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Refuting the hypothesis that the acquisition of germ plasm accelerates animal evolution

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Primordial germ cells (PGCs) give rise to the germ line in animals. PGCs are specified during embryogenesis either by an ancestral mechanism of cell–cell signalling (induction) or by a derived mechanism of maternally provided germ plasm (preformation). Recently, a hypothesis was set forth purporting that germ plasm liberates selective constraint and accelerates an organism's protein sequence evolution, especially for genes from early developmental stages, thereby leading to animal species radiations; empirical validation has been claimed in vertebrates. Here we present findings from global rates of protein evolution in vertebrates and invertebrates refuting this hypothesis. Contrary to assertions of the hypothesis, we find no effect of preformation on protein sequence evolution, the evolutionary rates of early-stage developmental genes, or on species diversification. We conclude that the hypothesis is mechanistically implausible, and our multi-faceted analysis shows no empirical support for any of its predictions.

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PGCs in animals typically form by one of two modes: first, the evolutionarily conserved mode known as induction (sometimes called epigenesis[1]), wherein PGCs are induced from presumptive mesoderm in the embryo; or second, the derived mode known as preformation (sometimes called inheritance), wherein PGCs are determined by preformed germ plasm inherited by maternal or early embryonic tissues[1,2]. On the basis of the phylogenetic distribution of these mechanisms across metazoans, induction is thought to be the ancestral animal mode of PGC specification, with preformation having arisen convergently multiple times in various animal phyla[1,3]. However, the selective pressures that could favour repeated evolution of the preformation mode are a matter of current debate. A recent hypothesis (referred to hereafter as the PGC-specification hypothesis) claims that preformation accelerates evolution as compared to induction[2,4–6]. This hypothesis posits that in organisms with induction, the requirement for induction of PGCs by neighbouring somatic cells, would act as a constraint on the early embryonic somatic tissues, and ultimately the fates and morphogenesis of an organism's somatic gene networks including those involved in late embryos and postembryonic stages[4,5]. In turn, under preformation, distinguishing somatic from germ line fates at the onset of development or even before fertilization would liberate constraint on genes and cellular behaviours involved in somatic tissue specification, patterning and morphogenesis[6]. This hypothesis thus predicts that organisms with preformation should exhibit enhanced 'evolvability' of proteins and morphology, as compared with animals 'constrained' under the induction mode[2,6].

The PGC-specification hypothesis has several predictions, each of which has profound consequences for animal evolutionary biology. First, a central prediction of the hypothesis is that preformation leads to elevated rates of changes in proteins, at a level that is observable at a genome-wide level (suggested to be up to 32% of the protein-coding sequences in a taxon[6]). Accordingly, this would mean that PGC-specification mode is a major factor shaping the evolution of coding-DNA, and thus crucial to our understanding of how animal genomes evolve. A secondary facet of this hypothesis is that the rapid evolution of proteins under preformation is most pronounced for genes expressed in early embryogenesis as compared with later developmental stages[6], since major tissue types are specified, patterned and shaped largedly at early stages of development. This would mean that PGC-specification mode is also an essential contributor to the evolution of early developmental genes. Finally, the hypothesis predicts that the proposed liberation of selective constraint under preformation leads to freedom to evolve diverse morphologies (evolvability), and thus markedly enhances species radiations[6], a concept suggested to be supported by observations of elevated species richness in some vertebrate clades with preformation as compared with clades with induction[2,5]. Under this scenario, PGC-specification mode would be a predominant factor contributing to the evolution of new species throughout animal evolutionary history. Taken together, the PGC-specification hypothesis, if well supported, could have widespread implications in genome biology and evolution.

The only empirical study to date testing this hypothesis was recently conducted among four pairs of divergent vertebrates, with one member of each pair displaying preformation, and the other displaying induction (anurans versus urodeles, birds versus crocodiles/turtles, snakes versus lizards and one clade of ray-finned fishes (Teleostei) versus another (Acipenseriformes))[6]. However, that study had notable limitations. First, rapid protein evolution, as inferred from incongruent gene trees, was observed for the preformation lineage (as compared with induction) for only two of the four main taxon contrasts. Second, protein evolution was studied using only first and second codon positions and third nucleotide positions of codons were excluded from the analysis due to saturation, since the clades being compared were too divergent in genome sequence to allow inclusion of the third codon position. As a change at the first and second positions of codons nearly always results in an amino-acid change (based on the genetic code, a change at the second position always, and at the first position usually (96%), causes an amino-acid substitution[7]), analysing only these two codon positions yields a statistic that loosely reflects the nonsynonymous substitution rate (dN). However, this approach cannot provide information on the synonymous substitution rate (dS; silent changes), nor most importantly about selection, which requires the ratio dN/dS[8,9]. By excluding dS (and thus dN/dS), one cannot ascertain whether observations of high dN result from an elevated mutation rate, and thus neutral evolution in a lineage, or from the liberation of selective pressures[8–10]. Third, the taxa used for each sequence analyses (for example, birds, crocodiles, mammals and an outgroup), were massively divergent, causing saturation, potentially making sequence alignments and substitution rate estimates unreliable[11]. Fourth, the assessment included many paired contrasts of preformation and induction species that were not phylogenetically independent. As an example, a large number of overlapping contrasts of anuran species versus urodele species were treated as independent data points, an approach known to cause tenuous correlations due to pseudoreplication[12]. Moreover, some of the species chosen only had substantially fewer than 500 partial-coding regions available for study, which does not represent a substantial part of the genome, and were derived from expression data sets from particular tissues, likely causing biases towards certain types of genes or functions (for example, brain, gonads and venom). Finally, invertebrates, which comprise over 97% of animals on earth[13], were excluded from analysis. Thus, it remains unknown whether the hypothesis of rapid protein evolution across a major portion of the genome under preformation holds for a broad range of animals, under analyses not limited by these methodological caveats. Moreover, the secondary facets of this hypothesis, namely the notion that preformation accelerates evolution of early-expressed developmental proteins as compared with those expressed at later stages, and that preformation promotes animal speciation, each warrant further evaluation.

Here based on comparative molecular evolutionary analysis in a wide range of animals, we show that the PGC-specification hypothesis is evolutionarily improbable, and that our empirical analysis provides no evidence in favour of any of its predictions.

Results

Preformation does not affect protein sequence evolution. For our analyses, we assessed whether preformation, but not induction, correlated with accelerated protein sequence evolution in a manner detectable across the genome in animals, including vertebrates and invertebrates. The PGC-specification mode does not typically vary within a single genus/family in animals (Fig. 1); this impedes common methods such as contrasts of dN/dS among species with preformation and induction mode across a single phylogeny due to saturation[9], but does differ between genera within a phylum (Fig. 1). Here we measured dN/dS between orthologues for pairs of species within the same genus for taxa with preformation and induction modes[14] as shown in Fig. 1 and Table 1. Genera were chosen based on strong cytological or experimental support for the mode of PGC-specification mode (Supplementary Table 1), availability of whole-genome sequence data for two species within the same genus (Supplementary Tables 2 and 3), and whenever possible, a

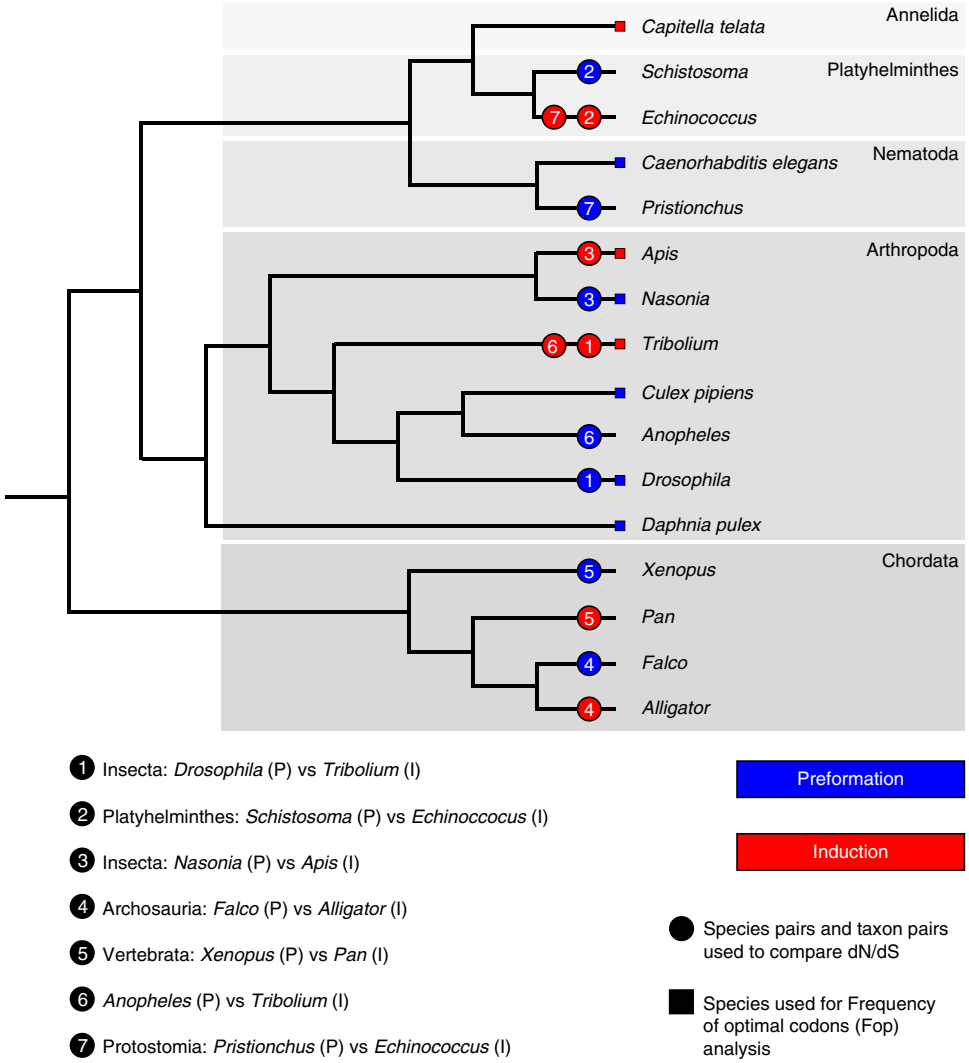

Figure 1 | The phylogenetic relationships among vertebrate and invertebrate taxa analysed. The mode of PGC formation (preformation (P): blue, induction (I): red) is shown on the branches. With respect to dN/dS, 12 genera were studied (two species per genus), and thus all comprise independent data points. In addition, the 12 genera were grouped into paired comparisons: pairs 1–5 represent phylogenetically independent contrasts (no overlap in the phylogeny between contrasts) and 6–7 are supplementary (non-independent) contrasts (Table 1).

second genus from the same phylum matching these criteria with an opposite PGC-specification mode. Using these criteria, we identified 12 animal genera for study: the invertebrate genera *Drosophila*; *Tribolium*; *Schistosoma*; *Echinococcus*; *Nasonia*, *Apis*; *Anopheles*; and *Pristionchus*, and the vertebrate genera *Falco*, *Alligator*, *Xenopus* and *Pan*. As dN/dS was determined between pairs of species within a genus, each of these 12 genera comprises an independent data point that is comparable to all other genera[14]. As a secondary assessment, we grouped the genera into five non-overlapping phylogenetically independent intergeneric contrasts of closely related pairs with opposite PGC modes (preformation versus induction) from the same phylum (see 'Primary dN/dS contrasts' Table 1; Fig. 1). We also included two supplemental contrasts (*Pristionchus* versus *Echinococcus*, and *Anopheles* versus *Tribolium*) with the important recognition that these were complementary tests (and not phylogenetically independent, and the former case spanned phyla) to our primary analysis. Given that all 12 within-genus species-pairs under study are closely related and independent, this approach avoids limitations of saturation, alignments across highly divergent taxa, and non-independence of contrasts[12,15–17], while providing a signal of rates of protein evolution across the genome[14]. In additon, this approach measures the current/ongoing rates of divergence (between two species in a genus), and avoids the potential misleading influence of bursts of rapid evoution that could occur anywhere on the branch from the last ancestor, which could afflict studies performed with highly divergent organisms[6].

Analysis of dN/dS in the 12 genera provides no evidence that the preformation specification mode accelerates molecular evolution in these animals. Typically $dN/dS<1$, $dN/dS=1$ and $dN/dS>1$ indicate purifying selection, neutral evolution and positive selection, respectively[9]. Because whole-gene dN/dS ratios are conservative measures of selection, even when $dN/dS<1$, genes with elevated values suggest events of relaxed selection or adaptive evolution. CDS were placed into one of four bins based on magnitude of dN/dS ($dN/dS<0.5$, $0.5\leq dN/dS<0.75$, $0.75\leq dN/dS<1$ and $dN/dS\geq 1$) as shown in Fig. 2a. As each of the 12 genera in Fig. 2a (within-genus species pairs) are independent data points, we compared the dN/dS profiles across all taxa. For all genera, including preformation and induction organisms (Fig. 1, Table 1), the vast majority of CDS had dN/dS values <0.5, consistent with strong purifying selection (Fig. 2a). Further, there was no tendency for more genes to evolve rapidly

Table 1 | The 12 within-genus species pairs used to measure dN/dS and the pairs of between-genus contrasts.

Paired between-genus contrasts	Genus*	Within-genus species pairs	PGC-specification mode
1	*Drosophila*	*D. melanogaster* and *D. simulans*	Preformation
	Tribolium	*T. castaneum* and *T. freemani*	Induction
2	*Schistosoma*	*S. japonicum* and *S. haematobium*	Preformation
	Echinococcus	*E. granulosus* and *E. multilocularis*	Induction
3	*Nasonia*	*N. vitripennis* and *N. giraulti*	Preformation
	Apis	*A. florea* and *A. mellifera*	Induction
4	*Falco*	*F. cherrug* and *F. peregrinus*	Preformation
	Alligator	*A. mississippiensis* and *A. sinensis*	Induction
5	*Xenopus*	*X. laevis* and *X. tropicalis*	Preformation
	Pan	*P. troglodytes* and *P. paniscus*	Induction
Supplemental contrasts			
6	*Anopheles*	*A. darlingi* and *A. gambiae*	Preformation
	Tribolium	*T. castaneum* and *T. freemani*	Induction
7	*Pristionchus*	*P. pacificus* and *P. exspectatus*	Preformation
	Echinococcus	*E. granulosus* and *E. multilocularis*	Induction

PGC, primordial germ cell.
*All 12 within-genus species pairs are independent and thus comparable across genera. The independent genera have been grouped into five phylogenetically independent between-genus contrasts (1–5), as well as two supplemental non-independent contrasts (6 and 7). For citations of evidence for PGC mode see Supplementary Table 1. Note that *Tribolium* and *Echinococcus* were used in two paired between-genus contrasts, for a total of 12 genera under study.

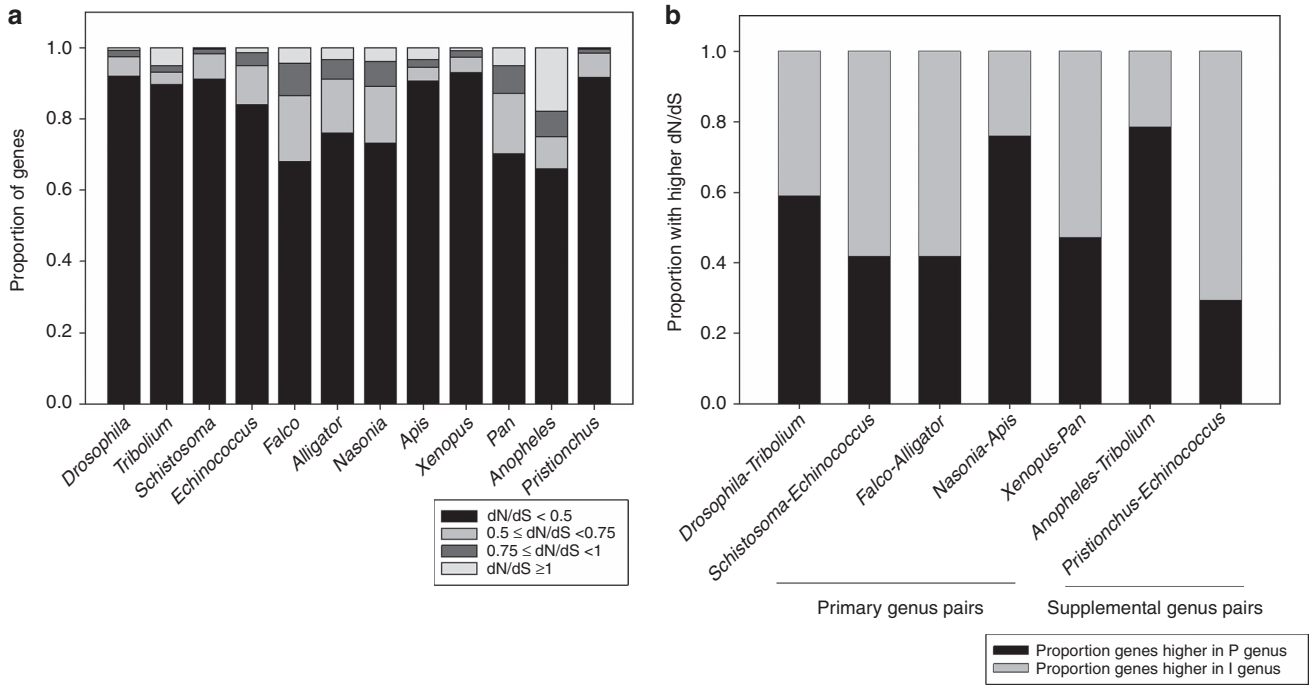

Figure 2 | The relationship between dN/dS and PGC-specification mode. (**a**) The profile of dN/dS for each vertebrate and invertebrate species pair per genus under study. Genes have been divided into four distinct dN/dS categories based on magnitude. (**b**) The proportion of orthologous genes with ≥1.5-fold higher dN/dS in preformation versus induction genera for intergeneric contrasts. P, preformation; I, induction.

under preformation. For example, the six genera with the fastest evolving genomes (highest proportion of genes per genome with dN/dS > 0.5) were *Echinococcus* (induction), *Falco* (preformation), *Alligator* (induction), *Nasonia* (preformation), *Pan* (induction) and *Anopheles* (preformation). As this sample contains three preformation and three induction taxa, it demonstrates that among those organisms with the highest proportion of genes with enhanced 'evolvability', or dN/dS > 0.5, there is not even a slight tendency (> 50%) for the taxa to use the preformation mode, rather than induction, in these animals.

Marginal differences were observed in genome-wide dN/dS profiles between the genera in Fig. 2a; however, these were unrelated to preformation or induction modes in a consistent way. For example, in the invertebrate *Drosophila* (preformation) > 90% of CDS had values < 0.5 (also see ref. 18), nearly identical to its sister taxon *Tribolium* (induction). Further, a lower fraction

of genes had dN/dS > 1 in *Drosophila* (0.68%) than in *Tribolium* (5.0%), suggesting positive selection is more common under induction (Fig. 2a). Strikingly similar dN/dS profiles were observed between *Schistosoma* (preformation) and *Echinococcus* (induction), with a marginally higher level (7%) of genes with dN/dS > 0.5 for the induction taxon, rather than the preformation taxon. *Nasonia* (preformation) exhibited an elevated level of CDS with relatively high dN/dS compared with its sister taxon *Apis* (induction), with 26.8% and 9.5% having dN/dS > 0.5, respectively, but had a similar proportion of CDS with dN/dS > 1. Collectively, dN/dS does not show any consistent relationship to PGC mode in these invertebrates.

Within vertebrates, a *Xenopus* (frog) versus *Ambystoma* (salamander) comparison is often invoked in discussion of PGC-specification mode due to strong evidence of preformation and induction modes, respectively[1,6,19]. However, the small data sets for the latter taxon used in Evans *et al.*[6] were deemed unsuitable for study here (Methods). We therefore compared *Xenopus* (preformation) versus *Pan* (induction); although divergent chordates, a strong effect of preformation on protein evolution in *Xenopus*, as reported by Evans *et al.*[6] should still be evident. We found four times as many genes in the induction genus had dN/dS > 0.5 compared with the preformation genus, implying that if anything, induction is associated with accelerated protein sequence evolution. In fact, *Xenopus* had the highest percentage of dN/dS < 0.5 (92.9%) among all 12 genera under study, consistent with the lowest level of evolutionary change (fewest fast-evolving proteins). The vertebrates *Falco* (preformation) and *Alligator* (induction) exhibited among the highest percentage of CDS (> 24%) with dN/dS > 0.5, suggesting both genera exhibit greater propensity for relaxed or positive selection than the other remaining genera (Fig. 2a). However, only a marginal difference (< 8%) was observed in the fraction of CDS per genome with high dN/dS (> 0.5).

Mann–Whitney *U* (MWU)-tests of genome-wide dN/dS per genus were statistically significantly different for four of the five between-genera pairs outlined in Table 1. The differences were as follows: *Drosophila* > *Tribolium*, *Schistosoma* < *Echinococcus*, *Nasonia* > *Apis*, *Xenopus* < *Pan* ($P < 10^{-15}$ for each contrast), with no difference for *Falco* and *Alligator* ($P = 0.13$; Supplementary Note 1), thus showing no consistent effect of PGC-specification mode. Supplementary contrasts of *Anopheles* (preformation) versus *Tribolium* (induction) and *Pristionchus* (preformation) versus *Echinococcus* (induction) revealed the preformation and induction taxa respectively, evolved more rapidly (MWU-tests $P < 10^{-15}$; Fig. 2a; Supplementary Note 1), again showing no relevant effect of PGC-specification mode.

Between-genus orthologues show no effect of preformation. Next we studied dN/dS among specific orthologues matched across five pairs of genera (Table 1, Fig. 1); we identified those orthologues with at least a 1.5-fold difference in dN/dS between the genus with induction and preformation (per between-genus pair). We found that dN/dS in the orthologous CDS sets was unrelated to PGC-specification mode. For instance, for *Nasonia* (preformation) and its sister taxon *Apis* (induction), 76.0% of the 2,161 orthologues exhibiting a ≥1.5-fold difference in dN/dS between taxa had a higher value in the preformation taxon, which may appear consistent with more genes in this CDS subset evolving rapidly under preformation. However, for *Drosophila* (preformation) and *Tribolium* (induction), 58.9% of the 2,921orthologues with a ≥1.5-fold difference had higher dN/dS under preformation), a difference level inconsistent with globally rapid CDS under preformation. Further, for *Schistosoma* (preformation) versus *Echinococcus* (induction), 58.2% of the orthologues with at least a 1.5-fold difference in dN/dS ($N = 1{,}321$) had higher values in the induction taxon (Fig. 2b), not the preformation taxon. Altogether, these results in invertebrates, consistent with the findings across all genes (Fig. 2a), show no pattern with respect to PGC-specification mode and fail to support the prediction that germ plasm accelerates protein sequence divergence.

For vertebrates, the *Falco* (preformation) and *Alligator* (induction) contrast showed rapid evolution was more commonly observed under induction than preformation: 58.1% of the 2,537 CDS exhibiting > 1.5-fold difference had elevated dN/dS for the induction taxon) The dN/dS values for the two *Falco* species (*F. cherrug* and *F. peregrinus*) correspond with prior findings for these taxa (mean dN/dS herein $= 0.36 \pm 6.2 \times 10^{-5}$, mean therein 0.39), where it was shown they exhibit high dN/dS within the bird clade[20]. Despite having high dN/dS within birds, they still exhibit no notable elevation with respect to alligators (Fig. 2b). Bird genes have previously been found to exhibit lower (as well as higher), dN/dS than their orthologues in other induction taxa such as mammals, which largely depends on the ontology class[21] and thus not PGC mode; further confirming no major role of PGC-specification mode in birds (Supplementary Note 2). In *Xenopus* (preformation) versus *Pan* (induction), 47.3% of orthologues with 1.5-fold difference ($N = 2{,}471$) had elevated dN/dS under preformation, and 52.7% had higher values under induction (Fig. 2b), inferring marginally higher rates when genes evolve under induction. Altogether, the two vertebrate contrasts show no signal of rapid sequence divergence under preformation. The supplemental contrasts of *Anopheles* (preformation) versus *Tribolium* (induction) and *Pristionchus* (preformation) versus *Echinococcus* (induction) revealed that more genes evolved rapidly for the preformation and the induction taxon, respectively (Fig. 2b), and thus no effect of PGC-specification mode.

While we cannot exclude that species-specific factors obscure a mild PGC mode effect, it is evident that if preformation liberates selective constraint and broadly enhances protein sequence evolution in animals, we would expect a detectable signal from the 12 independent genera data points (Fig. 2a) and from the five paired between-genera contrasts (Fig. 2b). As discussed in Supplementary Note 3, we exclude an effect of divergence times, and population size on our results. In addition, it is important to note that since dN/dS was determined within genera, dS was well below saturation levels (< 1) for all taxa under study herein, as shown in the bar and whisker plots provided in Supplementary Fig. 1. Thus, our collective results of dN/dS across genera in Fig. 2a,b show no pattern with respect to PGC-specification mode and fail to support the prediction that germ plasm accelerates protein sequence divergence.

As a complementary test to dN/dS, we assessed the frequency of optimal codons (Fop) relative to PGC-specification mode for various animals. Optimal codon usage has been employed in *Drosophila* and other eukaryotes to detect rapidly evolving proteins[22–24], as proteins that evolve rapidly tend to have low Fop[22–26]. We identified or verified the optimal codon lists for the taxa in Supplementary Table 4, and subsequently examined Fop for the preformation taxa *Caenorhabditis elegans*, *Culex pipiens* and *Daphnia pulex* and the induction species *Capitella teleta* (Supplementary Note 4; Supplementary Tables 5 and 6; and Supplementary Figs 2 and 3). No notable trends departing from normality were observed in the distributions of Fop for all three preformation species (Supplementary Fig. 2), indicating no tendency for rapid protein evolution under preformation. Similarly, for *C. teleta* (induction), there were no notable trends toward high Fop in the distribution that would suggest a broad

tendency for slowed protein evolution under induction (Supplementary Fig. 2).

Preformation is unlinked to divergence of early-stage genes. A second facet of the PGC-specification hypothesis is that preformation releases selective constraint more frequently in genes expressed at early embryogenesis, as compared with later developmental stages, a phenomenon not inherent to induction; this has been purported to be empirically supported in vertebrates[6]. In that assessment, the authors identified CDS with high dN in any of the preformation taxa studied, asked when the mouse or zebrafish orthologues of these genes were expressed during embryogenesis, and asserted that the orthologues were mainly expressed in early stages of development. However, no comparable assessment was conducted for genes that appeared to evolve rapidly in induction taxa. Here we investigated expression of all identifiable orthologues in the *Drosophila–Tribolium* and *Nasonia–Apis* contrasts (preformation-induction, respectively), which were the two (out of five) between-genus pairs with some sign of elevated dN/dS under preformation (Fig. 2b). There were two CDS sets per contrast: the set with 1.5-fold higher dN/dS in the preformation taxon and the non-overlapping set with 1.5-fold higher dN/dS (referred to hereafter as high dN/dS CDS sets) in the induction taxon. Using the comprehensive developmental expression database in *Drosophila* (Methods; http://www.flybase.org (ref. 27), the expression profile of the high dN/dS CDS were examined across 10 developmental stages/phases from 0 to 6 h embryos up until adulthood (Fig. 3).

In *Drosophila*, we found a lower percentage (78.0%) of the high dN/dS CDS set was expressed in 0–6 h embryos than in all nine later developmental stages (between 86.4 and 97.7%; χ^2 $P<0.001$ for all paired contrasts), inconsistent with preferential expression of fast-evolving CDS in early developmental stages under preformation. Further, the high dN/dS set from *Drosophila* (preformation) and its counterpart in *Tribolium* (induction) had nearly identical profiles with respect to development (Fig. 3a; the difference was $<1.2\%$ for each of 10 developmental stages (χ^2 $P>0.63$) and the percentages across stages were highly correlated between genera (Spearman's $R=0.985$, $P<2\times10^{-7}$; Supplementary Note 5).

For further stringency, we asked whether the high dN/dS CDS in *Drosophila* (preformation) were more commonly expressed at elevated levels (>50 reads per kilobase million (RPKM); defined as 'high' expression based on the whole transcriptome in Flybase, http://www.flybase.org) in early embryos as compared to the set from *Tribolium* (induction), as these genes may be most apt to be linked to crucial functions. Within the high dN/dS CDS from *Drosophila* and from *Tribolium*, the 0–6 embryos each exhibited a mildly (maximum of 14.0% difference) greater percentage of CDS with >50 RPKM (28.4%, in the 0–6 h embryos in both *Drosophila* and *Tribolium*), than the nine later developmental stages, with values between 14.4 and 23.7% (Fig. 3b; χ^2 $P<0.001$ for each contrast per taxon). The proportions, however, were in effect identical for the preformation and the induction taxa for 0–6 h embryos (χ^2 $P=1$, Fig. 3b), and were highly correlated between taxa across all developmental stages (Spearman's $R=0.840$, $P<2.0\times10^{-7}$), and thus disagree with the PGC-specification hypothesis.

The second independent assessment on the *Nasonia* (preformation) and *Apis* (induction) contrast yielded virtually identical results. For *Nasonia*, a lower percentage of high dN/dS CDS (>1.5-fold higher dN/dS in *Nasonia*) were expressed in 0–6 h embryos (83.7%) than all later stages (90.9 to 98.6%, χ^2 $P<0.001$ for all contrasts, Fig. 3c). The proportions of the *Nasonia* and *Apis* high dN/dS CDS sets expressed at each stage were nearly identical ($<1.7\%$ difference across all stages, χ^2 $P>0.19$ for all contrasts, Fig. 3c) and highly correlated (Spearman's $R=0.985$, $P<2.0\times10^{-7}$). In turn, the proportion of high dN/dS CDS with >50 RPKM in 0–6 h embryos was nearly identical between *Nasonia* and *Apis*, (30.2% and 30.1%, respectively, χ^2 $P=0.98$), and values highly correlated across development between genera ($R=0.778$, $P<2.0\times10^{-7}$, Fig. 3d).

Collectively, neither of the *Drosophila–Tribolium* or *Nasonia–Apis* contrasts, the only two contrasts (of five main between-genus contrasts, Table 1) that showed some tendency for more genes to evolve rapidly under preformation (Fig. 2b), support the notion that fast-evolving genes under preformation are preferentially linked to early development. We therefore conclude that at least for these two pairwise comparisons of induction versus preformation taxa: (1) fast-evolving genes under preformation are not linked to early development; and (2) developmental expression profiles of fast-evolving genes are nearly identical under preformation versus induction.

Developmental genes. As preformation has been proposed to release constraint on development and allow greater morphological variation that could contribute to speciation[2], we assessed evolutionary rates of developmental genes. We chose genes that are known to play important roles in the development in animals, have well-supported annotations, known functions, expression profiles and complete CDS (without unknown sites) in the model *D. melanogaster* (flybase.org), and with well-defined orthologues in *D. simulans* to allow assessment of dN/dS. Using these criteria, we identified 121 developmental genes for analysis (Supplementary Table 7). As shown in Fig. 4a, all 121 studied genes were expressed in at least one developmental stage, and $>95\%$ of this gene set was expressed all developmental stages. The average dN/dS for this developmental gene set was 0.118 ± 0.014 (median of 0.076), which was statistically significantly lower than for the remainder of CDS in the genome (Average $=0.189\pm0.002$, MWU-test $P<0.001$, Fig. 4b), indicating strong purifying selection, as may be expected for genes involved in crucial and multi-stage functions[28–30]. Further, no differences were detected in dN/dS of the matching putative orthologues between *Drosophila* (preformation) and the *Tribolium* (induction) genus (MWU-test $P>0.55$). In summary, we extend our conclusions that preformation does not enhance dN/dS of CDS at levels detectable across the genome (Fig. 2a,b), including CDS expressed at early stages (Fig. 3a–d), to also include genes specifically involved in development (Fig. 4a,b).

Discussion

The collective results herein do not support the hypothesis that the acquisition of germ plasm accelerates animal evolution. First, the 12 independent within-genus estimates of genome-wide dN/dS (Fig. 2a), as well as five paired intergeneric contrasts of matched orthologues in taxa with distinct PGC-specification modes (Fig. 2b), failed to support the assertion that germ plasm causes accelerated protein divergence (high dN/dS). If germ plasm broadly released morphological and sequence constraint[2,6], all preformation taxa studied herein, including the vertebrate taxa suggested by Evans *et al.*[6], should have exhibited fast rates of evolution in protein-coding genes across the genome (Fig. 2a,b). Instead, we observed not even a slight tendency in favour of this hypothesis: preformation and induction taxa were equally represented among the six genera with the fastest evolving genomes (Fig. 2a), and in the paired between-genus contrasts, fewer than half of the preformation genera showed any inclination for genes to evolve more rapidly than in induction

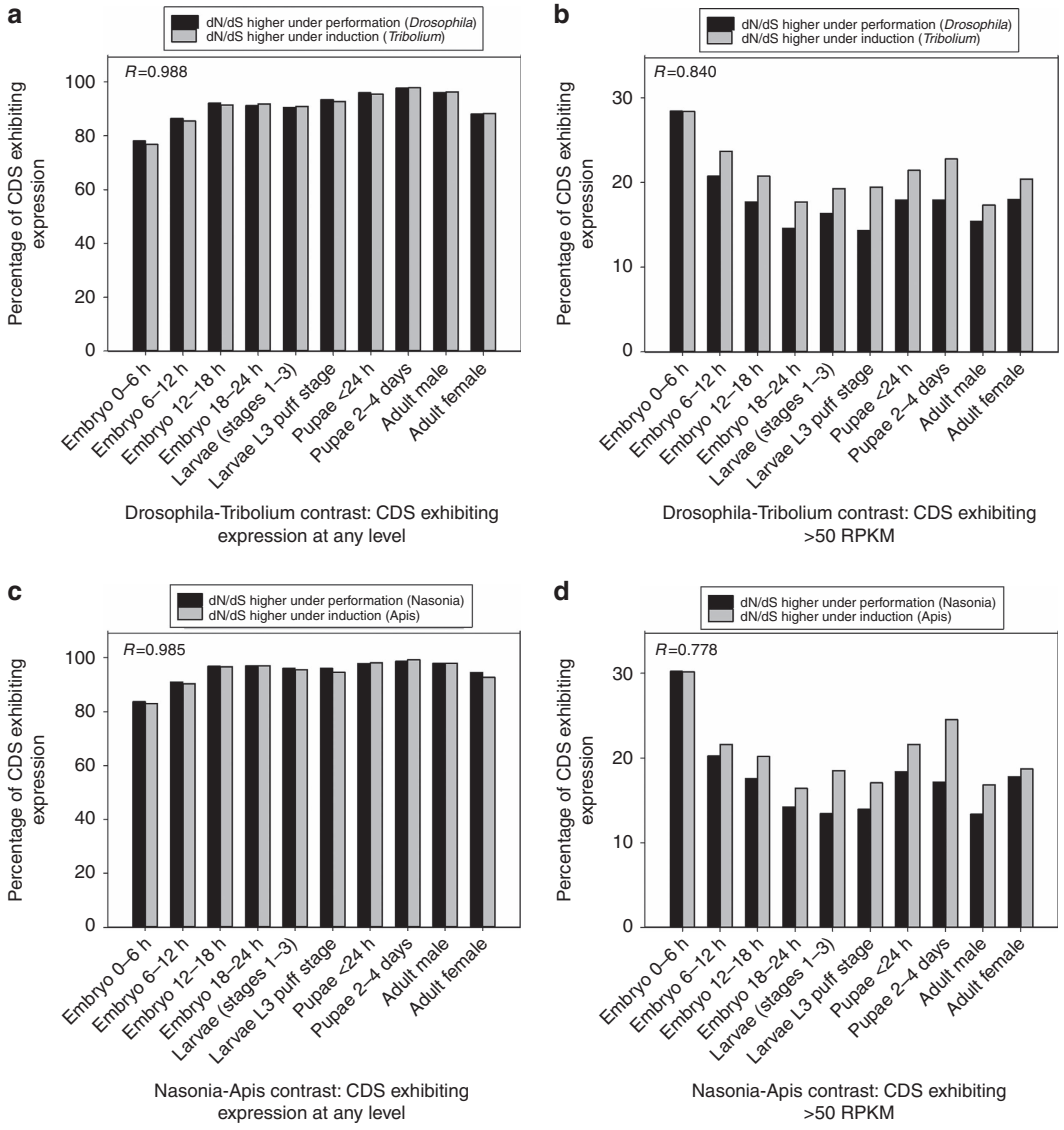

Figure 3 | The developmental expression profiles of CDS with high dN/dS within the *Drosophila–Tribolium* and the *Nasonia–Apis* contrasts. (**a**) The percentage of the high dN/dS CDS set ($\geq$1.5-fold difference in dN/dS) for genera from *Drosophila* (preformation: black bars) and from *Tribolium* (induction: grey bars) expressed at each developmental stage, and (**b**) the per cent expressed at $>$50 RPKM at each developmental stage. (**c**) The percentage of the high dN/dS CDS set from *Nasonia* (preformation: black bars) and from *Apis* (induction: grey bars) expressed at each developmental stage, and (**d**) the per cent expressed at $>$50 RPKM at each developmental stage. Spearman correlations (*R*) among the preformation and induction taxa across developmental stages are shown ($P<2.0\times10^{-7}$ for all *R* values). Note 2 of the 10 stages/data points were adult males and females.

genera (Fig. 2b). Second, our findings that early developmental genes were not evolving rapidly under preformation, and that the more rapidly evolving genes had nearly identical developmental expression profiles in both preformation and induction taxa (Fig. 3a–d), also counter the PGC-specification hypothesis of Evans *et al.*[6]. Importantly, although the Evans *et al.* hypothesis addresses only vertebrate evolution, our analyses provide no support for this hypothesis in either vertebrates or invertebrates.

A third facet of the PGC-specification hypothesis is that the acquisition of germ plasm, and fast evolution of protein-sequences, leads to enhanced speciation[2,6]. Anecdotal data based on species richness in vertebrate clades has been taken as support for this proposal[2,6]. For instance, it has been contended that the much higher number of species in some vertebrate clades with preformation, such as frogs (number of species estimated as 4,800), ascidians (3,000), teleosts (25,000) and birds (10,000), than in other groups with induction, including turtles (300), lancelets (23), non-teleost actinopterygians (44), salamanders (515) and hemichordates (100) provides evidence of higher speciation rates[2]. However, a rigorous assessment of species diversification rates would require large-scale phylogenetic data sets and multi-faceted intensive techniques, including assessments of clade-age and birth-deaths, approaches which are still largely under development, testing and refinement[31–33]. Methodological or data set challenges notwithstanding, anecdotal examples of species richness alone cannot be used to make strong conclusions about speciation rates.

Acknowledging that species-richness alone[2,6] comprises a relatively weak non-analytical approach to assessing diversification rates with respect to PGC-specification mode[34], even if one uses that approach, there are many anecdotal examples in the literature that support the opposite trend, of large radiations under induction. For instance, mammals (induction, 5,400 species[2]) and lizards (induction, $>$6,100

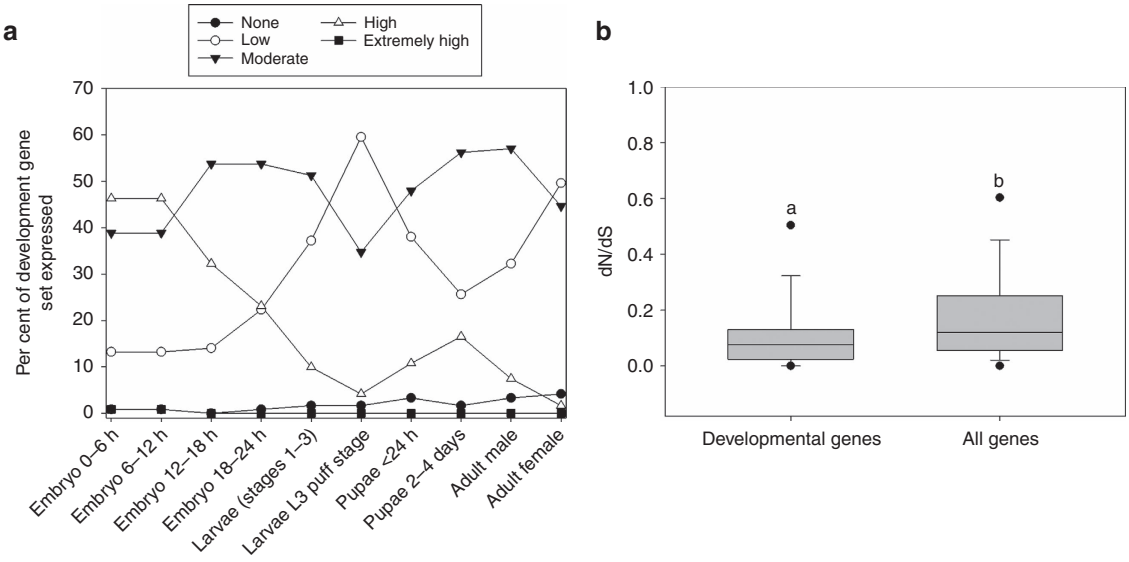

Figure 4 | The dN/dS of developmental genes in *Drosophila*. (**a**) The percentage of the 121 developmental genes with no, low, moderate, high or extremely high expression at each of ten stages of development in *Drosophila*. (**b**) Box and whisker plots (showing the 25th percentile/median/75th percentile and whiskers representing maximum and minimum excluding outliers) of dN/dS for the developmental genes and all remaining genes in *Drosophila* (*D. melanogaster* and *D. simulans*) genome. Different letters indicate a statistically significant difference using the MWU-test ($P<0.001$). Expression levels for (**a**) are as follows: none (0 RPKM), low (>0 RPKM ≤ 10), moderate (>10 RPKM ≤ 50), high (>50 RPKM $\leq 1{,}000$) or extremely high ($>1{,}000$).

species; www.reptile-database.org) also exhibit high levels of diversification. Further, an available diversification-rate assessment based on clade-age and birth-death analysis from 44 clades of jawed-vertebrates suggests despite their high species richness, frogs (preformation) do not exhibit an elevated (non-typical) diversification rate in this taxonomic group, including as compared with salamanders, counter to prior predictions for these sister taxa based on species richness alone[2]. Further, high diversification rates occur in clades using preformation such as some birds, teleosts and snakes as well as in clades with induction such as lizards and eutherian mammals[6,35], together suggesting diversification rates are unrelated to PGC-specification mode in those vertebrates.

Among insects, the order Diptera (Supplementary Table 3) comprises a large diverse group of $>240{,}000$ species that specify germ cells using preformation[36,37]. However, its sister clades Lepidoptera and Coleoptera, with many taxa exhibiting induction[38] also exhibit remarkable species diversity, with estimates of $>174{,}000$ (refs 39,40) and 390,000 described species, respectively[41]. In fact, the Coleoptera, containing numerous induction species[38] is the most speciose insect order[41]. Even within the family level of these insects, we find no consistent trends suggestive of higher species richness under preformation than under induction. As an example, the family Drosophilidae (preformation) contains about 4,000 species[42], while other families of Diptera (for example, Nemestrinidae; also with preformation) contain as few as 300 species[43]. In turn, the Coleopteran family Tenebrionidae (containing the induction species *Tribolium castaneum*[44,45] and *Tenebrio molitor*[46,47]) represents $>20{,}000$ species[48], while the Lepidopteran family Bombycidae (containing *Bombyx mori*, also with the induction mode, as cited in Supplementary Table 4 (refs 49–54) consists of 21 genera with just 150 species. Importantly, as noted by Wiegmann *et al.*[55], the Diptera (preformation), Coleoptera (induction), Hymenoptera (*Apis* (preformation) and *Nasonia* (induction)), and the Lepidoptera (induction) are four superradiators in insects, and account for the majority of animal life on earth. Additional examples are provided in Supplementary Note 6. Most importantly, given that we observed no molecular evolutionary evidence of release of constraint, or rapid protein sequence divergence, under preformation (Figs 2–4) the underlying mechanism contributing towards enhanced diversification in clades with germ plasm[2] is unlikely to exist in animals. Taken together, there is no current rationale to anticipate higher genome or species diversification under preformation across animals.

We propose that the fact that germ plasm has evolved convergently across animal lineages does not necessitate a general trend towards liberated constraint and rapid protein evolution, and rather likely results from other mechanisms. For instance, convergent evolution of a germ plasm-driven mechanism for specifying PGCs could result from advantageous mutations in a small subset of genes, or from gene expression changes[56,57] involved in the acquisition of germ plasm[3]. An alternate theory that has been proposed to explain the convergent evolution of germ plasm (preformation), is that it is simply a side-effect, or spandrel[58], of a heterochronic shift[59] in body plan specification mechanisms generally, from late to early development[60,61]. Organisms displaying the preformation mode of PGC specification also tend to have much of their early axial patterning and body plan specification determined maternally, by asymmetric deposition of regional determinants within the oocyte during oogenesis and early embryogenesis[62]. Under this hypothesis, germ plasm would be simply one of many such maternally supplied determinants, ensuring that the germ line, as well as, for example, the dorsoventral and anteroposterior axes, were established before or immediately following fertilization, without requiring extensive zygotic genome activity or zygotic cell–cell signalling. Quantitative empirical tests of this hypothesis, beyond establishing the strength of the correlation between germ plasm and other body plan determinants that appears to hold at least for well-established model organisms[62], may prove challenging. However, with our study we have sought to highlight the fact that as with all convergently derived traits, the mechanism of specification of the animal germ line may not itself be a direct target of selection, but rather an indirect consequence of selection for a distinct trait or mechanism.

While herein we found no evidence supporting the PGC-specification hypothesis, PGC-specification mode could affect other parameters related to molecular evolution, such as the evolutionary rates of a small number of genes, or sites within genes, involved in the mechanisms of preformation or induction. For example, evolution of germ plasm related genes such as *oskar*, *vasa*, *nanos*, *piwi*, *tudor*, *pie-1* and others might well differ from those shown to be instrumental to induction, such as BMP or Wnt signalling pathway members[63–66]. To test this, further studies should assess the molecular evolutionary dynamics of specific PGC genes and pathways using large-scale phylogenetic analysis across many species per genus with preformation and those with induction, allowing measurements of site-specific positive and negative selection[67]. Other molecular evolutionary parameters that PGC-specification mode might plausibly impact are mutation frequency in germ lines[68,69]. Future research should assess population-level frequencies of mutations to test for adaptive evolution and relaxed selection in specific PGC genes[70,71]. The rapid expansion of genome-wide sequence data sets in invertebrates[72] will allow assessment of positive selection in genes involved in germ plasm formation using phylogenetic approaches that span a wide range of taxa in the future.

Methods

Data extraction. For each taxon under study, CDS sequences were either downloaded directly from a public database, or extracted from genomic data (Supplementary Tables 1 and 2). In organisms where genomic DNA was available as assembled scaffolds (Supplementary Table 2), the CDS regions were extracted using Augustus[73] set at default parameters, and trained using a related species from the same genus with annotated genome data. To ensure accurate identification of CDS from scaffolds, open reading frames were verified using codons with ORF predictor[74]. For our analyses, we removed any CDS with unknown or ambiguous nucleotides, or with one or more internal stop codons.

Orthology identification and measurements of dN/dS. For the identification of orthologues among species pairs listed in Table 1 and Fig. 1, we used BLASTX[75] of the genome-wide CDS, where the match with the lowest *e*-value (and $e<10^{-6}$) in reciprocal BLASTX searches was identified as the orthologue. For genes with more than one similar isoform (varying by an exon, or point mutations), this method yields the longest isoform per gene among taxa. Genes not having the same match in both reciprocal BLASTX searches were excluded from further analysis. Intergeneric identification of orthologues was also conducted by reciprocal BLASTX.

Alignments of gene sequence across species were conducted at the codon level using the program MUSCLE[76]. The dN and dS values were determined using the Nei–Gojobori method after exclusion of all gaps[77]. MUSCLE alignments and dN and dS were each determined using MEGA-CC[78]. All CDS per species pair (Table 1, Fig. 1) with $dS>0$ were retained for analysis of dN/dS. As it has been posited that ambiguous alignments from distant organisms, and sequencing errors due to low coverage, could inflate or alter molecular evolution parameters reported in the literature, including dN and dS[11,79], we examined only closely related species with full CDS herein. Further, in the interest of prudency, we repeated our entire analyses in Figs 2–4 excluding all genes having dS values above the 90th percentile, which are most apt to exhibit segments of misalignment, imprecise orthology matches across taxa, and/or an abundance of sequence errors (each which can affect measures of molecular evolution parameters[79], and obtained results nearly identical to those reported in each figure (data not shown)). This cutoff prevented exclusion of high dN genes unless its matching dS was also unusually elevated.

Expression profiling. Expression levels of high dN/dS CDS across development in *Drosophila* were determined using modENCODE RNA-seq data in FlyBase (www.flybase.org)[27]. Expression levels for high dN/dS CDS sets across the ten analogous developmental stages in *Tribolium*, *Nasonia* and *Apis* were inferred[80] from the orthologues from the relatively closely related insect *Drosophila* (Fig. 1). We propose that this is a reasonable inference since (a) the general developmental progression of these insects is quite similar[81,82] and (b) the developmental gene expression profiles in *Drosophila* are highly conserved even with divergent invertebrates from non-Arthropod phyla[80], and thus apt to be similar in such closely related insects.

Identification of taxa for study. Phylogenetic independence among the invertebrates studied in Table 1; Supplementary Table 1; Fig. 1 was determined using phylogenies derived from large-scale sequence data[83–85].

The animal genera under study in Table 1 and Fig. 1 were chosen based on a well-established mode of PGC specification, public availability of whole-genome DNA sequences for two species from a single genus at the commencement of our analyses (September to October 2014), and lack of saturation in dS. The taxa we identified matching these criteria, and having suitable data for another genus to allow comparison within the same phylum (one exception, contrast 7), were included in our analysis of dN/dS (Table 1). We note that while frogs (*Xenopus* (anurans)) versus salamanders (*Ambystoma* (urodeles)) comprised a primary contrast used by Evans *et al.*[6], and represents a well-established case of preformation and induction respectively, we believe the available urodele sequence data sets are currently not suitable for large analyses representative of the genome, and are unsuitable for calculation within-genus dN/dS (Table 1, Fig. 1). This is because sequence data for salamanders (Ambystoma *mexicanum* and *A. tigrinum*) mainly comprise modest-sized expressed sequence tags data sets (ESTs) (~20,000 ESTs and 1,700 other nucleotide sequences) for *A. tigrinum* (National Center for Biotechnology Information, NCBI; http://www.ncbi.nlm.nih.gov; search by taxon name) and a mix of genomic, RNA-seq and ESTs for *A. mexicanum* (Evans *et al.*[6]), which after assembly and orthology identification in two species (per genus) yields small partial gene sets for study. For instance, Evans *et al.*[6] reported 6,679 and 2,078 CDS after assembly for each of *A. mexicanum* and *A. tigrinum*, respectively, many of which did not have a start or stop codon and thus were partial CDS (covering only part of the reading-frame) (http://www.nottingham.ac.uk/~plzloose/phyloinc). We found that only 523 partial CDS were available to study after orthology searches between the two salamander species using TBLASTX (cutoff, $e<10^{-6}$; NCBI http://www.ncbi.nlm.nih.gov/BLAST/blast_program.shtml). Further, since the CDS list in each species is incomplete, the CDS identified as predicted orthologues between species are most likely to be best hits between CDS lists, rather than true orthologues (since many true orthlogous CDS are likely absent due to poor expression, or small sequence sample size). In addition, the contigs are inherently biased towards highly expressed genes from the specific tissues used to create the complementary DNA libraries that these EST or RNA-seq collections were derived from (for *A. tigrinum*, ESTs were from various tissues such as brain or pooled tissues, while for *A. mexicanum*, the transcriptome was generated from a combination of oocytes, embryos and ESTs from various tissues such as the tail and limb blastema (NCBI; http://www.ncbi.nlm.nih.gov[6]), and are not an unbiased sample of CDS in the genome.

While teleosts (preformation) and cartilaginous fish (induction) were also major systems studied in Evans *et al.*[6], we consider that the within-genus data sets are too small to study here and claim that they are a representative sample of the genome. As an example, the salmon/trout (teleost) species *Oncorhynchus mykiss*, *O. nerka*, *O. tshawytscha* and *O. kisutch* had 5,745, 2,582 and 1,520 and 707 CDS/contigs (many not covering the complete CDS; http://www.nottingham.ac.uk/~plzloose/phyloinc) respectively, and thus no pairing between two of these species would yield sufficient orthologous CDS for analysis. Similar to the problem with the salamander data, these sequences would likely provide few true orthologues among the species in this genus (and rather best hits). Similarly, a paired within-genus contrast for the cartilaginous fish (Acipenseriformes) *Acipenser ruthenus* with *A. transmontanus* or *A. sinesis* using sequence data that were examined in that investigation[6] was not feasible as the latter two taxa had only 281 and 152 partial CDS available, respectively. Thus, despite the fact that these groups are of interest because of the compelling evidence regarding their modes of PGC specification, the assessments of evolutionary rates across the genome for these genera cannot be robustly performed at the moment, but must await the availability of whole genomic DNA sequence data. Note that all citations to the number of CDS or contigs per species studied in Evans *et al.*[6] were obtained by downloading the fasta files from http://www.nottingham.ac.uk/~plzloose/phyloinc.

Data availability. The genomic sequences studied herein are all publicly available and their locations are provided in Supplementary Table 2. The data that support the findings of this study are available from the corresponding author on request.

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Author contributions

C.A.W. and C.G.E. conceived the study, devised the methods, conducted the analysis and wrote the manuscript.

Additional information

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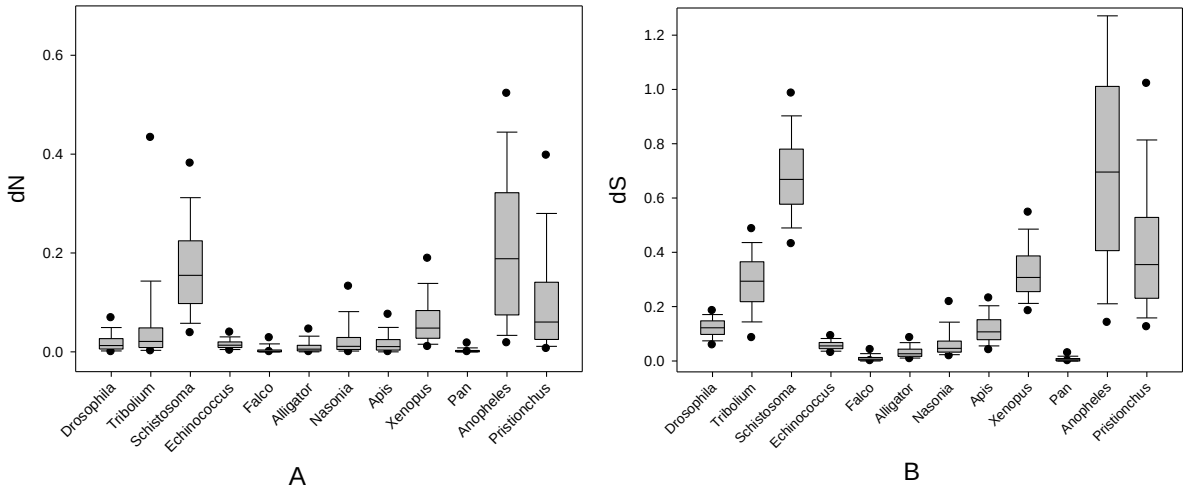
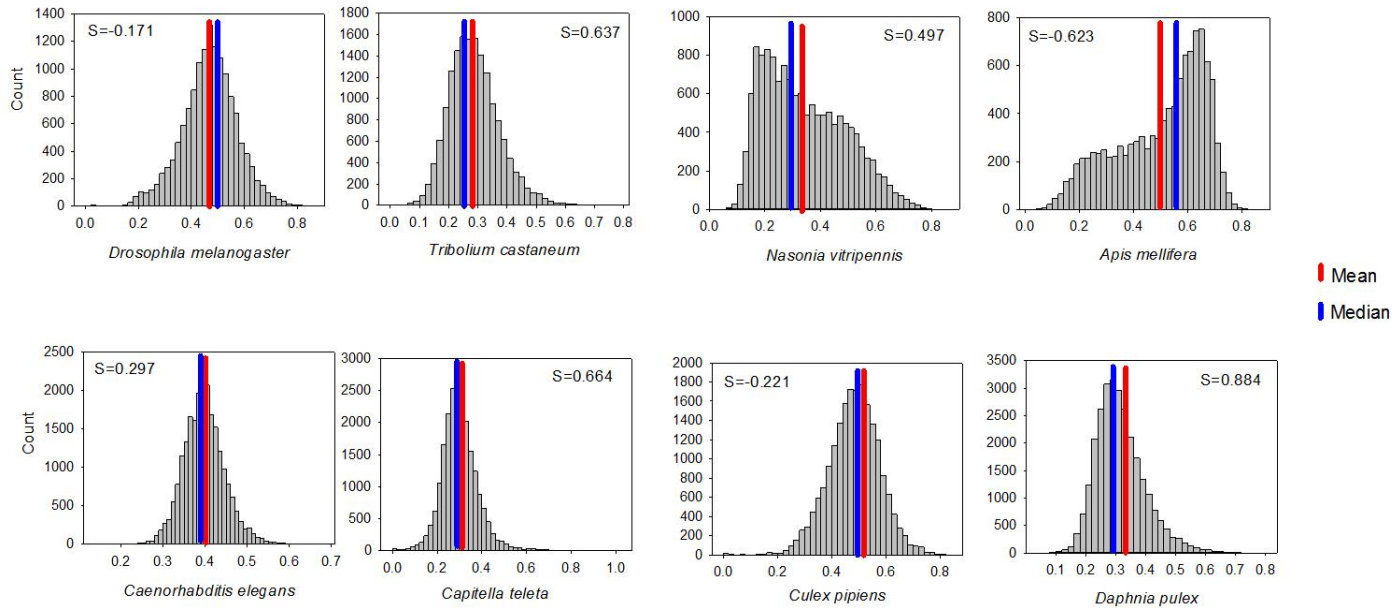
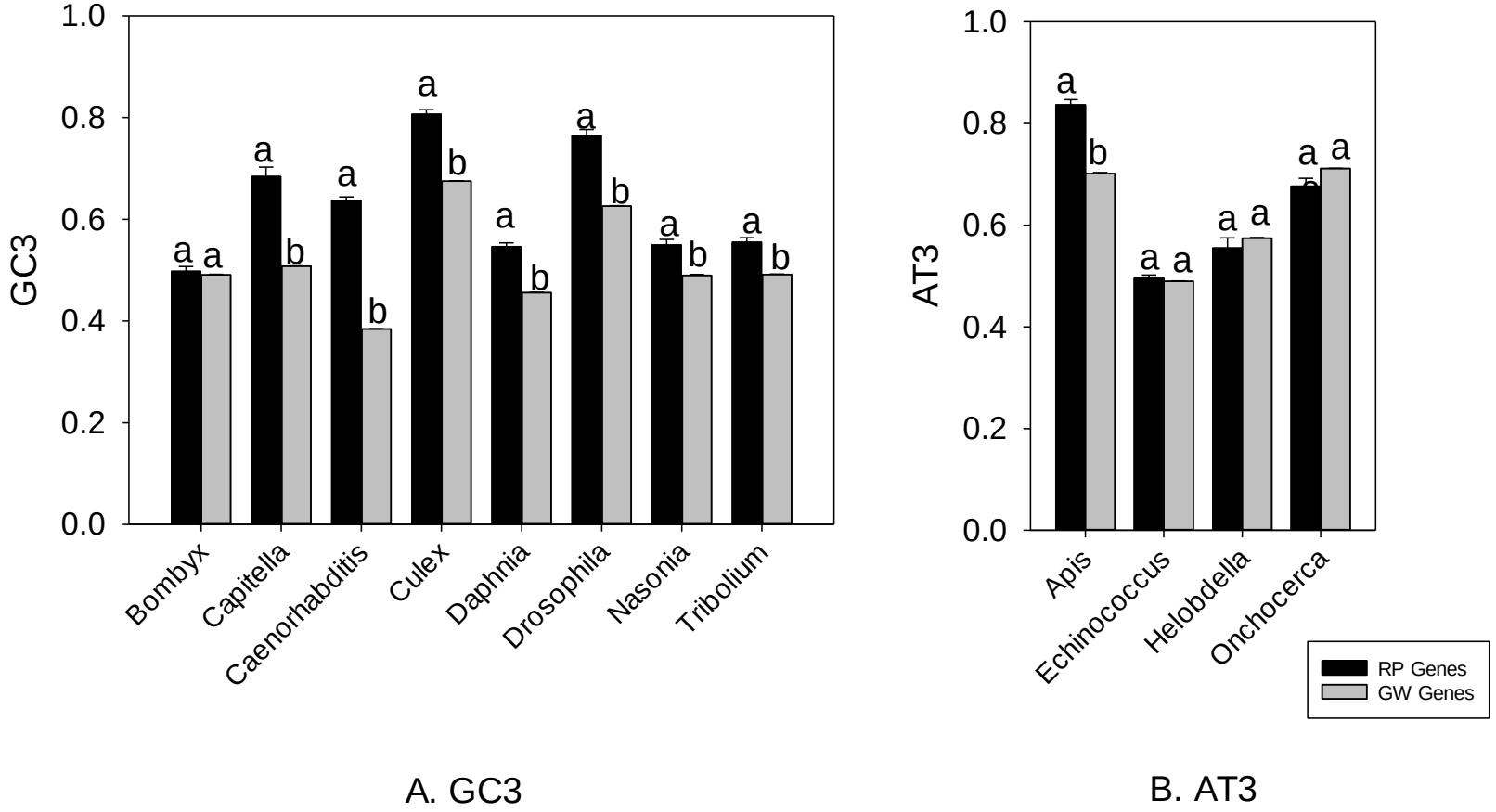
Supplementary Information



Supplementary Fig. 1. Box-whisker plots for the distribution of genome-wide (A) dN and (B) dS values for each of the twelve genera used in the dN/dS analysis.



Supplementary Fig. 2. The frequency distribution of Fop per gene for each species under study.



Supplementary Fig. 3. (A) The average GC3 content of ribosomal-protein genes (RP: black bars) and for all genes in the genome (GW: grey bars). Species listed are those wherein the putative optimal codons end in G or C (Supplementary Table 5). (B) The AT3 content for ribosomal protein genes (RP: black bars) and the genome-wide level (GW: grey bars) for species showing favoritism toward A- or T-ending codons (or no favoritism). Bars represent standard error. Species names are abbreviated using genus names.

Supplementary Table 1. The intrageneric species pairs and the intergeneric pairs used to compare dN/dS in the present study.

	Genera	Within-Genus Species Pair	PGC Specification Mode	Citation for PGC Specification Mode
Between Genus-Contrasts				
1	Drosophila	*D. melanogaster* and *D. simulans*	Preformation	[1-4]
	Tribolium	*T. castaneum* and *T. freemani*	Induction	[5,6]
2	Schistosoma	*S. japonicum* and *S. haematobium*	Preformation	[7-10]
	Echinococcus	*E. granulosus* and *E. multilocularis*	Induction	[11]
3	Nasonia	*N. vitripennis* and *N. giraulti*	Preformation	[12-14]
	Apis	*A. florea* and *A. mellifera*	Induction	[15-18]
4	Falco	*Falco cherrug* and *Falco peregrinus*	Preformation	[19]
	Alligator	*A. mississippiensis* and *A. sinensis*	Induction	[20]
5	Xenopus	*X. laevis* and *X. tropicalis*	Preformation	[21-26]
	Pan	*P. troglodytes* and *P. paniscus*	Induction	[27-29]
Supplemental Contrasts				
6	Anopheles	*A. darlingi* and *A. gambiae*	Preformation	[30]
	Tribolium	*T. castaneum* and *T. freeman*	Induction	See above

7	Pristionchus	*P. pacificus* and *P. exspectatus*	Preformation	[31,32]
	Echinococcus	*E. granulosus* and *E. multilocularis*	Induction	See above

Supplementary Table 2. The organisms examined in the present study and the location of their sequence datasets. Species were used in either dN/dS analysis and/or codon usage analysis. All datasets represent those versions available during the period of June to November 2014. Complete CDS were downloaded whenever possible, or were extracted from scaffolds. Note that genome Version Number is abbreviated as v.

Taxon	**Location of CDS or Scaffold Data**
dN/dS Analysis	
Alligator mississippiensis	**NCBI:** http://www.ncbi.nlm.nih.gov/bioproject/PRJNA221578 (Project ID PRJNA221578)
Alligator sinensis	**NCBI:** http://www.ncbi.nlm.nih.gov/bioproject/PRJNA221633 (Project ID PRJNA221633)
Anopheles darlingi	**Ensembl Genome:** http://metazoa.ensembl.org/Anopheles_darlingi/Info/Index (v. AdarC3.23)
Anopheles gambiae	**NCBI:** http://www.ncbi.nlm.nih.gov/bioproject/PRJNA163
Apis florea	**NCBI:** http://www.ncbi.nlm.nih.gov/refseq/ (Refseq v. 67, Organism *Apis florea*; Accessed Oct. 2014)
Apis mellifera	**Ensembl Genome:** http://metazoa.ensembl.org/Apis_mellifera/Info/Index (v. GCA_000002195.1.25)
Drosophila melanogaster	**FlyBase:** http://www.flybase.org (v. 5.57)
Drosophila simulans	**FlyBase:** http://www.flybase.org (v. r1.4)
Echinococcus granulosus	**Sanger:** http://www.sanger.ac.uk/resources/downloads/helminths/ (Accessed Oct. 2014)
Echinococcus multilocularis	**Sanger:** http://www.sanger.ac.uk/resources/downloads/helminths/ (Accessed Oct. 2014)
Falco cherrug	**NCBI:** http://www.ncbi.nlm.nih.gov/refseq/ (Refseq v. 67, Organism *Falco cherrug,* Accessed Oct. 2014*)*
Falco peregrinus	**NCBI:** http://www.ncbi.nlm.nih.gov/refseq/ (Refseq v. 67, Organism: *Falco peregrine;* Accessed Oct. 2014)
Nasonia giraulti	**NCBI:** http://www.ncbi.nlm.nih.gov/bioproject/PRJNA20223 (Project ID PRJNA2022; scaffolds)
Nasonia vitripennis	**Ensembl:**http://metazoa.ensembl.org/Nasonia_vitripennis/Info/Index **(**v. GCA_000002325.2.22)

Pan troglodytes	**Ensembl**: http://www.ensembl.org/Pan_troglodytes/Info/Index (v. CHIMP 2.1.4)
Pan paniscus	http://www.ncbi.nlm.nih.gov/refseq/ (Refseq v. 67, Organism: *Pan paniscus*; Accessed Oct. 2014)
Pristionchus pacificus	**Wormbase:** ftp://ftp.wormbase.org/pub/wormbase **(**v. WS246)
Pristionchus exspectatus	**Wormbase:** ftp://ftp.wormbase.org/pub/wormbase **(**v. WS246)
Schistosoma haematobium	**SchistoDB:** http://schistoDB.net/ (Accessed Oct. 2014)
Schistosoma japonicum	**SchistoDB:** http://schistoDB.net/ (Accessed Oct. 2014)
Tribolium castaneum	**Beetle Base:** http://beetlebase.org/ (http://metazoa.ensembl.org/Tribolium_castaneum)
Tribolium freeman	**Beetle Base:** http://beetlebase.org/ (Scaffold file name: tfre.scaffold0.fa; ftp://ftp.bioinformatics.ksu.edu/pub/BeetleBase/latest/)
Xenopus laevis	**Xenbase***:* http://xenbase.org (v. 6)
Xenopus tropicalis	**JGI:** http://genome.jgi-psf.org/Xentr4/Xentr4.info.html (v. 4)

Additional Taxa For Codon Usage Analysis

Apis mellifera	See above
Bombyx mori	**Silkdb:** http://www.silkdb.org/silkdb/doc/download.html
Caenorhabditis elegans	**Wormbase:** http://www.wormbase.org/ (WBcel235.75)
Capitella teleta	**Joint Genome Institute (JGI):** http://genome.jgi-psf.org/Capca1/Capca1.download.ftp.html (v. 1)
Culex pipiens	**Broad Institute:** http://www.broadinstitute.org/annotation/genome/culex_pipiens.4/MultiDownloads.html (v. 4)
Daphnia pulex	**JGI:** http://genome.jgi-psf.org/Dappu1/Dappu1.download.ftp.html (v. 1)
Drosophila melanogaster	See above
Echinococcus granulosus	See above
Helobdella robusta	**Ensembl**: http://metazoa.ensembl.org/Helobdella_robusta/Info/Index (v. GCA_000326865.1)

Nasonia vitripennis	See above
Onchocerca volvulus	**Wormbase**: ftp://ftp.wormbase.org/pub/wormbase/releases/WS245/species/o_volvulus/PRJEB513/ **(**v. WS246)
Tribolium castaneum	See above

Supplementary Table 3. The taxa examined in the present study, their phylum, class, order and family, and the number of putative orthologs within genera. For dN/dS, two pairs of species were examined per genera. Genera with opposite PGC modes were grouped into five phylogenetically independent contrasts (numbered in leftmost column). The number of orthologous CDS was determined after reciprocal BLASTX and removal of all sequences with any ambiguous nucleotides or internal stop codons. The identified paired putative orthologs per genus were processed and analyzed as described in Methods and Supplementary Note 1.3. See Table 1 for citations for PGC specification mode for each genus.

	Genera	Species Pair per Genera	Phylum, Class, Order, Family	PGC Specification Mode	No. of Putative Orthologous CDS
Primary dN/dS Contrasts					
1	Drosophila	*D. melanogaster* and *D. simulans*	Kingdom: Animalia Phylum: Arthropoda Subphylum (hexapods) Class Insecta Order Diptera Family: Drosophilidae	Preformation	11,896
	Tribolium	*T. castaneum* and *T. freemani*	Kingdom: Animalia Phylum: Arthropoda Subphylum: Hexapoda Class: Insecta Order: Coleoptera Family: Tenebrionidae	Induction	5,656
2	Schistosoma	*S. japonicum* and *S. haematobium*	Kingdom: Animalia Phylum: Platyhelminthes Class: Trematoda Subclass: Digenea Order: Strigeidida Family: Schistosomatidae	Preformation	6,189

	Echinococcus	*E. granulosus* and *E. multilocularis*	Kingdom: Animalia Phylum: Platyhelminthes Class: Cestoda Order: Cyclophyllidea Family: Taeniidae	Induction	9,208
3	Nasonia	*N. vitripennis* and *N. girault*i	Kingdom: Animalia Phylum: Arthropoda Class: Insecta. Order: Hymenoptera Family: Pteromalidae	Preformation	7,058
	Apis	*A. florea* and *A. mellifera*	Kingdom: Animalia Phylum: Arthropoda Class: Insecta Order: Hymenoptera Family: Apidae	Induction	6,869
4	Falco	*Falco cherrug* and *Falco peregrinus*	Kingdom: Animalia Phylum: Chordata Class: Aves Order: Falconiformes Family: Falconidae	Preformation	8,659
	Alligator	*A. mississippiensis* and *A. sinensis*	Kingdom: Animalia Phylum: Chordata Class: Reptilia Superorder: Crocodylomorpha Order: Crocodilia Family: Alligatoridae	Induction	11,376

5	Xenopus	*X. laevis* and *X. tropicalis*	Kingdom: Animalia Phylum: Chordata Class: Amphibia Order: Anura Family: Pipidae Subfamily: Xenopodinae	Preformation	8,926
	Pan	*P. troglodytes* and *P. paniscus*	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Primates Family: Hominidae	Induction	10,479
Supplemental dN/dS Contrasts					
6	Anopheles	*A. darlingi* and *A. gambiae*	Kingdom: Animalia Phylum: Arthropoda Subphylum (hexapods) Class Insecta Order Diptera Family: Culicidae	Preformation	7,483
	Tribolium	*T. castaneum* and *T. freeman*	See above	Induction	
7	Pristionchus	*P. pacificus* and *P. exspectatus*	Kingdom: Animalia Phylum: Nematoda Class: Chromadorea Order: Rhabditida Family: Diplogastridae	Preformation	8,829

Echinococcus	*E. granulosus* and *E. multilocularis*	See above	Induction

Supplementary Table 4. The species studied for optimal codon usage and their PGC specification mode.

	Genus	Species	Phylum, Class, Order, Family	PGC Specification Mode	Citation for PGC Specification Mode
1	Apis	*Apis mellifera*	Phylum: Arthropoda Class: Insecta. Order: Hymenoptera. Family: Apidae	Induction	15-18
2	Bombyx	*Bombyx mori*	Phylum: Arthropoda Subphylym (hexapods) Class Insecta Order Lepidoptera Family: Bombycidae	Induction	33-39
3	Caenorhabditis	*Caenorhabditis elegans*	Phylum: Nematoda Class: Chromadorea Order: Rhabditida Family: Rhabditidae	Preformation	40-42
4	Capitella	*Capitella teleta*	Phylum: Annelida Class: Polychaeta Subclass: Scolecida Family: Capitellidae	Induction	43-45
5	Culex	*Culex pipiens*	Phylum: Arthropoda Subphylum (hexapods) Class Insecta Order Diptera Flies: Culicidae	Preformation	30,46
6	Daphnia	*Daphnia pulex*	Phylum: Arthropoda Subphylum: Crustacea Class: Branchiopoda Order: Cladocera Family: Daphniidae	Preformation	47
7	Drosophila	*Drosophila melanogaster*	Phylum: Arthropoda Subphylum (hexapods) Class Insecta Order Diptera Family: Drosophilidae	Preformation	1-4

8	Echinococcus	*Echinococcus granulosus*	Phylum: Platyhelminthes Class: Cestoda. Order: Cyclophyllidea. Family: Taeniidae	Induction	[11]
9	Helobdella	*Helobdella robusta*	Phylum: Annelida Class: Clitellata Subclass: Hirudinea Order:Rhynchobdellida Family:Glossiphoniidae	Induction	[48,49]
10	Nasonia	*Nasonia vitripennis*	Phylum: Arthropoda Class: Insecta. Order: Hymenoptera Family: Pteromalidae	Preformation	[12-14]
11	Onchocerca	*Onchocerca volvulus*	Phylum: Nematoda Class: Secernentea Order: Spirurida Family: Onchocercidae	Preformation	[32,50]
12	Tribolium	*Tribolium castaneum*	Phylum: Arthropoda Subphylym (hexapods) Class Insects Order Coleoptera Family: Tenebrionidae	Induction	[5,6]

Supplementary Table 5. The putative optimal codons per amino acid for the 12 taxa under study herein. Putative optimal codons were determined using ΔRSCU values from CDS sequences of genes with the highest versus the lowest 3% of ENC values (high versus low codon usage bias (CUB)). The preference for GC3 or AT3 codons is also shown. The putative optimal codon per amino acid is in bold for each taxon. P-values of t-tests among genes with high versus low ENC after correction for multiple tests are shown with asterisks: *10^{-10}>P<0.05; **P≤10^{-10}. The "+" symbol indicates a gain in frequency of a codon in highly biased genes, while " –" indicates reduced level of the codon. Species names correspond to those presented in Supplementary Table 4 and have been abbreviated by genus name. Preformation has been abbreviated as P and induction as I. This table should be taken in conjunction with Supplementary Fig. 3 as described in Supplementary Note 1.4.

		Taxon											
Optimal Codons		**Apis**	**Bombyx**	**Capit.**	**Caeno.**	**Culex**	**Daphnia**	**Droso.**	**Echin.**	**Helob.**	**Nason.**	**Oncho.**	**Tribo.**
Number per Taxon		18	17	8	15	17	16	18	0	6	18	11	11
GC3 or AT3 Biased		AT	GC	GC	GC	GC	GC	GC	-	AT	GC	AT	GC
PGC Mode		I	I	I	P	P	P	P	I	I	P	P	I
		Δ RSCU Values											
Amino Acid	**Codon**												
Ala	GCT	+0.47**	-0.44**	-0.2*	+0.51**	-0.47**	+0.04	-0.37**	-0.08	+0.17	-0.72**	+0.1	-0.11
Ala	GCC	-0.77**	+0.3*	**+0.3***	**+0.66****	**+1.05****	**+0.4****	**+1.34****	+0.01	-0.38*	**+1.25****	-0.33*	**+0.41****
Ala	GCA	**+1.14****	-0.25*	-0.18*	-0.63**	-0.69**	-0.26*	-0.68**	-0.04	**+0.3***	-0.79**	**+0.46***	-0.27*
Ala	GCG	-0.94**	**+0.34***	-0.08	-0.6**	-0.12	-0.22*	-0.25*	-0.03	-0.19*	+0.27*	-0.35*	-0.05
Arg	CGT	+0.07	-0.36*	-0.04	+1.2**	+0.12	-0.01	+0.37*	+0.24	-0.13	-0.42*	-0.06	-0.25*
Arg	CGC	-0.52**	**+1.01****	**+0.34***	**+0.45***	**+1.03****	**+0.78****	**+2.08****	+0.01	-0.33*	**+1.24****	-0.28*	**+0.24***
Arg	CGA	-0.36*	-0.4**	-0.59**	-0.91**	-0.78**	-0.37*	-0.87**	-0.21	-0.33*	-0.66**	**+0.63***	-0.2*
Arg	CGG	-0.52**	+0.31*	-0.23*	-0.64**	+0.13	-0.22*	-0.33*	-0.22	-0.32**	+0.43**	-0.21	+0.05
Arg	AGA	**+2.13****	-0.26	-0.03	+0.24	-0.33*	-0.11	-0.82**	-0.07	**+1.19****	-0.94**	+0.27	-0.01
Arg	AGG	-0.83**	-0.33*	+0.12	-0.61**	-0.42**	-0.25*	-0.55**	+0.15	-0.19	+0.33*	-0.46*	+0
Asn	AAT	**+0.67****	-0.39**	-0.15*	-0.58**	-0.56**	-0.25**	-0.66**	-0.07	+0.07	-0.84**	**+0.28***	-0.24*

Asn	AAC	-0.69**	**+0.31****	-0.01	**+0.54****	**+0.47****	**+0.15***	**+0.67****	-0.08	-0.11*	**+0.79****	-0.28*	**+0.13***
Asp	GAT	**+0.65****	-0.43**	-0.17*	-0.25*	-0.45**	-0.21**	-0.39**	-0.08	+0.1	-0.8**	+0.11	-0.2*
Asp	GAC	-0.68**	**+0.37****	+0.01	+0.09	**+0.29****	**+0.1***	**+0.37****	-0.05	-0.19*	+0.77**	-0.22*	+0.08
Cys	TGT	**+0.71****	-0.33**	-0.38**	-0.39**	-0.41**	-0.34**	-0.53**	-0.04	+0	-0.61**	+0.01	-0.27*
Cys	TGC	-0.78**	**+0.26***	-0.14	**+0.17***	+0.04	-0.05	**+0.44****	-0.15	-0.3**	**+0.57****	-0.18	-0.09
Gln	CAA	**+0.49****	-0.34**	-0.37**	+0.12	-0.58**	-0.23**	-0.76**	-0.08	+0.1	-0.77**	+0.11	-0.1
Gln	CAG	-0.53**	**+0.28***	**+0.11**	-0.22*	**+0.48****	**+0.12***	**+0.73****	-0.1	-0.26**	**+0.76****	-0.24*	+0
Glu	GAA	**+0.68****	-0.41**	-0.26**	-0.35**	-0.49**	-0.16*	-0.75**	-0.1	+0.12*	-0.86**	+0.24*	-0.18*
Glu	GAG	-0.69**	**+0.37****	+0.07	**+0.26***	**+0.38****	**+0.11***	**+0.71****	+0.02	-0.24**	**+0.84****	-0.27*	+0.08
Gly	GGT	+0.39*	-0.39*	-0.11	-0.39**	-0.14	-0.16*	-0.23*	+0.04	+0.08	-0.66**	+0.33	-0.16
Gly	GGC	-0.79**	**+0.54****	**+0.28***	-0.63**	**+0.26***	**+0.31***	**+1.05****	-0.09	-0.2*	**+1.44****	-0.4*	+0.15
Gly	GGA	**+0.81****	-0.29*	-0.17*	**+1.5****	-0.18	-0.02	-0.37*	-0.12	+0.17	-0.6**	**+0.33**	+0.03
Gly	GGG	-0.51**	+0.07	-0.27*	-0.44**	-0.23*	-0.22*	-0.44**	-0.07	-0.31**	-0.15*	-0.24*	-0.13
His	CAT	**+0.69****	-0.37**	-0.19*	-0.31**	-0.47**	-0.32**	-0.5**	-0.15	+0	-0.7**	+0.1	-0.18*
His	CAC	-0.74**	**+0.28***	-0.09	+0.07	**+0.24***	**+0.12***	**+0.5****	-0.04	-0.24*	**+0.72****	-0.23*	+0.04
Ile	ATT	+0.41**	-0.43**	-0.3*	-0.44**	-0.57**	-0.24*	-0.5**	-0.01	+0.09	-0.82**	**+0.29***	-0.21*
Ile	ATC	-0.86**	**+0.56****	**+0.3***	**+0.82****	**+0.83****	**+0.36****	**+1.19****	+0.02	-0.17*	**+1.36****	-0.25*	**+0.31***
Ile	ATA	**+0.46****	-0.23*	-0.18*	-0.45**	-0.39**	-0.25**	-0.71**	-0.1	+0.01	-0.55**	-0.09	-0.24*
Leu	TTA	**+2.7****	-0.37*	-0.1	-0.54**	-0.26*	-0.32**	-0.61**	-0.06	+0.51**	**-0.78****	**+0.86****	-0.19
Leu	TTG	-0.71**	-0.48**	-0.13	-0.4*	-0.63**	**+0.3***	-0.67**	+0.06	+0.16	-0.93**	+0.26	+0.17
Leu	CTT	-0.05	-0.41**	-0.41**	+1.01**	-0.62**	-0.26*	-0.65**	-0.03	-0.12	-0.71**	-0.28*	-0.15
Leu	CTC	-0.77**	+0.45*	-0.11	**+1.27****	-0.12	+0.24*	+0.1	+0.25	-0.33**	**+1.82****	-0.2	+0.13
Leu	CTA	-0.21*	-0.29*	-0.24*	-0.56**	-0.44**	-0.3**	-0.58**	-0.22*	-0.02	-0.62**	-0.23	-0.34**
Leu	CTG	-0.94**	**+1.08****	**+0.83****	-0.76**	**+2.08****	+0.27*	**+2.42****	-0.02	-0.32*	+1.23**	-0.44*	**+0.33***
Lys	AAA	**+0.57****	-0.42**	-0.39**	-0.7**	-0.58**	-0.19*	-0.73**	-0.11	**+0.12***	-0.87**	**+0.2***	-0.17*
Lys	AAG	-0.57**	+0.32**	+0.24**	**+0.65****	**+0.52****	**+0.1***	**+0.73****	-0.02	-0.16*	**+0.87****	-0.22*	**+0.13***
Phe	TTT	**+0.79****	-0.34**	-0.29**	-0.67**	-0.43**	-0.29**	-0.8**	-0.08	+0.11	-0.75**	**+0.26***	-0.28*

Phe	TTC	-0.82**	**+0.27***	+0.01	**+0.59****	**+0.34****	**+0.14***	**+0.79****	-0.03	-0.27**	**+0.72****	-0.27*	**+0.13***
Pro	CCT	+0.28*	-0.37*	-0.43**	-0.58**	-0.45**	-0.18*	-0.39**	-0.02	-0.05	-0.69**	-0.26	-0.27*
Pro	CCC	-0.49**	**+0.28***	+0.19	-0.54**	+0.11	**+0.21***	**+1.17****	+0	-0.4**	**+1.03****	-0.14	**+0.33***
Pro	CCA	**+1.19****	-0.36*	-0.2*	**+1.83****	-0.62**	-0.1	-0.59**	+0.08	**+0.4***	-0.92**	+0.25	-0.2
Pro	CCG	-0.93**	+0.28*	+0.05	-0.8**	**+0.58****	-0.07	-0.2*	-0.18	-0.41**	+0.52**	-0.02	-0.09
Ser	TCT	+0.81**	-0.27*	-0.01	+0.44*	-0.4**	+0	-0.44**	+0.07	+0.12	-0.76**	-0.14	-0.26*
Ser	TCC	-0.72**	**+0.39***	+0.09	**+1.16****	**+0.58****	+0.08	**+1.09****	-0.05	-0.33**	+0.47**	-0.27*	**+0.29***
Ser	TCA	**+1.17****	-0.45*	-0.21*	-0.29*	-0.56**	-0.31*	-0.69**	+0.03	+0.19	-0.74**	**+0.83****	-0.26*
Ser	TCG	-1.01**	+0.39*	-0.04	-0.41**	+0.49*	+0	+0.26*	-0.03	-0.38**	+0.48**	-0.24	+0
Ser	AGT	+0.56**	-0.34*	-0.07	-0.71**	-0.51**	-0.14*	-0.69**	+0.01	+0.21	-0.71**	+0.1	+0
Ser	AGC	-0.87**	+0.15	+0.1	-0.21*	+0.19	**+0.33***	+0.5**	-0.05	+0.04	**+1.26****	-0.27*	+0.07
Thr	ACT	+0.55**	-0.33*	-0.14	-0.01	-0.53**	-0.17	-0.54**	+0.04	+0.1	-0.79**	+0	-0.21*
Thr	ACC	-0.81**	+0.25*	**+0.29***	**+1.13****	**+0.87****	**+0.41****	**+1.49****	+0.05	-0.28*	**+1.18****	-0.2*	**+0.34***
Thr	ACA	**+1.25****	-0.35*	-0.3*	-0.68**	-0.51**	-0.2*	-0.8**	-0.03	**+0.43****	-0.81**	**+0.33**	-0.28*
Thr	ACG	-1.02**	**+0.28***	-0.12	-0.54**	+0.02	-0.11*	-0.22*	-0.11	-0.35**	+0.38*	-0.25*	+0.06
Tyr	TAT	**+0.78****	-0.37**	-0.15*	-0.47**	-0.41**	-0.3**	-0.67**	-0.11	+0.14	-0.66**	**+0.21***	-0.21*
Tyr	TAC	-0.77**	**+0.28***	-0.16*	**+0.44****	**+0.3****	+0.11	**+0.66****	-0.12	-0.29*	**+0.67****	-0.2*	+0.1
Val	GTT	+0.75**	-0.43**	-0.35**	+0.11	-0.45**	-0.13*	-0.59**	-0.07	**+0.29***	-0.84**	+0.26	-0.19
Val	GTC	-0.71**	+0.04	**+0.23***	**+0.88****	**+0.65****	**+0.3***	+0.38**	-0.13	-0.38**	**+1.17****	-0.32*	-0.01
Val	GTA	**+0.87****	-0.3*	-0.16*	-0.47**	-0.49**	-0.23*	-0.6**	-0.05	+0.06	-0.64**	+0.23	-0.09
Val	GTG	-0.9**	**+0.64****	+0.07	-0.54**	+0.23*	-0.01	**+0.79****	+0.18	-0.1	+0.31*	-0.25	**+0.23***

Supplementary Table 6. The GC3 content of genes with the upper 3% codon usage bias (lowest ENC) and for the genome-wide CDS in the 13 taxa under study. The CDS were concatenated prior to calculation of GC3.

	Apis	Bombyx	Caeno.	Capit.	Culex	Daphnia	Droso.	Echin.	Helob.	Nason.	Oncho.	Tribo.
GC3 of 3% Most-Biased Genes	0.09	0.77	0.52	0.58	0.73	0.62	0.78	0.51	0.35	0.89	0.21	0.55
GC3 of Genome-Wide CDS	0.32	0.49	0.37	0.51	0.68	0.46	0.62	0.49	0.429	0.5	0.28	0.49

Supplementary Table 7. A sample of 121 known developmental genes used in our study. The FlyBase identification number, gene name and gene symbol are shown for each gene. The expression profiles and dN/dS values are shown in Fig. 4.

FB ID	Gene Name	Gene Symbol
FBgn0000014	*abdominal A*	*abd-A*
FBgn0000015	*Abdominal B*	*Abd-B*
FBgn0010379	*Akt1*	*Akt1*
FBgn0000097	*anterior open*	*aop*
FBgn0031458	*anterior pharynx defective 1*	*aph-1*
FBgn0262739	*Argonaute-1*	*AGO1*
FBgn0004569	*argos*	*aos*
FBgn0000117	*armadille*	*arm*
FBgn0000114	*arrest*	*aret*
FBgn0000119	*arrow*	*arr*
FBgn0024491	*Bicoid interacting protein 1*	*Bin1*
FBgn0000179	*bifid*	*bi*
FBgn0014135	*branchless*	*bnl*
FBgn0005592	*breathless*	*btl*
FBgn0261787	*brunelleschi*	*bru*
FBgn0004856	*Bx42*	*Bx42*
FBgn0000250	*cactus*	*cact*
FBgn0262975	*cap-n-collar*	*cnc*
FBgn0000251	*caudal*	*cad*
FBgn0036827	*CG6843*	*CG6843*
FBgn0013764	*Chip*	*Chi*
FBgn0000382	*corkscrew*	*csw*
FBgn0000339	*cornichon*	*cni*
FBgn0014143	*crocodile*	*croc*
FBgn0000394	*crossveinless*	*cv*

FBgn0004859	*cubitus interruptus*	*ci*
FBgn0000405	*Cyclin B*	*CycB*
FBgn0000490	*decapentaplegic*	*dpp*
FBgn0000439	*Deformed*	*Dfd*
FBgn0000524	*deltex*	*dx*
FBgn0000157	*Distal-less*	*Dll*
FBgn0010269	*Downstream of raf1*	*Dsor1*
FBgn0004638	*downstream of receptor kinase*	*drk*
FBgn0000576	*empty spiracles*	*ems*
FBgn0004875	*encore*	*enc*
FBgn0003731	*Epidermal growth factor receptor*	*Egfr*
FBgn0000611	*extradenticle*	*exd*
FBgn0001085	*frizzled*	*fz*
FBgn0001078	*ftz transcription factor 1*	*ftz-f1*
FBgn0001079	*fused*	*fu*
FBgn0001077	*fushi tarazu*	*ftz*
FBgn0250823	*gilgamesh*	*gish*
FBgn0024234	*glass bottom boat*	*gbb*
FBgn0001148	*gooseberry*	*gsb*
FBgn0264495	*grappa*	*gpp*
FBgn0001139	*groucho*	*gro*
FBgn0001137	*gurken*	*grk*
FBgn0004644	*hedgehog*	*hh*
FBgn0015805	*Histone deacetylase 1*	*HDAC1*
FBgn0263782	*HMG Coemzyme A reductase*	*Hmgcr*
FBgn0001235	*homothorax*	*hth*
FBgn0004864	*hopscotch*	*hop*
FBgn0261434	*huckebein*	*hkb*
FBgn0001180	*hunchback*	*hb*

FBgn0037657	*hyrax*	*hyx*
FBgn0001320	*knirps*	*kni*
FBgn0001319	*knot*	*kn*
FBgn0001325	*Kruppel*	*Kr*
FBgn0002522	*labial*	*lab*
FBgn0011278	*ladybird early*	*lbe*
FBgn0002552	*lines*	*lin*
FBgn0002736	*mago nashi*	*mago*
FBgn0011648	*Mothers against dpp*	*Mad*
FBgn0011656	*Myocyte enhancer factor 2*	*Mef2*
FBgn0038872	*Negative elongation factor A*	*Nelf-A*
FBgn0017430	*Negative elongation factor E*	*Nelf-E*
FBgn0261617	*nejire*	*nej*
FBgn0039234	*nicastrin*	*nct*
FBgn0004647	*Notch*	*N*
FBgn0004102	*oceliless*	*oc*
FBgn0002985	*odd*	*odd skipped*
FBgn0003002	*odd paired*	*opa*
FBgn0025360	*Optix*	*Optix*
FBgn0261885	*osa*	*osa*
FBgn0020622	*Pi3K21B*	*Pi3K21B*
FBgn0003089	*pip*	*pipe*
FBgn0019947	*Presenilin*	*Psn*
FBgn0053198	*presenilin enhancer*	*pen-2*
FBgn0004595	*prospero*	*pros*
FBgn0000273	*Protein kinase, cAMP-dependent, catalytic subunit 1*	*Pka-C1*
FBgn0003165	*pumilio*	*pum*
FBgn0043900	*pygopus*	*pygo*
FBgn0033649	*pyramus*	*pyr*

FBgn0037364	*Rab23*	*Rab23*
FBgn0003079	*Raf oncogene*	*Raf*
FBgn0004390	*Ras GTPase activating protein 1*	*RasGAP1*
FBgn0003205	*Ras85D*	*Ras oncogene at 85D*
FBgn0024194	*rasp*	*rasp*
FBgn0004795	*retained*	*retn*
FBgn0004635	*rhomboid*	*rho*
FBgn0003300	*runt*	*run*
FBgn0003345	*scalloped*	*sd*
FBgn0003463	*short gastrulation*	*sog*
FBgn0027363	*Signal transducing adaptor molecule*	*Stam*
FBgn0016917	*Signal-stansducer and activator of transcription protein at 92E*	*Stat92E*
FBgn0004666	*single-minded*	*sim*
FBgn0024291	*Sirtuin 1*	*Sirt1*
FBgn0003430	*sloppy paired 1*	*slp1*
FBgn0003450	*snake*	*snk*
FBgn0001965	*Sons of sevenless*	*Sos*
FBgn0261648	*spalt major*	*salm*
FBgn0020767	*Sprouty-related protein with EVH-1 domain*	*Spred*
FBgn0263396	*squid*	*sqd*
FBgn0030869	*Suppressor of Cytokine signaling at 16D*	*Socs16D*
FBgn0041184	*Suppressor of Cytokine Signaling at 36E*	*Socs36E*
FBgn0033266	*Suppressor of Cytokine Signling at 44A*	*Socs44A*
FBgn0005355	*Suppressor of fused*	*Su(fu)*
FBgn0004837	*Suppressor of Hairless*	*Su(H)*
FBgn0039734	*Tace*	*Tace*
FBgn0033652	*thisbe*	*ths*
FBgn0262473	*Toll*	*Tl*

FBgn0003867	*torso-like*	*tsl*
FBgn0265974	*tout-velu*	*ttv*
FBgn0086356	*tumbleweed*	*tum*
FBgn0003900	*twist*	*twi*
FBgn0003944	*Ultrabithorax*	*Ubx*
FBgn0004003	*windbeutel*	*wbl*
FBgn0004360	*Wnt oncogene analog 2*	*Wnt2*
FBgn0036141	*wntless*	*wls*
FBgn0016078	*wunen*	*wun*
FBgn0041087	*wunen-2*	*wun-2*

Supplementary Notes

Supplementary Note 1 (related to Fig. 2a)

Mann-Whitney U-tests across whole genome dN/dS support no consistent trends with respect to preformation and induction. dN/dS tended toward significantly higher values for preformation genera in only two cases (Drosophila (preformation) versus Tribolium (induction) and Nasonia (preformation) versus Apis (induction)), but was significantly higher for induction genera than preformation genera in two other cases (Echinococcus (induction) versus Schistosoma (preformation), and Pan (induction) versus Xenopus (preformation); $P<10^{-15}$ for all contrasts), and showed no significant difference between Falco (preformation) and Alligator (induction) (P=0.13). In summary, multiple independent paired contrasts of genome-wide dN/dS distributions across metazoans do not support a trend of rapid gene evolution under preformation.

We report in Fig. 2a that the taxa Anopheles (preformation) and Pan (induction) had among the highest fraction of their CDS with dN/dS >0.5 (>29%), and >1 (>4%) of all genera under study. These trends indicate that highly similar dN/dS distributions can occur across organisms with opposite PGC modes. For Anopheles in particular, the unusually high fraction of genes with accelerated protein evolution could be explained by a number of life history traits that are independent of PGC specification mode, for example, its role as a vector in malaria transmission, which likely requires rapid adaption to the host and gene evolvability [51,52]. Pristionchus (preformation) exhibited a similar dN/dS profile to that observed in numerous other organisms with varying PGC specification modes, including Drosophila (preformation), Tribolium (induction), Schistosoma (preformation), Apis (induction) and Xenopus (preformation), again suggesting no link between PGC specification mode and the global rate of evolution of protein sequences. Pristionchus (preformation) also had fewer CDS with dN/dS>0.5 than Echinococcus. Collectively, the genome-wide profiles of dN/dS provide no evidence for a tendency towards rapid genome evolution in preformation organisms in invertebrates nor in vertebrates.

Supplementary Note 2 (related to Fig. 2b Falco versus Alligator)

A total of 58.1% of the 2,537 CDS exhibiting >1.5 differences between the vertebrates Falco (preformation) and Alligator (induction), had elevated dN/dS in the induction taxon rather than the preformation taxon. Nevertheless, the two Falco species under study (*F. cherrug* and *F. peregrines*) have been shown to exhibit rapid evolution of orthologs as compared to other birds such as chicken, turkey and zebra finch [53]. Thus, even closely related species with preformation, can exhibit relatively fast or slow gene evolution within a single class (Aves) [53,54]. Moreover, recent findings indicate that alligators exhibit very slow rates of sequence evolution per unit time, as compared to birds [55]. Indeed, after converting our dN and dS values to rates per unit time using divergence time of at least 23 (Paleogene period) and 2.1 Mya, respectively [53,56], we obtained a more than 8 fold lower substitution rate in alligators than birds for each parameter (MWU-test $P<10^{-15}$; note that using the upper limit of 66mya for the Paleogene period, yields a 2.8 fold lower rate in alligators than birds). This agrees with the notion that alligators have an exceptionally low mutation rate, in fact the lowest found among vertebrates to date [55]. Nevertheless, our data show that dN/dS distributions exhibit no notable differences among birds and alligators (Fig. 2) at broad scale, suggesting a comparable propensity for relaxed or positive selection under preformation and induction in these vertebrates. We do not exclude differences in these taxa for specific groups of genes (or for any of the taxon pairs studied), but the results suggest no broad effect observable across the genome with respect to PGC-specification mode.

It is worth noting that birds, which have extensive publicly available intergeneric genome data, have been shown to exhibit variable dN/dS among lineages [57], have lower dN/dS than mammals, (induction) for genes from many GO classes [57], and their mtDNA dN/dS has been shown to be typically lower than crocodiles, proposed to result from their endothermic nature [58]. None of these observations is consistent with PGC-specification mode being a major factor shaping protein evolution in this vertebrate taxon.

Supplementary Note 3 (Excluding a Role of Saturation, Divergence time, and Population Size on Results in Fig. 2)

We address three important factors that could be hypothesized to account for the patterns we observed in dN/dS in our paired contrasts. First, for the analyses in Figs. 2ab, we verified that genome-wide dN and dS were unsaturated for all interspecies contrasts within genera. The mean and median of dN and dS values were well below 1 for each genus (Supplementary Fig. 1). Nonetheless, any genes identified as substantial outliers (dS >3) between putative orthologs (Supplementary Table 3) were excluded from analysis. For further stringency, we repeated all our entire analyses (Figs. 2-4) excluding all those genes per genus (per species-pair) with dS values above the 90th percentile to avoid any potential effect of saturation (as well as avoiding putative misalignments or orthology mismatches, see Methods) and found nearly identical results for all of our figures (results not shown). Thus overall, our results from dN/dS analyses of genome-wide unsaturated and independent contrasts of preformation and induction genera (Figs. 2ab) suggest no consistent connection between PGC specification mode and molecular evolution. We note that the species pair for Falco and for Pan had the lowest divergence in dN or dS among all species pairs (Supplementary Fig. 1). For these species pairs, similar to all other species pairs, we presented all orthologs with dN≥0 and dS>0 in Fig. 2, noting dN=0 were most common in these taxa. The median dS for genes studied (dS>0) with dN =0 (Median Falco=0.006, Median Pan=0.006) closely matched the median across studied genes (Median $Falco_{All\ Genes}$=0.007, Median $Pan_{All\ Genes}$=0.007); suggesting the cases with a zero value for dN were the result of purifying selection, rather than to insufficient evolutionary time to accumulate detectable mutations.

Second, it has been suggested that dN/dS in bacteria may be elevated for more closely related than distantly related species pairs, due to a time lag in removal of slightly deleterious mutations [59]. Such a phenomenon cannot explain the present results in the eukaryotes studied here. For example, for Drosophila (preformation), the species examined (*D. melanogaster* and *D. simulans*) have a divergence time of about 1.2 Mya [60] whilst the Tribolium (induction) species (*T. castaneum* and *T. freemani*) diverged >11.6 mya [61]. The fact that we found only a very marginal proportion of genes with elevated dN/dS in Drosophila rather than Tribolium (Figs. 2ab), despite the potential for the shorter divergence time in the preformation genus to enhance dN/dS, strengthens our conclusions. Similarly, divergence times are lower for the two species of Nasonia (preformation) (~1 Mya, [62,63] than for Apis (preformation) (approximately Miocene, 5-25 Mya, [64]. Thus, if divergence time affected dN/dS, the marginally higher values observed in Nasonia would be an overestimate, again strengthening our conclusions. The two Falco (preformation) species (Table 1) have a shorter divergence time (~2.1mya; [53]) than those from Alligator (induction) (>23 mya) [56], but despite a short divergence time that could possibly increase dN/dS for the preformation taxon, we still observed higher values under induction (Fig. 2b). Finally, the divergence time of the two species of Pan is lower (<1.6mya [65]) than that of the Xenopus species (50mya, [66]), and divergence times are, to our knowledge, not established for Schistosoma (preformation) and Echinococcus (induction) species studied here. Thus, we cannot formally exclude the possibility that the tendency for lower dN/dS under preformation than induction in these two cases was partly due to shorter divergence times for species with induction (Fig. 2b). However, we suggest this is unlikely given the lack of an

effect observed in all the other contrasts. Collectively, these trends point toward the conclusion that our results cannot be explained by divergence time variation.

Third, small effective population sizes (Ne) can enhance dN, and thus dN/dS, mainly for the subset of genes in the genome with large negative selection coefficients, by allowing more frequent fixation of deleterious amino acids [67]. We consider the role of population size here, and do not exclude the possibility that Ne had an effect on dN/dS. Rather, we argue that Ne could not explain our results. For instance, in the contrasts that opposed the preformation/induction theory (i.e., exhibited similar dN/dS under preformation and induction, or had higher dN/dS under induction), namely Drosophila (preformation) versus Tribolium (induction), Schistosoma (preformation) versus Echinococcus (induction), Pristionchus (preformation) versus Echinococcus (induction), Falco (preformation) versus Alligator (induction), and Xenopus (preformation) versus Pan (induction), the induction taxon could have had a history of smaller Ne or experienced more bottlenecks over its evolutionary history, leading to elevated dN/dS values for the induction taxon. However, this appears unlikely to have occurred for all five independent contrasts, and particularly for the insects Drosophila (preformation) and Tribolium (induction), and for the two contrasts involving Schistosoma (preformation), Echinococcus (induction) and Pristionchus (preformation) which all represent short-lived free-living or parasitic worms. Ne could have an effect for the comparison of Falco (preformation) versus Alligator (induction), where smaller populations or more bottlenecks may have occurred in the evolutionary history of the latter taxon (but this remains debatable [53,68]). Population size could also play a role in Xenopus (preformation) versus Pan (induction), wherein the latter taxon has a longer generation time (15 years, Stone et al. 2010; and is four months to two years in Xenopus, http://www.xenbase.org), which typically corresponds to a smaller population size [65,67,69]. However, even if Ne were smaller for the induction taxon in these two latter cases, if preformation is indeed the predominant factor accelerating protein evolution and liberating selective constraint, as concluded by Evans et al. [70], then it would be expected to counteract any effect of a small-Ne in the compared induction species; thus closing any gap in dN/dS values among preformation and induction or even yielding higher dN/dS under preformation. Taken together, we conclude that our findings are unlikely to be explained by population size, and that preformation does not accelerate dN/dS in the animals studied herein

Supplementary Note 4 (Frequency of Optimal Codons and PGC Mode)

As a complementary test to dN/dS, we studied the frequency of optimal codons (Fop) and report that this parameter is also uncorrelated to PGC specification mode. Optimal codons may not be present in every organism, but have been reported a wide range of animal systems, including Drosophila, Caenorhabditis, and Tribolium [71-73]. Analysis of optimal codon usage has been employed in Drosophila and other eukaryotes to detect rapidly evolving proteins [74-76], as proteins that are evolving rapidly appear to have low Fop [74-78]. The explanation for this relationship is twofold. First, purifying selection often affects proteins and codon usage similarly [77,79,80]. Thus, relaxed purifying selection on proteins may be detected as reduced Fop [77,80]. Second, positive selection on a protein sequence can reduce Fop due to selective sweeps, leading to fixation of non-optimal codons at linked gene sites[81-83]. Under the hypothesis of liberation of selective constraint on proteins from preformation species proposed by Evans et al. [70], which presumably includes relaxed selection and/or positive selection, we would expect to detect losses of optimal codons in organisms with preformation.

To test whether Fop is connected to PGC mode, we first needed to verify, or in some cases identify, the list of optimal codons for each taxon under study (see below "Identification of Optimal Codons" in Section 1.4). For this, we examined whole genome-CDS for twelve taxa that have publicly available large-scale DNA sequence datasets and a known mode of PGC formation (Supplementary Table 4). Within this species list, we included *D. melanogaster*, *T. castaneum*, *Nasonia vitripennis* and *Apis mellifera* as controls to compare to our

dN/dS findings, and eight additional species listed in Supplementary Table 4. In summary, we found optimal codons for the four aforementioned taxa as well as for the species *C. elegans*, *C. teleta*, *Culex pipiens*, and *Daphnia pulex* (for further details, see below "Identification of Optimal Codons"). Most of these species had putative optimal codons ending in GC3, but *A. mellifera* had AT3 putative optimal codons (Supplementary Table 5, also verified with ribosomal protein gene analysis, see "Identification of Optimal Codons"). Four of the twelve species studied had inconclusive or had no evidence of optimal codons. As species without optimal codons are not informative with regard to selection relative to PGC specification mode, these species were not included in subsequent analyses.

Using the optimal codon list for each of eight taxa, we studied the frequency distributions of gene Fop values across the genome (Supplementary Fig. 2). If an increased rate of protein sequence evolution arises due to relaxed and/or positive selection after an evolutionary transition to the preformation mode of PGC formation, then one would predict that a major portion of gene sequences should exhibit lowered Fop relative to the genome-wide Fop in such taxa [74,76]. Instead, we found that for all eight species under study, regardless of PGC specification mode, Fop appeared approximately normally distributed. This distribution profile is consistent with patterns previously observed for Fop (GC3) in Drosophila [84]. Nevertheless, for each species, Fop exhibited mild skewing, with mildness defined as $0<S<1$ for positive skewing, or $0>S>-1$ for negative skewing [85] (P-value of Kolmogorov-Smirnov test (K-S) of normality <0.05 for all species). While the absolute value of skewness (S) was <1 for each species, no severe cases of skewness (S>2) were observed.

The Fop distribution varied mildly among taxa. For instance, in our control species (those in which we had both dN/dS and Fop data) *D. melanogaster* (preformation) and *T. castaneum* (induction) very weak skewing was observed in each taxon (Supplementary Fig. 2), and agrees with the absent/very mild genome-wide differences detected between these taxa in dN/dS (Figs. 2ab). For *N. vitripennis* we found an abundance of low Fop values that clustered below the average (Supplementary Fig. 2), whilst *A. mellifera* showed on opposite clustering toward high Fop values. This is also consistent with the dN/dS analysis, which showed that a marginally greater portion of gene sequences had elevated dN/dS in *N. vitripennis* relative to *A. mellifera* (Fig. 2). Together, these results indicate that Fop reflects the patterns of genome-wide protein evolution as revealed by dN/dS analysis in these taxa [74,77,78]. Thus, we used Fop as a proxy for protein evolution in the remaining four organisms, which are described in the main text for Supplementary Fig. 2.

Identification of Optimal Codons (used to calculate Fop above)

We confirmed, or identified, optimal codon lists for twelve animal species in our study. Taxa and their PGC mode are listed in Supplementary Table 4, and include species of Drosophila. Tribolium, Nasonia, Apis, Bombyx, Capitella, Caenorhabditis, Culex, Daphnia, Echinococcus, Helobdella and Onchocerca. Putative optimal codons can be identified by asking which synonymous codons increase in frequency per amino acid as genes become more biased in codon usage [72], followed by verification of their abundance in highly expressed genes, such as ribosomal protein genes [86]. The effective number of codons (ENC) provides measure of the degree of codon usage bias irrespective of the type of bias (e.g., AT3 or GC3). When codons are all used at similar levels, the ENC has a high value (up to 61) whilst a greater bias results in a low ENC (as low as 20) [72,87]. Accordingly, to identify optimal codons in each taxon, we studied codon usage in the CDS with the highest 3% lowest ENC values versus those with the lowest 3% highest values. For each gene per gene set, we determined the relative synonymous codon usage (RSCU). A higher RSCU value for a codon in a synonymous codon family (amino acid) denotes increased usage [72,88]. Codons with biased usage were identified as those with the greatest change in RSCU among highly biased and low biased genes ($\Delta RSCU = RSCU_{High\ ENC} - RSCU_{Low\ ENC}$) [60,71,89] using t-tests corrected for multiple contrasts (Supplementary Table 5). As a second step, to confirm the

optimal codons were associated with gene expression, rather than mutational pressure, we examined ribosomal protein genes (RPGs), which are typically among the highest expressed and most conserved genes in most organisms [86,90]. In particular, we assessed whether codon usage in the highly expressed RPGs supported a role of selection in the optimal codons identified per organism [86].

Using *Drosophila melanogaster* and *Caenorhabditis elegans* wherein optimal codons have been identified a priori [60,71], we confirmed the effectiveness of the above approach to find optimal codons. For *D. melanogaster* and *C. elegans*, our results showed a strong preference for GC-ending codons (GC3): 100% of the optimal codons end in G or C (Supplementary Table 5). Further, the optimal codon list for *D. melanogaster* matches precisely that previously reported for this taxon (18 of 18 optimal codons) [60,71,72] For *C. elegans* we identified 15 of 18 the optimal codons previously reported for this taxon. Excluding our strict correction for multiple comparisons, an additional two optimal codons were identified for this taxon ($P<0.05$), which correspond to the same codons previously shown to exhibit a weak signal as optimal codons [60]. Thus, 17 of 18 optimal codons in this taxon match those previously reported using gene expression analyses [60]. Our RPG analyses also support the identity of optimal codons. For instance, for *D. melanogaster* ($N_{RPGs}=87$) and *C. elegans* ($N_{RPGs}=82$), GC3 content was statistically significantly higher in the RPGs than the genome-wide average (Supplementary Fig. 3). As optimal codons end in GC3 in these taxa, this suggests that selection is shaping their codon usage.

As codon usage studies from invertebrates other than *D. melanogaster* and *C. elegans* are less common, or absent, we determined the optimal codon list for the remaining species herein using the above approach. We report that optimal codon usage was evident within the Diptera, wherein ΔRSCU revealed that *Culex pipiens* (and *D. melanogaster*) each have a preference for GC3 optimal codons across synonymous codon families (Supplementary Table 5). Further, GC3 was statistically significantly higher for RPGs than for the genome-wide CDS (Supplementary Fig. 3), suggesting that the optimization of codon usage is shaped by expression-related selection in these organisms.

In the Hymenoptera, *Apis mellifera* and *Nasonia vitripennis* showed signals of having optimal codons (Supplementary Table 5). In *A. mellifera*, ΔRSCU indicated that the favored codons ended in A or T (AT3), and the association with expression was confirmed using RPGs (Supplementary Fig. 3). This differs from a recent report suggesting primarily GC3 optimal codons in this taxon (Carlini and Makowski 2015). However, as acknowledged in that assessment, *A. mellifera* showed a weak signature of optimal (or preferred as named therein) codon usage, lower than all other species studied, and the analysis of optimal codons did not include expression data. Hence, since we observed clear signals of AT3 optimal codons using ribosomal protein genes (as a measure of high expression) (Supplementary Fig. 3), we used our current optimal codon list for analysis. Nonetheless, future large-scale transcriptome datasets will confirm the definitive optimal codon list in this taxon . In *N. vitripennis*, putative optimal codons ended in G or C (GC3) (Supplementary Table 5, Supplementary Fig. 3); this agrees with a recent report for *N. vitripennis* [91]. We found that while *N. vitripennis* has substantial AT3 levels in CDS regions (50%, Supplementary Table 6), its optimal codons in highly biased genes are comprised of GC3 codons (Supplementary Table 5, Supplementary Fig. 3). In fact, for *N. vitripennis*, GC3 was 78% higher in the highly biased gene set (3% lowest ENC) than the genome-wide CDS (Supplementary Table 6), representing the strongest signal for the optimal codons among the organisms under study. This phenomenon parallels trends observed in Caenorhabditis where the genome-wide CDS has been reported to be AT3 rich [92], as observed here (AT3=0.63, Supplementary Table 6), but the optimal codons typically exhibit GC3 (Supplementary Table 5; also see [60,71]).

For the taxon *Bombyx mori* (Lepidoptera), we found evidence of biased codon usage, but the codon profiles appeared unlikely to be driven by selection. Specifically, ΔRSCU revealed preferential usage of GC3

codons for 17 of the 18 amino acids with synonymous codons in *B. mori* (Supplementary Table 5). However, the RPGs showed similar levels of GC3 as those observed in the genome-wide CDS (Supplementary Fig. 3), implying that codons with elevated ΔRSCU were common in these highly expressed genes. One possible explanation for this result is that RPGs exhibit uncharacteristically lowered expression in this taxon. To assess this possibility, we assembled a database using all *B. mori* ESTs available at NCBI, representing the testis, hemocyte, malphigian tubule, midgut or ovary tissues. We then compared the expression level of the RPG's and the 3% most biased genes for *B. mori*. Using the number of EST hits per gene as a measure of gene expression level [71,89], we found that the RPGs were highly expressed, and even had higher expression levels than the average for the 3% most biased genes (Average ESTs per 1,000 per gene= 1.62 and 0.37 respectively; t-test preformation value=$6.2X10^{-6}$). In contrast, the ribosomal proteins genes exhibited relatively low bias in codon usage, with an average ENC=52.4 (±0.68). In sum, we conclude that whilst selection might play some role in *B. mori* codon usage [93], no clear signal was evident herein, suggesting that other factors, such as mutational pressure, play a significant role in this particular taxon. This is consistent with recent reports for codon usage this taxon [94].

The taxon *Daphnia pulex* showed bias towards for GC3 codons (Supplementary Table 5). For *D. pulex*, the GC3 content of RPGs was greater than the genome-wide average, consistent with a role of expression-related selection in this taxon (Supplementary Fig. 3). Some taxa had moderate numbers of amino acids with an optimal codon including *Tribolium castaneum* (Arthropoda), and *Capitella teleta* (Annelida). For *T. castaneum* and *C. teleta* ΔRSCU showed a preference for GC3 in 11 and 8 amino acids, respectively. Further, GC3 was statistically significantly higher for RPGs than the genome-wide CDS in each taxon, indicating that these are indeed likely optimal codons shaped by selection (Supplementary Fig. 3). In *T. castaneum* a recent study assigning optimal codons as those with the strongest correlation values to expression, suggested favored codons end in GC, agreeing with our results, but suggested that preferences were found for 16 of 18 amino acids [73]. However, the effect was weak for some of the codons [86]. Nonetheless, due to the high stringency herein, we consider our putative optimal codon lists conservative.

Among the remaining organisms, *Helobdella robusta, Echinococcus granulosus,* and *Onchocerca volvulus* showed no evidence of selection mediated optimal codon usage. Although *H. robusta* (Annelida) showed six codons with preferential usage of AT3 (Supplementary Table 5), no difference was detected among RPGs and genome-wide AT3 (Supplementary Fig. 3), suggesting that this mild bias is not driven by selection. For *O. volvulus*, which favored AT3 codons, the AT3 of RPGs was showed no difference or was lower, respectively, than for the genome-wide AT3, inconsistent with the presence of optimal codons (Supplementary Fig. 3). The taxon *E. granulosus* (Platyhelminthes) was the only organism with no evidence of biased codon usage using ΔRSCU (Supplementary Table 5). Taken together, it is evident that RPGs suggest a role of selection in shaping optimal codon usage for eight of the twelve species studied, including *A. mellifera*, *C. elegans*, *C. pipiens*, *C. teleta*, *D. melanogaster*, *D. pulex*, *N. vitripennis*, and *T. castaneum*, with no or inconclusive signals of optimal codons for *E. granulosus*, *H. robusta*, *B. mori* and *O. volvulus*. Further studies using genome-wide expression will be needed to include/exclude optimal codons in those organisms. As species without signals of optimal codons are uninformative with regard to selection, we studied optional codon usage in the eight species with evidence of optimal codons, in order to evaluate whether PGC mode influences molecular evolution.

We note that for our four "control" species, *D. melanogaster*, *T. castaneum*, *Nasonia vitripennis* and *Apis mellifera* (used as controls to discern a relationship between dN/dS and Fop), we found that after binning of dN/dS into the four classes used in Fig. 2a ($dN/dS<0.5$, $0.5\leq dN/dS<0.75$, $0.75\leq dN/dS<1$, and $dN/dS\geq 1$), there was an inverse correlation between dN/dS and Fop for *D. melanogaster*, (Spearman R=-1,P=0.017), *A.*

mellifera (R= -0.9 P<0.047), *N. vitripennis* (R=-1, P=0.017), and *T. castaneum* (R=-0.299, P=0.68), similar to trends suggested in other organisms [74,77,78]. In *T. castaneum*, whilst this correlation did have a negative R value, it was not statistically significant, perhaps because this taxon had fewer optimal codons than other species, making Fop values less strong than other species (Supplementary Table 5).

Supplementary Note 5 (dN/dS and Developmental Stage)

We note that whilst the percentage of high dN/dS CDS expressed at each developmental stage is the same between Drosophila and Tribolium, the absolute number of CDS with high dN/dS is slightly higher for Drosophila across all developmental stages, simply because the Drosophila-Tribolium contrast was one of two (among our five contrasts; the second such contrast was Nasonia-Apis) that had a marginally higher number of high dN/dS in the preformation taxon (MWU-tests $P<10^{-15}$, see Results for Fig. 2ab).

Supplementary Note 6.(Additional Examples of Speciation Under Preformation and Induction)

Among the two Platyhelminthes taxa studied here (Fig. 1), the genus Schistosoma (preformation) has 21 recognized species [95] and Echinococcus (induction) has nine species [96], thereby suggesting very low genus-level species richness under both PGC modes. The Annelida, a group that originated more than 516 Mya, is a highly diverse phylum with a minimum predicted 26,000 species [97]. The two divergent Annelid species examined here (Capitella and Helobdella, Fig. 1) both exhibit induction mode (Supplementary Table 4), suggesting that this mode of PGC formation (in at least some lineages) did not impede its high radiations. Other invertebrates also suggest PGC mode is unrelated to radiation across protostomes. For example, the Daphniidae (containing Daphnia, preformation [47,98,99]) have just 121 described species [100], while Aphididae (containing a number of preformation species including *Acyrthosiphon pisum* [101-104]) has approximately 4,300 [105]. Together, this suggests the preformation mode can be linked to low or high levels of radiation, based solely on family level species diversity. Collectively, these examples indicate that preformation and induction modes are uncorrelated to species radiations in invertebrates.

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