoints during L4) for the analyses that include all genes ($P < 0.0001$). Genes identified by Reinke et al. (2004) to have enriched expression during spermatogenesis (sperm genes) account for much of the elevation in average rate of protein evolution during the L4 and young adult stages, whereas oocyte and germline-related genes partially account for the lower rates of evolution of genes more commonly expressed in L3 (fig. 1D). However, the elevated evolutionary rate in L4 and adult is still significant for somatic genes alone (fig. 1D) ($P \leq 0.0003$ for all p). Furthermore, genes that change their expression from L3 to adult (“modulated” genes [Reinke et al. 2004]) evolve faster than nonmodulated genes ($\chi^2 = 5.1$, $df = 1$, $P = 0.024$).

Evolution in Early Embryogenesis

Our analysis of the embryonic expression data of Baugh et al. (2003) shows no unidirectional trend in evolutionary rate over the course of approximately 165 hours in early development: expression of embryonic genes is associated with high evolutionary constraint overall (fig. 2). However, genes with transiently elevated expression in early embryogenesis (ETmd and METd) exhibit significantly higher rates of protein evolution than genes with other embryonic expression profiles ($F_{2,3661} = 21.0$, $P < 0.0001$ [table 2]). These genes represent embryo-produced transcripts expressed temporarily during the transition between maternal and embryonic control of development.

Evolution of Gender and Gamete Loci

We calculated rates of synonymous and nonsynonymous site substitution from ortholog comparisons between *C. elegans* and *C. briggsae* and subdivided them among the mutually exclusive categories of gamete and gender associations identified by Reinke et al. (2004) (see *Methods*). Proteins encoded by sperm genes in both males and hermaphrodites evolve more rapidly than most other types of genes (table 3). By contrast, genes with ubiquitous expression in males, but not in sperm, fall into the slowest evolving gene category, and oogenesis-related and

hermaphrodite-related genes generally have average rates of evolution (table 3). The average rate of evolution for male genes associated with the soma is comparable to other gene classes, but they have a particularly high variance, suggesting that this group may contain subsets of genes subject to a variety of selection pressures. Unlike those sperm genes that are expressed disproportionately in males and/or hermaphrodites, sperm genes that do not show elevated expression in either sex (“other sperm”) evolve at rates comparable to most genes in the genome (table 3). Sperm and male genes also tend to have short coding sequences, high codon bias, and disproportionate representation among gene orphans (figure 3, and figure 2 in Supplementary Material online).

Rates of protein evolution for male-related genes on the X chromosome are significantly lower than on the autosomes (all male $\chi^2 = 8.8$, $df = 1$, $P = 0.003$; soma only $\chi^2 = 5.5$, $df = 1$, $P = 0.019$). This can be partly explained by the slower evolution of X-linked loci overall ($\chi^2 = 14.8$, $df = 1$, $P < 0.0001$), but male X-linked loci evolve even more slowly than other loci on the X chromosome ($\chi^2 = 4.7$, $df = 1$, $P = 0.03$ [table 4]). Male-related genes are not disproportionately represented on autosomes in *C. elegans* (Reinke et al. 2004), unlike *D. melanogaster* (Parisi et al. 2003). There are few sperm genes on the X chromosome (Reinke et al. 2000, 2004), yet these few genes show the same pattern as the male genes of slower evolution on the X chromosome ($\chi^2 = 5.7$, $df = 1$, $P = 0.017$). In contrast, rates of evolution of X-linked hermaphrodite-related and oogenesis genes show slight but nonsignificant trends in the opposite direction ($P > 0.05$).

Evolution of Gene Function and Codon Usage Bias

When subdivided among Gene Ontology classifications, evolutionary profiles followed three general patterns

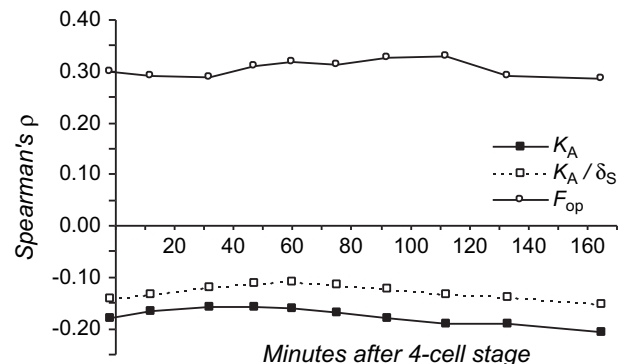


FIG. 2.—Evolutionary rates across embryonic development. Correlation coefficient interpretations of evolutionary rates as for figure 1C.

Table 2
Substitution Rates for Genes from Mutually Exclusive Embryonic Expression Clusters

Expression Cluster	n	Median K_A	Mean K_A (SE)	Median K_A/δ_S	Mean K_A/δ_S (SE)
METd	336	0.110	0.127 (0.005) ^a	0.075	0.087 (0.003) ^a
ETmd	75	0.109	0.140 (0.012) ^a	0.081	0.094 (0.007) ^{ab}
No cluster	624	0.098	0.123 (0.004) ^a	0.079	0.091 (0.002) ^a
MDET	21	0.095	0.109 (0.016) ^{abc}	0.066	0.077 (0.009) ^{abc}
Emdt	228	0.090	0.107 (0.004) ^{ab}	0.071	0.082 (0.003) ^{ab}
Mdet	975	0.090	0.115 (0.003) ^{ab}	0.072	0.085 (0.002) ^a
MDet	425	0.089	0.114 (0.004) ^{ab}	0.069	0.081 (0.003) ^{abc}
MDEt	340	0.077	0.096 (0.004) ^{bc}	0.058	0.069 (0.002) ^c
MEdt	640	0.074	0.099 (0.003) ^c	0.067	0.077 (0.002) ^{bc}
METd + ETmd	411	0.110	0.129 (0.005) ^a	0.075	0.088 (0.003) ^a
Other clusters	2629	0.085	0.107 (0.002) ^b	0.068	0.080 (0.001) ^b
No cluster	624	0.098	0.123 (0.004) ^a	0.079	0.091 (0.002) ^a

NOTE.—Superscripted letters indicate Tukey honestly significant differences; significance based on $\log_{10}$ transformed values (K_A top, K_A bottom, K_A/δ_S top, and K_A/δ_S bottom): $F_{8,3655} = 10.6$, $P < 0.0001$; $F_{2,3661} = 21.0$, $P < 0.0001$; $F_{8,3655} = 7.1$, $P < 0.0001$; $F_{2,3661} = 13.6$, $P < 0.0001$. M = maternal, E = embryonic, T = transient; D = degradation; uppercase = present, lowercase = absent (Baugh et al. 2003).

across worm development from L3 to adult (Reinke et al. 2004) (figure 4A, and figures 1B and 3 in Supplementary Material online). Collagen, chromatin, and protein synthesis genes exemplify one pattern of strong evolutionary constraint in L3 and L4 followed by elevated evolutionary rates later in development. In contrast, other functional classes showed a more modest shift in rates of protein evolution over time (e.g., neural, signaling, and transcription-factor genes) or fairly strong conservation among all larval and adult stages (e.g., degradation, metabolism, and RNA-synthesis genes). These patterns of evolutionary dynamics over ontogeny are not directly related to the mean rates of protein evolution. For example, chromatin-related genes have the highest mean K_A , and collagen genes have the lowest mean K_A , yet both classes exhibit dramatic change in the rate of evolution of their constituent genes that are differentially expressed over the course of development (figures 1B and 3C in Supplementary Material online).

To investigate variation in the strength of selection for translational efficiency across development, we compared the correlation between codon bias (F_{op}) and gene expression level at each developmental timepoint (Stenico, Lloyd, and Sharp 1994; Duret 2000). There is a high and

consistent correlation between codon bias and gene expression level throughout embryogenesis (fig. 2), indicative of strong selection for efficient translation (Duret 2000). The weaker association in L3, L4, and adults suggests that selection for optimal codon usage is typically stronger for genes expressed predominantly during embryogenesis (figs. 1C and 2). In addition to the expected negative association between codon bias and synonymous site substitution ($\rho = -0.40$, $P < 0.0001$), codon bias correlates negatively with the rate of nonsynonymous site substitution ($\rho = -0.32$, $P < 0.0001$), presumably reflecting stronger purifying selection on both the protein sequence and codon usage of highly expressed genes.

Codon usage bias values for *C. elegans* genes are tightly correlated with the values of their *C. briggsae* orthologs ($r^2 = 0.77$, $F_{1,8773} = 28738.0$, $P < 0.0001$) (fig. 4B), indicating that overall selection on codon usage (and, therefore, on transcription level) is likely similar between orthologs of these two species. However, codon bias values in *C. briggsae* are higher on average, suggesting either that selection for translational efficiency may be more efficient or that mutational effects differ. *C. briggsae*'s greater genetic variation is consistent with it having a larger effective size and, therefore, more effective selection

Table 3
Site Substitution Rates for Genes with Primarily Reproduction-Related Gene Expression

Category	n	Median K_A	Mean K_A (SE)	Median dS	Mean δ_S (SE)	Median K_A/δ_S	Mean K_A/δ_S (SE)
Male sperm	21	0.197	0.226 (0.028) ^{ab}	1.39	1.479 (0.084) ^{abcd}	0.148	0.158 (0.019) ^a
Herm. sperm	32	0.183	0.191 (0.018) ^{abc}	1.50	1.492 (0.072) ^{abcd}	0.129	0.129 (0.011) ^{ab}
Shared sperm	149	0.159	0.171 (0.007) ^a	1.45	1.496 (0.027) ^{abc}	0.112	0.122 (0.005) ^a
Shared oocyte	43	0.141	0.156 (0.015) ^{abcd}	1.59	1.606 (0.049) ^{ab}	0.098	0.111 (0.011) ^{abc}
Shared intrinsic	38	0.120	0.142 (0.005) ^{bc}	1.48	1.528 (0.014) ^a	0.081	0.092 (0.002) ^{bc}
Herm. oocyte	474	0.117	0.131 (0.004) ^c	1.49	1.528 (0.014) ^a	0.079	0.088 (0.002) ^{bc}
Male soma	116	0.111	0.157 (0.022) ^{abcde}	1.54	1.598 (0.050) ^{ab}	0.087	0.105 (0.012) ^{abcd}
Herm. intrinsic	486	0.107	0.121 (0.006) ^{bcd}	1.32	1.396 (0.029) ^{bcd}	0.077	0.088 (0.004) ^{bc}
Herm. soma	160	0.104	0.118 (0.007) ^{cde}	1.29	1.327 (0.029) ^d	0.081	0.092 (0.005) ^{bc}
Herm. ubiquitous	158	0.097	0.119 (0.006) ^{cde}	1.37	1.406 (0.025) ^{bcd}	0.067	0.081 (0.004) ^{cd}
Other sperm	123	0.097	0.135 (0.011) ^{cde}	1.33	1.363 (0.031) ^{cd}	0.086	0.115 (0.009) ^{abc}
Other	5011	0.089	0.112 (0.001) ^{de}	1.32	1.370 (0.005) ^d	0.071	0.084 (0.001) ^{cd}
Other oocyte	325	0.085	0.110 (0.005) ^{de}	1.43	1.459 (0.019) ^{abc}	0.060	0.077 (0.003) ^d
Male ubiquitous	142	0.083	0.102 (0.006) ^c	1.29	1.365 (0.027) ^{cd}	0.082	0.094 (0.005) ^{bcd}

NOTE.—Superscripted letters indicate Tukey honestly significant differences; significance for K_A and K_A/δ_S based on $\log_{10}$ transformed values. Herm. = hermaphrodite.

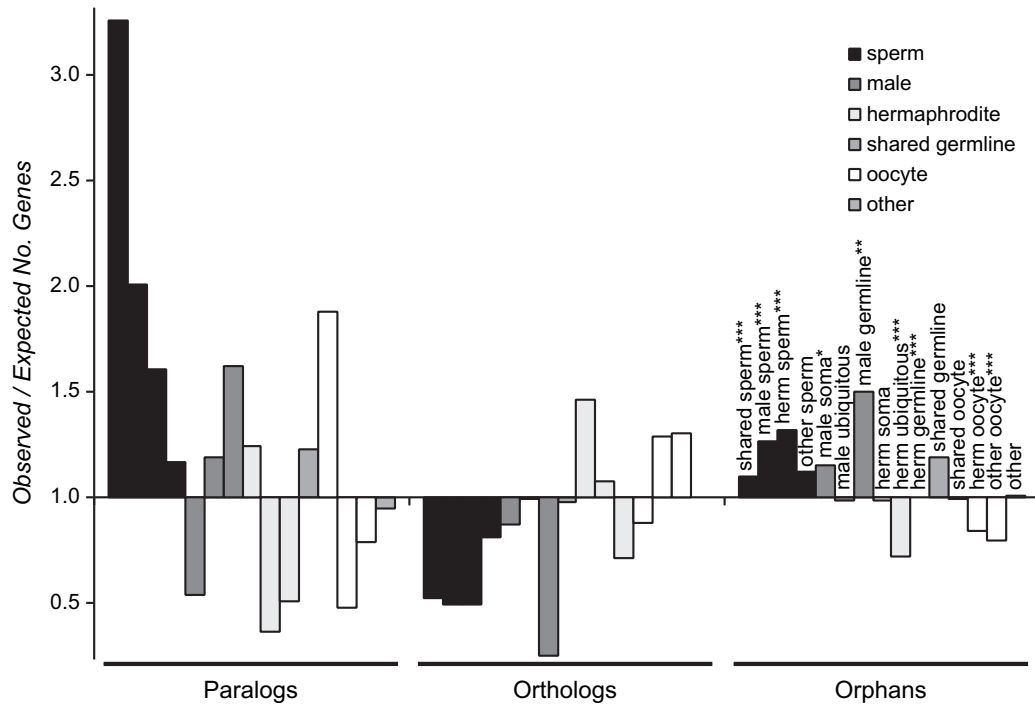


FIG. 3.—Distribution of observed/expected numbers of genes in each phenotype category among paralogs, orthologs and orphan genes. *G*-test significance levels among homology categories: * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

(Graustein et al. 2002), yet its higher G+C content relative to *C. elegans* could support a model of stronger mutational bias in *C. briggsae* (Stein et al. 2003).

Discussion

Ontogenetic Molecular Evolution and Aging

C. elegans high intrinsic growth rate and its optimization of sperm production to maximize growth rate signify that efficient, rapid development through ontogeny is at a premium and, therefore, subject to strong selection (Byerly, Cassada, and Russell 1976; Hodgkin and Barnes 1991; Barker 1992; Cutter 2004). Consequently, responses to varying selection pressure across development should be especially conspicuous in this species, although predictions and evidence for general patterns of evolutionary constraint over development have proved controversial (Galis, van Dooren, and Metz 2002).

Across nematode development from the third larval stage (L3) to adult, genes expressed predominantly later in development evolve more rapidly. Sperm-genes account for part of this elevation in L4 and young adult (when spermatogenesis occurs), but most of the elevation is

found among genes primarily expressed in the soma after the onset of reproductive maturity. This result is consistent with several interpretations: (1) more extensive developmental system drift after maturity (True and Haag 2001), (2) operation of the pleiotropy model of developmental evolution expanded to very late developmental stages (Raff 1996), (3) widespread positive selection on genes expressed primarily in adults, (4) higher mutation rates in adult-expressed genes, and (5) relaxed selection on genes expressed after the onset of maturity as in the mutation-accumulation model of aging (Medawar 1952; Promislow and Tatar 1998).

The first two interpretations given above offer little in the way of specific predictions that we can test with these data, so we focus on the latter explanations. With respect to positive selection on adult-specific genes, no obvious change in *C. elegans* ecology or morphological ornamentation coincides with the onset of maturity to provide reasonable candidates for increased adaptive evolution among adult-expressed genes. In addition, our analyses showed that the shift in evolutionary rates at maturity was not solely caused by reproduction genes. Consequently, the shift in rates of protein evolution for collagen genes expressed at different stages is compelling, given their

Table 4
Site Substitution Rates for Genes on Autosomes and the X Chromosome

Chromosome	Genes Included	n	Median K_A	Mean K_A (SE)	Median δ_S	Mean δ_S (SE)	Median K_A/δ_S	Mean K_A/δ_S (SE)
Autosomes	All	6131	0.095	0.120 (0.001)	1.36	1.40 (0.005)	0.075	0.088 (0.001)
Autosomes	Male only	227	0.099	0.136 (0.007)	1.32	1.38 (0.023)	0.091	0.115 (0.006)
X	All	1150	0.090	0.106 (0.002)	1.36	1.41 (0.011)	0.068	0.081 (0.002)
X	Male only	55	0.076	0.085 (0.008)	1.32	1.34 (0.040)	0.073	0.082 (0.007)

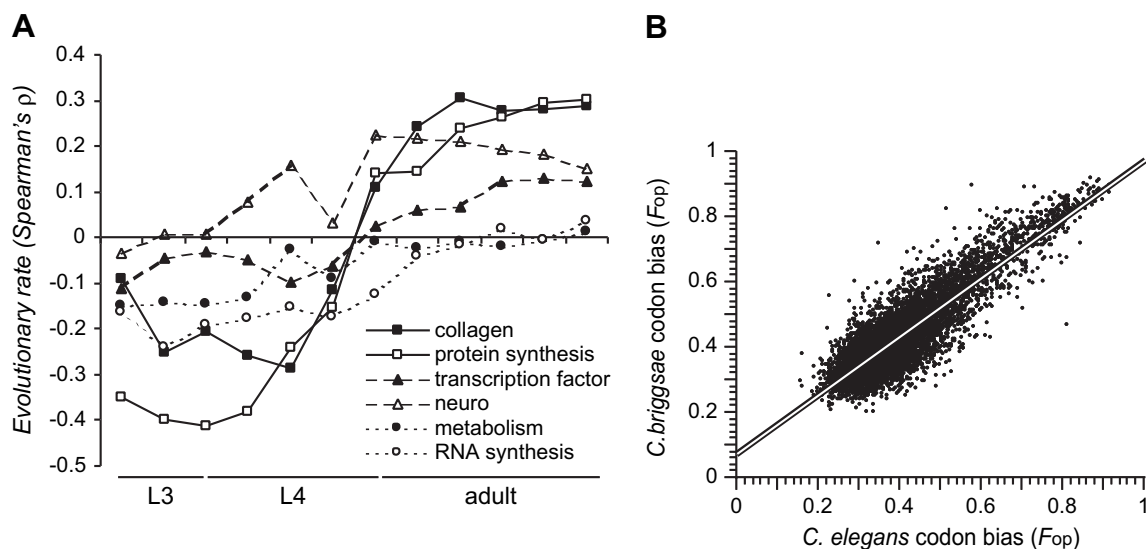


FIG. 4.—Functional groups of genes differ in their evolutionary conservation across development. (A) The protein conservation of collagen and protein synthesis genes expressed in the adult is much less than that of similar genes expressed in larvae, whereas metabolism genes and RNA-synthesis genes show little dependence on timing of expression in their level of constraint. (B) Codon bias is strongly correlated between *C. elegans* and *C. briggsae* orthologs ($r^2 = 0.77$, $F_{1,8773} = 28738.0$, $P < 0.0001$). The significant positive y-intercept (0.073; $P < 0.0001$) indicates higher average codon bias in *C. briggsae*.

association with the cuticle (Cox, Kusch, and Edgar 1981). However, collagen genes have low average substitution rates, making it difficult to ascribe the elevated substitution rates of collagen genes expressed in adults to positive selection.

Synonymous substitution rates are higher in L4-expressed and adult-expressed genes, suggesting that elevated mutation rates might contribute to the more rapid evolution of adult-expressed genes. Nevertheless, non-synonymous site substitution rates remain higher later in development after accounting for this effect. However, given the high and saturated rates of synonymous site substitution, δ_s may not accurately reflect variation in mutation rates among loci.

Assuming that the shift in protein conservation among genes expressed differentially across development is caused by relaxed selection in adults, these data support the mutation-accumulation model of aging. Experimental studies in *D. melanogaster* provide most of the evidence to date that is consistent with this model (Mueller 1987; Hughes and Charlesworth 1994; Charlesworth and Hughes 1996; Pletcher, Houle, and Curtsinger 1998). Whether the pattern of faster evolution among late-expressed genes is general across taxa remains to be seen; an analogous result occurs within the anthocyanin genetic pathway of some plants (Rausher, Miller, and Tiffin 1999; Lu and Rausher 2003) but is not observed in *C. elegans* embryogenesis. The increasing rates of molecular evolution in genes expressed from L3 to L4 to adult, in combination with the fact that few genes vary in expression across adulthood and into the postreproductive period in *C. elegans* (Lund et al. 2002), suggest that the principle shift in selective constraint centers around the onset of reproduction rather than the termination of reproduction. Work on aging also implicates important shifts at reproductive maturation, in terms of shared transcriptional features with distantly

related invertebrates and the effects of mitochondrial respiration and the insulin pathway (Dillin et al. 2002; Dillin, Crawford, and Kenyon 2002; McCarroll et al. 2004).

These data cannot directly test the competing and generally favored “antagonistic pleiotropy” model of aging, which enjoys experimental support in *C. elegans* (Williams 1957; Walker et al. 2000; Partridge 2001). The antagonistic pleiotropy model depends on the evolution of opposing fitness effects at different timepoints in development, which will be hampered by the pervasiveness of transient gene expression (Baugh et al. 2003; Reinke et al. 2004). However, adult-expressed genes provide a large temporal target for negatively pleiotropic effects because of their largely nonvariable expression in adults (Lund et al. 2002).

Molecular Evolution in Embryogenesis

The first suite of genes that are transiently expressed by the embryo evolve faster than embryonic transcripts with other expression profiles. Although it is not obvious why transiently expressed embryonic genes should evolve rapidly, a provocative hypothesis is that conflicts of interest between embryo and mother might promote protein divergence in early embryo-transcribed genes (Parker and MacNair 1979; Haig 1993). Alternatively, the pattern could be explained by nonlinear changes in the modularity of gene networks over the course of embryogenesis that generate reduced robustness to genetic changes during the transition from maternal to embryonic expression of genes (Goodwin, Kauffman, and Murray 1993; von Dassow et al. 2000). This finding of a temporary, intermediate elevation also contrasts with the pattern observed in *D. melanogaster*, in which the principle pattern reflects greatly reduced rates

of evolution among genes expressed during the second half of embryogenesis (J. Davis, personal communication).

We also corroborate the previous finding of no unidirectional trend in evolutionary rate among genes expressed across embryogenesis (Castillo-Davis and Hartl 2002). This suggests that the “pleiotropy model” of developmental evolution (Raff 1996; Galis and Metz 2001) does not define the overwhelming paradigm for protein sequence evolution across embryonic development in *C. elegans*.

Gender-Limited and Gametic Molecular Evolution

Loci with gamete-specific or gender-specific expression form an especially compelling suite of genes for evolutionary analysis. Many are likely to have a direct association with fitness and sexual selection can play a role in their evolution, as proposed for similar categories of genes in other taxa (Swanson and Vacquier 2002). In *C. elegans*, sperm and reproduction-related genes tend to cluster physically in the genome (Roy et al. 2002; Miller et al. 2004), and a trend toward faster evolution has been observed among several sex determination loci (Civetta and Singh 1998; Haag, Wang, and Kimble 2002). We identify faster rates among approximately 200 genes associated specifically with sperm and trends for elevated rates for some classes of germline and oocyte genes.

These classes of genes that evolve rapidly also have fewer one-to-one orthologs in *C. briggsae* than expected by chance, and sperm genes have more intraspecific paralogs, indicative of greater rates of duplication, divergence, and loss. Thus, the interpretation of faster sperm-gene evolution is reinforced by divergence both in terms of gene presence/absence and nucleotide substitution. The fitness premium placed on the speed of spermatogenesis in hermaphrodites (Hodgkin and Barnes 1991; Barker 1992; Cutter 2004), and also presumably in males, in combination with the large quantities of some sperm proteins required in these cells likely causes selection to favor the duplicate copies of many sperm genes (e.g., major sperm protein [Burke and Ward 1983; Klass, Kinsley, and Lopez 1984]). In contrast, most genes with oocyte-limited and hermaphrodite-limited expression evolve at average or slower than average rates and have more one-to-one orthologs in *C. briggsae* than do other gene classes.

Genes exhibiting an RNAi effect on fecundity in *C. elegans* tend to have orthologs in distantly related eukaryotes and slow rates of evolution when compared with *C. briggsae* orthologs, indicating strong evolutionary conservation of such genes (Kamath et al. 2003; Cutter et al. 2003). This pattern was interpreted as evidence of a small role for sexual selection in *C. elegans*, in contrast to some of the hypotheses outlined below. However, sperm genes are largely resistant to RNAi (Fraser et al. 2000; E. Davis and S. Ward, personal communication) and the RNAi protocol was applied only to L4-stage hermaphrodites (Kamath et al. 2003). These limitations, thus, restricted the RNAi-based identification of genes involved in fertility to genes associated with oocytes and the somatic gonad of hermaphrodites, masking the contrasting pattern among sperm genes recovered with gene expression microarrays.

Several alternative hypotheses can explain the patterns of molecular evolution among the sex and gamete categories of genes, although none seem sufficient individually. First, the rarity of males in *C. elegans* and *C. briggsae* populations could result in relaxed selection on genes expressed only in males (Chasnoff and Chow 2002; Cutter, Avilés, and Ward 2003), resulting in higher substitution rates. However, genes classified by Reinke et al. (2004) as “male ubiquitous” (i.e., present in male soma, germline, and gametes) show the slowest rates of protein evolution. This observation that male genes evolve no faster than other genes lends support to the notion that males confer an evolutionary advantage despite their numerical rarity, presumably by facilitating outcrossing (Stewart and Phillips 2002; Cutter, Avilés, and Ward 2003). Additionally, sperm genes in both sexes show elevated rates of evolution, not just male sperm genes. We can also probably reject the possibility that genes involved in spermatogenesis are somehow subjected to higher mutation rates because synonymous site substitution rates (either adjusted, δ_s , or unadjusted, K_s , for codon bias) of sperm genes are no higher than for other genes (Wilcoxon $P > 0.16$).

Second, competition between the sperm of males and hermaphrodites could lead to increased divergence in sperm genes that are specific to each sex, although this cannot explain the high divergence in sperm genes common to both males and hermaphrodites. There is great potential for an evolutionary response to sperm competition, as evidenced both by the experimental evolution of larger, more competitive sperm in obligately outcrossing experimental populations of *C. elegans* and by the presence of larger sperm in nematode species that outcross obligately (Lamunyon and Ward 1995, 1998, 1999; Singson, Hill, and L'Hernault 1999; Lamunyon and Ward 2002). Likewise, the larger size and greater competitive ability of male sperm relative to hermaphrodite self-sperm is consistent with this idea (Lamunyon and Ward 1995, 1998; Singson, Hill, and L'Hernault 1999). Male-male sperm competition could account for more rapid evolution of sperm genes that are specific to males or shared by both sexes, but not for elevated rates in hermaphrodite sperm genes. However, formal tests of positive selection are not feasible given the high synonymous site divergence between *C. elegans* and *C. briggsae*.

Third, more rapid evolution among sperm genes could result from antagonistic coevolution between sperm and oocytes and between males and hermaphrodites (Rice 1998). Given the coupled fitness interests of egg and sperm within an individual hermaphrodite, however, the elevated divergence among sperm genes shared between the two sexes seems inconsistent with this explanation. In addition, the paucity of sperm genes on the sex chromosome and the slow rate of evolution of X-linked male genes conflict with other predictions of this model (Rice 1984; Charlesworth, Coyne, and Barton 1987; Rice 1998). These observations and the lack of an X-autosome pattern in flies suggests that pressures on the sex chromosome unrelated to adaptive evolution or interlocus contest evolution may often outweigh these forces (Betancourt, Presgraves, and Swanson 2002). For example, factors involved with proper segregation and in sex chromosome silencing in the *C. elegans*

germline are probably much more potent agents of selection (Kelly et al. 2002).

Finally, independent origins of hermaphroditism in *C. elegans* and *C. briggsae* (Cho et al. 2004; Kiontke et al. 2004) could have lead to divergence via drift or lineage-specific selection in hermaphrodite reproduction genes. However, this does not provide a simple explanation for the more rapid evolution of male sperm genes.

In conclusion, a combination of evolutionary forces likely drives the patterns of molecular evolution in gamete and gender loci. For example, the co-occurrence of male-hermaphrodite and male-male sperm competition could explain much of the observed pattern of elevated rates of sperm-gene evolution. It is also important to recognize that many factors correlate with rates of protein evolution, including linkage, number of duplicates, expression level, mutation rate, and gene function (Li 1997; Williams and Hurst 2000; Castillo-Davis and Hartl 2003; Fraser and Hirsh 2004). Disentangling the partial effects of these various components presents a real challenge to ascribing a particularly important role to any given factor. Lastly, because our analyses use functional data derived only from *C. elegans*, future functional studies with *C. briggsae* and comparisons with genomic data from other relatives will facilitate discrimination of the relative importance and species specificity of these alternative interpretations.

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