Mating strategy predicts gene presence/absence patterns in a genus of simultaneously hermaphroditic flatworms

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Abstract

Gene repertoire turnover is a characteristic of genome evolution. However, we lack well-replicated analyses of presence/absence patterns associated with different selection contexts. Here, we study ~100 transcriptome assemblies across *Macrostomum,* a genus of simultaneously hermaphroditic flatworms exhibiting multiple convergent shifts in mating strategy and associated reproductive morphologies. Many species mate reciprocally, with partners donating and receiving sperm at the same time. Other species convergently evolved to mate by hypodermic injection of sperm into the partner. We find that for orthologous genes annotated as expressed in the body region containing the testes, sequences from hypodermically inseminating species diverge more rapidly from the model species, *M. lignano,* and have a lower probability of being observed in other species. For other genes, simpler models with a constant rate of similarity decay with increasing genetic distance from *M. lignano* match the observed patterns well. Thus, faster rates of sequence evolution for hypodermically inseminating species in testis-region genes result in higher rates of homology detection failure, yielding a signal of rapid evolution in gene presence/absence patterns. Our results highlight the utility of considering appropriate null models for unobserved genes, as well as associating patterns of gene presence/absence with replicated evolutionary events in a phylogenetic context.

Introduction

Gene repertoire turnover characterises the evolution of genomes throughout the tree of life (Albalat and Cañestro 2016; Fernández and Gabaldón 2020). A common observation at many taxonomic scales is that some lineages have no detectable homologs of genes present in other, related lineages (Zhaxabayeva et al. 2008; Vakirlis et al. 2020; Weisman et al. 2020). Often, the inference is that such patterns are the result of adaptive gene gain and/or loss. For example, the study of the origins of novel genes has received a lot of attention (e.g. Neme et al. 2017), and gene loss is often adaptive (Albalat and Cañestro 2016). However, a well-understood, but less exotic, explanation is that "missing" genes have simply evolved, at a constant rate, to the point of being unrecognisable by sequence-similarity approaches to homology detection, so-called "homology detection failure" (Zhaxabayeva et al. 2008; Vakirlis et al. 2020; Weisman et al. 2020). As many as 20-55% of so-called "lineage-specific" genes may be due to homology detection failure in outgroups (e.g. Zhaxabayeva et al. 2008; Vakirlis et al. 2020; Weisman et al. 2020). For genes with overall rapid rates of sequence evolution (e.g. reproduction-related genes, see below) homology detection failure may be more common and failure to consider it as a hypothesis will lead to errors of inference. Recent efforts have aimed to develop methods to test whether an observation of gene absence is expected on the basis of sequence similarity at different evolutionary distances from a focal species, when assuming a constant rate of sequence evolution (Weisman et al. 2020). Deviations from this "null" model can then be due to a variety of factors, including changes in the rate of evolution due to changes in selective pressures on existing genes or *bona fide* gene turnover (gain/loss; Weisman et al. 2020).

Gene presence/absence and the evolution of reproduction-related genes

Reproduction-related genes are typically found to be evolving at higher rates compared to the genomic background (Swanson and Vacquier 2002; Wilburn and Swanson 2016). A commonly proposed explanation for these observations is diversifying sexual selection (Swanson and Vacquier 2002; Dapper and Wade 2016; 2020; for reviews see also e.g., Vacquier and Swanson, 2011; Wilburn and Swanson, 2016). However, an alternative explanation is that relaxed selection is the driving force behind many of these patterns (Dapper and Wade 2016; 2020). Indeed, there are several reasons why reproduction-related genes might be expected to be under relaxed selection. First, in separate-sexed organisms, reproduction-related genes are often sex-biased or even sex-limited in expression (Dapper and Wade 2020). In the extreme case of sex-limited expression, the efficiency of selection is half that of a gene with unbiased expression, because it is only exposed to selection when residing in one sex (Dapper and Wade 2020). However, it is unclear to what extent sex-biases in expression could operate under other modes of reproduction (e.g. simultaneous

hermaphroditism, where individuals are both male and female at the same time; Wiberg et al. 2021). Second, also in separate-sexed organisms, reproduction-related genes are often sexually antagonistic, where the fitness effects differ, and are sometimes in opposition, in the two sexes. In this case the net selection is the average of that experienced in either sex. All else being equal, sexually antagonistic loci therefore necessarily experience lower net directional selection (with a similar effect to relaxed selection) compared to the rest of the genome. Finally, regardless of the mode of reproduction (i.e. hermaphroditism or gonochorism), genes involved in sperm competition or cryptic female choice experience weaker selection because the genetic variation exposed to selection within females is typically only a fraction of that present in the population at large, especially in mating systems with low female remating rates (Dapper and Wade 2016; 2020).

Be it due to relaxed selection or positive selection, the faster rates of evolution of reproduction-related genes would also predict higher rates of homology detection failure for such genes. A few studies have found evidence that, in addition to rapid rates of sequence evolution, turnover of gene repertoires is a characteristic feature of reproduction-related genes (Zhang et al 2004; Cutter & Ward 2005, Ellegren and Parsch 2007; Hahn et al. 2007; Ahmed-Braimah et al. 2017). In particular genes expressed in male reproductive tissues (e.g. the testes, the prostate, or accessory glands) often show high rates of turnover (Zhang et al 2004; Cutter & Ward 2005, Ellegren and Parsch 2007; Hahn et al. 2007; Ahmed-Braimah et al. 2017). In contrast, some studies, for example a recent study of 11 passerine species, found no evidence for higher turnover rates for male-biased reproduction-related genes (Rowe et al. 2020). However, compared to studies of rates of molecular sequence evolution, relatively little work has focused on patterns of presence/absence of reproduction-related genes in different lineages, especially in systems where a link can be drawn to consistent differences in the sexual selection context.

Although there is considerable taxonomic bias in the above studies, they suggest that, in general, reproduction-related genes may not only be rapidly evolving in sequence but also show higher rates of missing genes than other categories of genes. However, this pattern is not strictly universal, raising the question of why such patterns are observed in some systems but not others. Such a pattern is not easily explained as a simple consequence of the higher rates of sequence evolution due to relaxed selection at reproduction-related genes. Since sexual selection is thought to be an important force affecting the evolution of reproduction-related genes, an obvious explanation is that differences in the sexual selection context between systems might account for the observed differences in patterns of gain and loss. This is especially true since changes in copy number may be an important component in the evolution sexual dimorphism; redundant copies of genes are more free to evolve sex-specific expression patterns and functions, partially resolving intra-locus sexual conflict (Ellegren and Parsch 2007). For example, sex-biased expression, allowing sex-specific

phenotypes, seems to be more common at recently duplicated genes (Wyman et al. 2011). Moreover, turnover in sex-biased gene expression is associated with differences in the sexual selection context in birds (Harrison et al. 2015). Thus, both a reduced efficiency of selection at reproduction-related genes as well as changes in the intensity of selection at particular loci due to changing sexual selection contexts, might result in the loss of genes from a species' repertoire. To date, few studies have associated such changes in gene repertoires with changes in the sexual selection context.

A further technical complication is that it is often difficult to distinguish actual absence of a gene from artefacts arising from homology detection failure (e.g. Weisman et al. 2020) or poor gene assembly quality. Additionally, in the case of transcriptome data, variation in expression levels may also create apparently missing genes if the transcriptome is not sampled to high enough coverage or when expression of some genes is temporally or spatially restricted. Some of this variation in expression, at least across species, may also be a signal of adaptive differences (e.g. Harrison et al. 2015). It is clear that more work is needed to understand whether and how patterns of rapid evolution of reproduction-related genes translates into patterns of gene presence/absence. Additionally, there is a need for data from more taxonomic groups, ideally combined with replicated contrasts of different sexual selection contexts.

The genus Macrostomum

Free-living flatworms of the genus *Macrostomum* have become excellent models for studying mating behaviour and sexual selection, including sexual conflict (Schärer et al. 2004; Vizoso et al. 2010; Ramm et al. 2015; Giannakara et al. 2016; Giannakara and Ramm 2017; Marie-Orleach et al. 2017; Patlar et al. 2020; Singh et al. 2020a, 2020b). In particular *M. lignano* has been the focus of numerous experiments and has substantial genetic resources (Ladurner et al. 2005; Wudarski et al. 2020). For example, gene expression studies in *M. lignano* have provided information about body regions containing important reproductive tissues that can be used to annotate reproduction-related genes (Arbore et al. 2015; Brand et al. 2020). *Macrostomum* worms are simultaneously hermaphroditic, producing sperm and eggs at the same time. Hermaphroditism is expected to lead to conflicts between individuals over the sex roles they assume during copulation, with the role of sperm donor thought to be preferred over the role of sperm recipient in many scenarios (Charnov 1979; Michiels 1998; Schärer et al. 2015). In *Macrostomum* such conflicts likely explain much of the diversity of reproductive morphologies and behaviours that are observed among species (Schärer et al. 2011; Brand et al. 2022a).

One of the main aspects of this diversity is variation in the mating strategies. Many species of *Macrostomum* engage in reciprocal copulation, where both partners donate and receive sperm at

the same time (Schärer et al. 2004; Vizoso et al. 2010). Other species inseminate hypodermically by injecting sperm into the body of their partner using a "needle-like" stylet (Schärer et al. 2011; Ramm et al. 2015). These contrasting strategies have been shown to be strongly associated with other axes of variation in reproductive morphology and behaviour (Schärer et al. 2011; Brand et al. 2022a). In particular, reciprocally copulating species have sperm with stiff lateral bristles that likely serve as an anchoring mechanism to prevent postcopulatory sperm removal (Vizoso et al. 2010; Schärer et al. 2011). Meanwhile hypodermically inseminating species have simpler sperm that lack such bristles. A recent phylogenomic study has shown that hypodermic insemination has evolved at least nine times independently within the genus (Brand et al. 2022a; Brand et al. 2022b). A handful of species also show intermediate sperm morphologies, with short or reduced bristles, possibly representing a transitional state between the reciprocal and hypodermic mating strategies (Schärer et al. 2011; Brand et al. 2022a). The state of the sperm bristle (present, reduced, or absent) is therefore a good indicator of the mating strategy (Schärer et al. 2011; Brand et al. 2022b). Thus, contrasting mating strategies, themselves the result of conflicts over sex roles, represent different sexual selection contexts resulting in repeated evolution of different reproductive morphologies.

We have previously shown that reproduction-related genes evolve rapidly in *Macrostomum* (Brand et al. 2020; Wiberg et al. 2021), and that species with absent sperm bristles (taken as a proxy for hypodermic mating) show faster genome-wide rates of sequence evolution than species with bristles (Wiberg et al. 2021). However, an open question is whether the bristle state also predicts whether orthologs of reproduction-related genes are observed, or not, across species. Transcriptome assemblies show substantial variation in gene presence/absence patterns, with a conspicuous lack of representative sequences within a clade of exclusively hypodermically mating species (figure S1). A strong effect of bristle state would indicate that different sexual selection contexts alter the nature of selection acting on particular genes, sets of genes, or the genome as a whole. Such effects might inform us about candidate genes involved in, for example, sperm bristle development in the case of genes expressed in the testes. If shifts to a hypodermic mating strategy impose strong selection to lose the bristles, then the genes involved might degrade and be lost rapidly, making them difficult to detect in studies of the rate of sequence evolution (e.g. Brand et al. 2020 and Wiberg et al. 2021).

If there were no overall effect of bristle state then the reason for unobserved transcripts is more difficult to infer and could be linked to one of multiple, non-mutually exclusive factors. For example, the lack of an effect could indicate that there is no overall turnover of gene repertoires due to sexual selection. Technical effects due to the use of transcriptome data, such as variation in the quality of assemblies or low expression of certain genes, will lead to noise in presence/absence data (see above).

Here we use a set of previously established orthogroups (OGs – a collection of orthologous sequences across multiple species; Brand et al. 2022a; Wiberg et al. 2021) that have been annotated on the basis of reproduction-related genes from the model species *M. lignano* (Arbore et al. 2015; Brand et al. 2020). We then test for a signal of decay in similarity scores with increasing phylogenetic distance from the model species *M. lignano* and evaluate the probability of detection given the observed rates of decay (Weisman et al. 2020). Next, we apply phylogenetically controlled logistic regression to test the hypothesis that different sexual selection contexts predict the probability of observing a representative sequence in an OG. We also extend the annotation of these OGs using novel RNA-Seq data comparing gene expression between adults and hatchlings in the model species *M. lignano.* Elevated expression in adults presents an additional source of evidence for a reproduction-related function of a gene because only adults are reproductively active.

Methods

Orthogroups and pairwise similarity scores

We obtained the amino acid alignments corresponding to the codon alignments used in Wiberg et al. (2021). These alignments represent orthogroups (OGs) and are broadly annotated into reproduction-related categories, namely by their expression in the testis-, ovary-, and tail-region of the worm (Arbore et al. 2015; Brand et al. 2020; Wiberg et al. 2021; table S1 – sheet 2). There is also a randomly chosen set of OGs that are annotated as ubiquitously expressed with no obvious reproductive function that serves as a comparison to the reproduction-related OGs. A random subset, rather than all ubiquitously expressed genes, was chosen also to mitigate the inflated power of any statistical analyses that assume statistical independence between OGs. These amino acid alignments were trimmed using ZORRO (v. 1.0; Wu et al. 2012) scores, removing columns with a score ≤ 3. For each trimmed alignment, we then removed gap characters (-) and created protein blast databases (blast v. 2.9.0; Camacho et al. 2009). We also refined the annotation of these OGs using gene expression data from adults and hatchlings of *M. lignano* to identify genes that are more highly expressed in adults (see supplementary text T1 in the supporting information for more details as well as figure S2, table S2, and table S3).

Evaluating rates of homology detection failure

For all OGs we collected similarity scores (bit-scores) for all hits between the focal *M. lignano* sequence and all other species present in the OG using a BLASTP (v. 2.9.0; Camacho et al. 2009) search of all sequences in an OG against a database created from the same OG always treating the *M. lignano* sequence as the "target". We then applied an approach to model the decay in

the bit-score with increasing genetic distance from *M. lignano* (table S1 – sheet 3). Weisman et al. (2020) defined an exponential decay model based on a simple model of sequence evolution:

$$S(t) = Le^{-Rt} \qquad \text{(eqn. 1)}$$

Where $S(t)$ is the bit-score at a genetic distance t from the focal species (see table S1 – sheet 4 for the genetic distances of all species). L is the bit-score of the gene of the focal species with itself (and thus related to the protein's length), and R is the rate of decay in the bit-score. The expected variance at genetic distance t is given by:

$$\sigma^2(t) = L(1-e^{-Rt})(e^{-Rt})$$

Because linear and non-linear regression fitting approaches minimise slightly different sums of squares, and non-linear regression approaches require specified starting values for estimated parameters, we took a double-fitting approach to estimate the L and R parameters. First we fit a linear model to the log()-transformed bit-scores, and used the L and R parameter estimates as starting values to fit a non-linear least squared (nls) regression with the "nls()" R function. To fit these models, we only used species with bristles present, and only OGs with data from ≥ 5 species (table S1 – sheet 4). This approach allowed us to evaluate whether the species with reduced or absent bristles deviated from the patterns observed in the species with bristles present. We also created a weighting variable in an attempt to account for variation in assembly quality (*weight* = $1 - P_{miss}$; where P_{miss} is the proportion of BUSCO (v. 5.0.0; Simão et al. 2015; metazoa_odb10) genes that were reported missing from a transcriptome; see table S1 – sheet 3). Any OGs that produced R values > 0 (i.e. an estimated increase in similarity score with increasing distance from *M. lignano;* N = 7/999, 0.7%) were treated as spurious, likely due to noisy input data, and were not considered further. We next computed residuals for all species, including those that were not used to estimate the best-fit line, by subtracting the empirical bit-score from that predicted by the parameter estimates and the non-linear equation given above (eqn. 1). Because none of the species used in the model fitting had genetic distances to *M. lignano* > 0.7, the accuracy of predictions could be compromised when extrapolating to larger genetic distances. We therefore only used residuals computed for species with genetic distances to *M. lignano* < 0.7. This effectively excludes all species in a clade of exclusively hypodermically inseminating species (called the "hypodermic mating clade" in Brand et al. 2022a; yellow box in figure 1A). This had the added benefit that there was no longer a detectable systematic difference between the species with different bristle states in the genetic distance to *M. lignano* (figure S3A and B). Moreover, there was no systematic difference

in assembly "completeness", as measured by BUSCO, between species with different bristle states (figure S3C and D). We summarised the residuals for each bristle state within each OG as the median value across representative species (figure 1B).

Given this model, and the parameter estimates of L and R for each OG, it is then possible to compute the probability of detecting a homolog at some genetic distance t, by setting an e-value threshold, and assuming the sequence length. We set an e-value threshold of 0.001 (a commonly used value), and assume that the length of a hypothetical sequence is the same as that of the relevant *M. lignano* sequence. The database size was set to 853,740,050 amino acids, the size of the initial database Brand et al. (2022a) used for homology detection. We then computed the probability of detection (p_{detect}) as the proportion of a normal distribution with mean $S(0.7)$ and standard deviation $\sigma(0.7)$ that lies above the given bit-score threshold (Weisman et al. 2020; figure 1C). We identified OGs with a high probability of detection ($p_{detect} > 0.90$; table S1 – sheet 4). For such OGs with $p_{detect} > 0.90$, but for which representative sequences are unobserved for some species, alternative explanations can then be explored (see also next section).

The approach by Weisman et al. (2020) has some limitations. First, because bit-scores are dependent on the length of the sequences being compared, and sequence lengths in the alignments varied substantially, even after trimming, this led to substantial variation in the bit-scores (figure S4A). This is likely to be a source of increased variation in bit-scores more generally, especially in non-model systems with fewer and less well characterised genetic resources. Second, as in any comparative analysis, species may have similar bit-scores, and genetic distances to the focal species, because they are themselves closely related (i.e. phylogenetic non-independence; Felsenstein 1985; Uyeda et al. 2017). Thus, modelling approaches that explicitly account for phylogeny would be preferable. We therefore also modelled the rate of decay in the bit-scores with increasing phylogenetic distance from *M. lignano* in a way that tried to address these limitations. However, because these additional analyses produced very similar results to those above, we only present the relevant methods and results in the supplementary information (supplementary text T2; table S1 – sheet 5).

Gene presence/absence

Our above approach to compute residuals from the fitted decay models does not capture cases of complete absence of a sequence from an orthogroup, because in such cases there are no empirical bit-scores to compare against predicted values. We therefore took a complementary approach to analyse gene presence/absence data. We fit phylogenetically-controlled logistic regression models to gene presence/absence data for those OGs with a high probability of detecting homologs among species with bristles present ($p_{detect} > 0.9$; see above). These models test whether

the bristle state or reproduction-related annotations had an effect on the probability of observing a representative sequence. Because the decay rate analyses establish that simple models of similarity decay fit the data well also for species with reduced and absent bristles, any significant effect of bristle state on the probability can be attributable to other processes. To incorporate the phylogenetic relationships as a random effect, we fit these logistic regression models with the "MCMCglmm" package (v. 2.32; Hadfield 2010), using a probit link function (family = "categorical"). To incorporate the phylogenetic random effect, we transformed the phylogenetic tree (H-IQ-TREE from Brand et al. 2022a; see also figure 1A) to be ultrametric with a root depth of 1 using the penalised marginal likelihood approach (Sanderson 2002) in TreePL (Smith and O'Meara 2012), as in Brand et al. (2022b). As above, we excluded data from species with a genetic distance from *M. lignano* > 0.7. For a single OG model fitting was not possible because all species with genetic distances to *M. lignano* < 0.7 had a representative sequence. We summarised the effects of bristle state by collecting, for each OG, the posterior means (on the probit scale) for each bristle state (table S1 – sheet 6). We then tested for an overall difference in the posterior means between bristle states within each annotation category, and between annotations within bristle states, using Kruskal-Wallis rank-sum tests and Dwass-Steele-Critchlow-Fligner all-pairs tests for *post-hoc* contrasts. We also identified a set of candidate OGs by comparing the 95% confidence intervals of the posterior means between bristle states (table S1 – sheet 6). If the entire interval for species with absent bristles was below that for species with bristles present, indicating a much lower probability of observing a representative sequence, we called the posterior means significantly different.

Statistical software and packages

In addition to the above-named packages, several other R packages were used in the analysis pipeline of these data; "ddpcr" (v. 1.15; Attali 2020), "plyr" (v. 1.8.6; Wickham 2011), "ggplot2" (v. 3.3.3; Wickham 2016), "here" (v. 1.0.1; Müller 2020), as well as the packages for handling phylogenetic trees; "ape" (v. 5.5; Paradis & Schliep 2019), "ggtree" (v. 3.2.1; Yu et al. 2017; Yu et al. 2018; Yu 2020), "ggimage" (v. 0.3.0; Yu 2021), "TDbook" (v. 0.0.5; Yu et al. 2022) and "phytools" (v. 0.7-70; Revell 2012). Bioinformatic analyses involved cutadapt (v. 3.0; Martin 2011), salmon (v. 1.4.0; Patro et al. 2017), DESeq2 (v. 1.24.0; Love et al. 2014) as well as the *M. lignano* transcriptome assembly (Wudarski et al. 2017; Grudniewska et al. 2018; see supplementary text T1).

Results and Discussion

Decay in similarity scores with genetic distance

310 Overall, we were able to estimate decay rates for 959 OGs (figure S5 A-D; table S1 – sheet 4), and for 796 of these OGs (83%) a homologous sequence had a probability > 0.9 of being detected at a genetic distance of 0.7. This percentage varied among the reproduction-related OGs (Testis-region 81.7%; Ovary-region 83.6%; Tail-region 78.2%; Ubiquitously expressed 88.0%) As expected, OGs annotated as ubiquitously-expressed had the highest percentage (88%). The 315 ubiquitously expressed OGs are expected to be more conserved and thus probably have slower rates of evolution on average, making representative sequences easier to detect at greater genetic distances.

There were significant differences in the median residuals across bristle states for OGs with the testis-region annotations (Kruskal-Wallis rank-sum test, $\chi^2 = 53.51$, $p < 0.001$), with the pattern 320 being driven by more negative residuals in species with reduced and absent bristles (figure 2A). This suggests that the simple model of a constant rate of similarity decay estimated from species with bristles does not adequately explain similarity scores for species with either reduced or absent bristles. This is in line with previous observations that genes with testis-region annotations show faster rates of sequence evolution in species with absent bristles compared to species with bristles 325 (Wiberg et al. 2021). This also predicts that testis-region genes might show an effect of bristle states on the probability of observing a gene. Similarly, we found that among genes with ubiquitously expressed annotations there was a significant effect of bristle state ($\chi^2 = 12.95$, $p < 0.001$), also in line with previous findings (Wiberg et al. 2021). However, for ubiquitously expressed OGs the effect was driven by slightly more negative residuals in species with reduced bristles compared to 330 either species with present bristles or species with absent bristles (figure 2A), which is difficult to explain. This might be a spurious effect of the relatively low sample sizes for species with reduced bristles. This predicts that there should be no overall difference among bristle states in the probability of observing a gene for OGs annotated as ubiquitously expressed.

In contrast, we did not detect an effect of bristle state for ovary- ($\chi^2 = 4.53$, $p = 0.1$), or tail- 335 region ($\chi^2 = 2.78$, $p = 0.25$) OGs. For these annotation groups, the results therefore differ from previous analyses, where an effect of bristle state was observed for all reproduction-related annotation categories (Wiberg et al. 2021). However, given that the model of Weisman et al. (2020) employed here is a fairly simplistic way of describing sequence evolution, these contrasting results are not so surprising. Codon models, as applied in Wiberg et al. (2021), are likely much more 340 sensitive to variation in rates of sequence evolution, and we therefore consider these results largely consistent with those of previous work (Brand et al. 2020; Wiberg et al. 2021). On the other hand, the absence of a significant difference in the residuals between species with present bristles and those with absent bristles suggests that the probability of a homolog being detectable should also

not depend on the bristle state. Therefore, if we observed a significant effect of bristle state on the probability of observing a gene this is less likely to be attributable to homology detection failure.

Finally, when OGs were further subset to those with higher expression in adults than in hatchlings, significant differences between bristle states were restricted to OGs with testis-region annotations ($\chi^2 = 55.86$, $p < 0.001$), although the effect for OGs annotated as ubiquitously expressed appeared similar in magnitude, though no longer statistically significant ($\chi^2 = 5.11$, $p = 0.078$; figure 2B). Effects for ovary- ($\chi^2 = 1.1$, $p = 0.58$), and tail- ($\chi^2 = 2.44$, $p = 0.29$) region OGs remained non-significant.

Orthogroup occupancy

With the predictions about patterns of ortholog detection in hand from the results outlined above, we next turn to analyses of presence/absence. There were significant effects of bristle state on the probability of observing representative genes within OGs with testis-region annotations (Kruskal-Wallis rank-sum test; $\chi^2 = 22.07$, d.f. = 2, $p < 0.001$). This effect was driven by differences between species with bristles and those with either reduced or absent bristles (figure 3A). These results therefore mirror the results of the bit-score decay analysis, which indicated rapid rates of sequence evolution. The most likely explanation is that the more rapid rates of sequence evolution in species with reduced and absent bristles lead to higher rates of homology detection failure. This potentially has implications for interpreting previous findings in other taxa. Several studies have found male-biased genes, in particular those expressed in testes and the male germline, to show both high rates of sequence evolution and gene turnover. For example, in both *Caenorhabditis briggsae* (Cutter & Ward 2005) and *Drosophila melanogaster* (Zhang et al. 2004) there is evidence of high turnover for genes expressed in the testes and the male germline. Additionally, in a comparative study of *Drosophila*, Hahn et al. (2007) showed evidence for expansions in gene families associated with spermatogenesis in the lineage to *D. melanogaster*. Another comparative study of *Drosophila,* this time in the *D. virilis* group, found lineage-specific transcripts only for genes expressed in testes of *D. americana* and the ejaculatory bulb of *D. lummei* (Ahmed-Braimah et al. 2017). In the light of our findings, such results might be due to detection failure in some lineages due to the more rapid sequence evolution of genes expressed in testes as compared to other tissues. Our results suggest that this hypothesis should be considered and tested before explanations involving adaptive gain/loss are invoked.

For OGs annotated as ubiquitously expressed there was also an effect of bristle state ($\chi^2 = 8.31$, d.f. = 2, $p = 0.01$). This was driven entirely by species with reduced bristles having lower posterior mean probabilities than species with bristles (figure 3A). There were no detectable effects of bristle state for ovary- ($\chi^2 = 3.0$, d.f. = 2, $p = 0.22$) or tail-region ($\chi^2 = 4.37$, d.f. = 2, $p = 0.11$)

OGs. When OGs were subset to those that annotated as more highly expressed in adults than in hatchlings, the effect of bristle state only remained for OGs with testis-region annotations (χ^2 = 19.85, $p < 0.001$; figure 3B). These results therefore, again, mirror the results of bit-score decay detailed above and suggest that the strongest response in both analyses is for testis-region annotated OGs. In other taxa, different groups of reproduction-related genes also show interesting patterns of presence/absence. For example, for lineage-specific gene families unique to the *Drosophila* sub-genus, many of the genes unique to *D. melanogaster* were found to be expressed in the accessory glands (Hahn et al. 2007; Clark et al. 2007; Haerty et al. 2007). Accessory gland genes, for example, are also known to evolve particularly rapidly in *Drosophila* (Haerty et al. 2007). More recently, it was shown that several members of the CheB family of gustatory receptors, which show sexually dimorphic expression and are involved in modulating mating and aggression behaviours in *D. melanogaster,* show high rates of gene turnover (Torres-Oliva et al. 2016). Our results suggest that homology detection failure might provide an explanation more generally and gene turnover rates should be placed in the context of sequence evolution for a full picture.

Some important caveats should be borne in mind. There is substantial variation in assembly quality and completeness across species and this probably explains some of the variation in unobserved genes. For any given species we cannot exclude the possibility that a gene is unobserved due to it not being assembled, even though it occurs in the species. Unassembled genes might result for related technical reasons, with low-expression genes being less likely to be sampled by an RNA-Seq strategy. Moreover, gene expression levels may vary across species. Many of the analysed worms were caught in the field and are thus not standardised in condition or environment, which might also result in gene expression variation. In addition, turnover of expression across species, for example due to selection for different expression patterns, might also results in variation.

The above-mentioned caveats notwithstanding, a number of OGs (N = 15) can be highlighted as potentially interesting candidates for follow-up studies (table S1 – sheet 7) on the basis of non-overlapping confidence intervals between bristle states (see Methods) from logistic regression analysis in the presence/absence data (table S1 – sheet 6). Of these, 4 OGs are annotated as expressed in the testis region, all with significantly higher expression in adults compared to hatchlings, making them strong candidates for genes affecting reproduction-related functions. In particular, the gene Mlig016310.g1, which annotates OG0001273_1_MIortho10 as being expressed in the testis region, has been confirmed, by *in situ* hybridisation (ISH), to show expression in the testes of *M. lignano* (Arbore et al. 2015; called RNA815_7008 therein). Moreover, RNAi knockdown of this gene in *M. lignano* produces an aberrant sperm bristle phenotype (Arbore et al. 2015), in which the usually backwards pointing bristles are no longer stably anchored in that

position, pointing to a possible role in the formation of the bristle complex (Willems et al. 2004). While a previous study did identify orthologs of this gene both in a species with bristles (*M. spirale*) and two species with absent bristles (*M. pusillum*, and *M. hystrix*), all showing expression localised to the testes (Brand et al. 2020), no species with absent bristles are represented within this OG in this study. This discrepancy can probably be attributed to differences in the orthology detection approach. Brand et al. (2020) relied exclusively on the sequence similarity-based OrthoFinder pipeline (Emms and Kelly 2015), while we here use orthologs generated from the PDC pipeline of Yang et al. (2014), which incorporates phylogenetic information from gene trees of initial homolog groups. Thus, previously identified orthogroups may have contained spuriously identified paralogs with similar tissue expression patterns across species that are correctly identified as paralogs in more recent analyses including more species and tree-based methods (Yang et al. 2014; Brand et al. 2022a; Wiberg et al. 2021). Further validation of these findings will require a more thorough, manual investigation of ortholog and paralog relationships of genes with homology to Mlig016310.g1, which is outside the scope of this study. An additional 7, 3, and one candidate OGs are annotated as ubiquitously expressed, or specific to the ovary region and tail region, respectively. No further ISH data are currently available for the genes in these OGs. As above, these OGs will require more thorough validation, such as more targeted identification of homologs, orthologs, and paralogs, as well as ISH experiments.

Conclusions

We explored patterns of missing genes in ~100 transcriptome assemblies from diverse *Macrostomum* species with contrasting mating strategies. We also expand gene annotations for *M. lignano* and identify a more stringent set of reproduction-related genes with higher expression in adults compared to hatchlings. Our results are consistent with previous findings of rapid sequence evolution at reproduction related genes, at least for orthogroups annotated as testis-region specific, and in species with reduced and/or absent sperm bristles, a proxy for the hypodermic mating strategy. Our novel findings also show that species with absent bristles have a lower probability of observing a gene within OGs annotated as testis-region specific. We suggest that the most parsimonious explanation is that the faster rate of evolution seen also for these OGs results in a higher rate of homology detection failure for these genes, resulting in a signal of rapid evolution of reproduction-related genes also in gene presence/absence data. Our study highlights at least one exciting candidate OG for further study where representative genes have already been associated with phenotypic effects on sperm bristle phenotypes in *M. lignano*. Thus, our results highlight the utility of considering an appropriate null expectation for missing or unobserved genes as well as

associating patterns of gene presence/absence with replicated evolutionary events in a phylogenetic context.

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Author contributions: RAWW and LS conceived the study. RAWW conducted all analyses and led the writing of the manuscript with input from LS. GV performed RNA extractions and purification. All authors have commented on and approved the final manuscript.

Data accessibility: Transcriptome assemblies and annotated orthogroups forming the basis of these analyses are available from https://doi.org/10.5281/zenodo.4543289 and https://doi.org/10.5281/zenodo.4972188. Raw-sequencing files for the adult-hatchling contrast of *M. lignano* has been deposited with the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB51403 (https://www.ebi.ac.uk/ena/browser/view/PRJEB51403). Data and analysis scripts used in this study are available from https://doi.org/10.5281/zenodo.6478115

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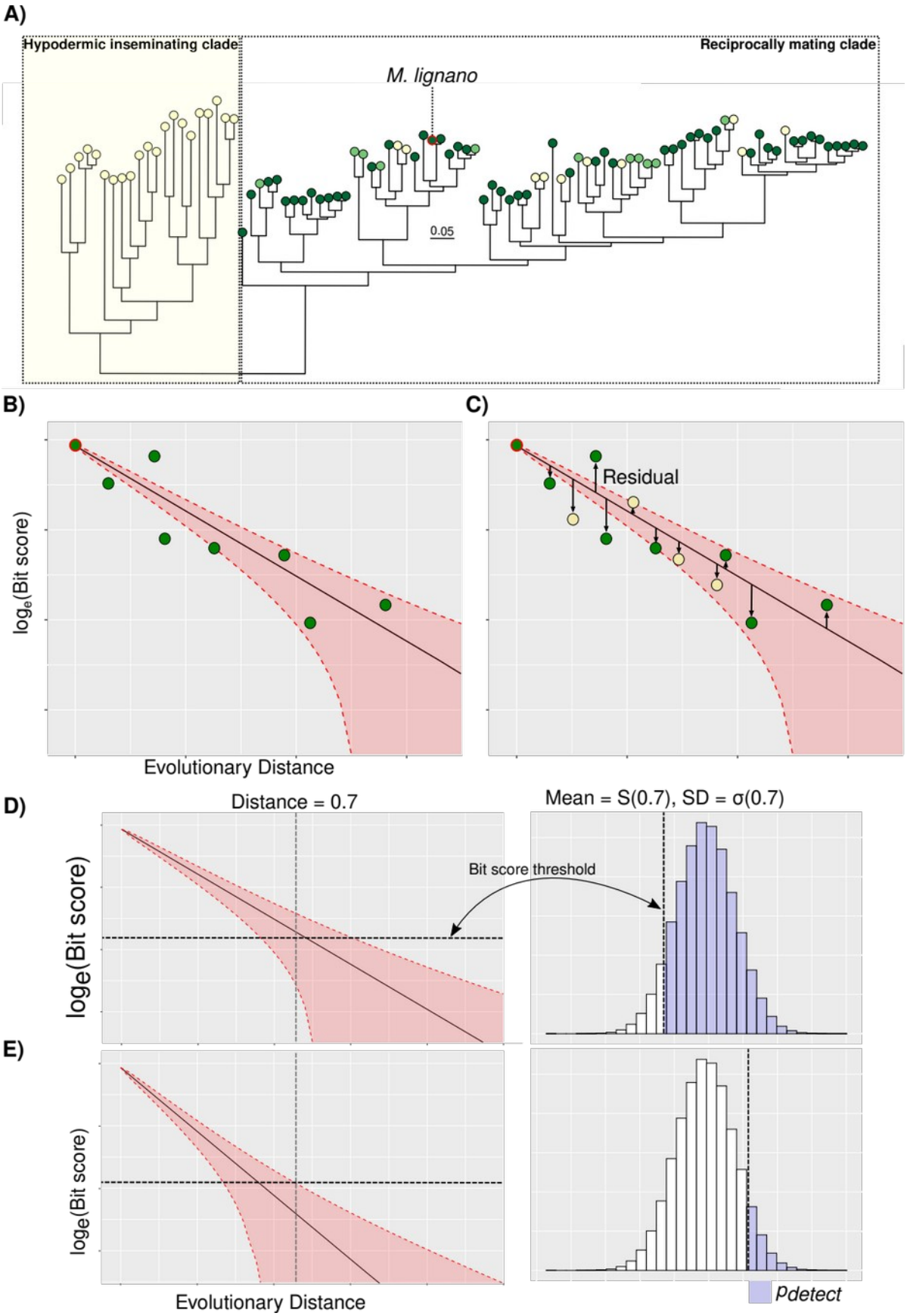
Figures, figure legends, and tables



605 **Figure 1. A)** A depiction of the phylogenetic distribution of species used in this study. The hypodermic mating clade, which is excluded from analyses (see Methods), is highlighted in the

yellow box, and the focal species (*M. lignano*) is outlined in red. Dark green, light green, and yellow circles at branch tips denote species with present, reduced, and absent sperm bristles, respectively. **B)** and **C)** are diagrams of the 2-step analysis approach taken for each orthogroup (see
610 Methods): **B)** species with bristles are used to fit a line describing the decay in bit scores as a function of evolutionary distance from the focal species (*M. lignano*). **C)** Residuals from other species (here only species with absent bristles are shown) to the line of best fit are computed and summarised. **D)** and **E)** are representations of the approach to compute the probability of detection (p_{detect} ; see Methods). A bit-score threshold is defined, and the proportion of a normal distribution
615 with mean S(0.7) and standard deviation σ(0.7) that lies above this threshold is defined as p_{detect}. **D)** upper two plots, show a high p_{detect}, where most of the predicted distribution of bit-scores lies above the threshold bit-score value **E)**, in contrast, demonstrate a low p_{detect}

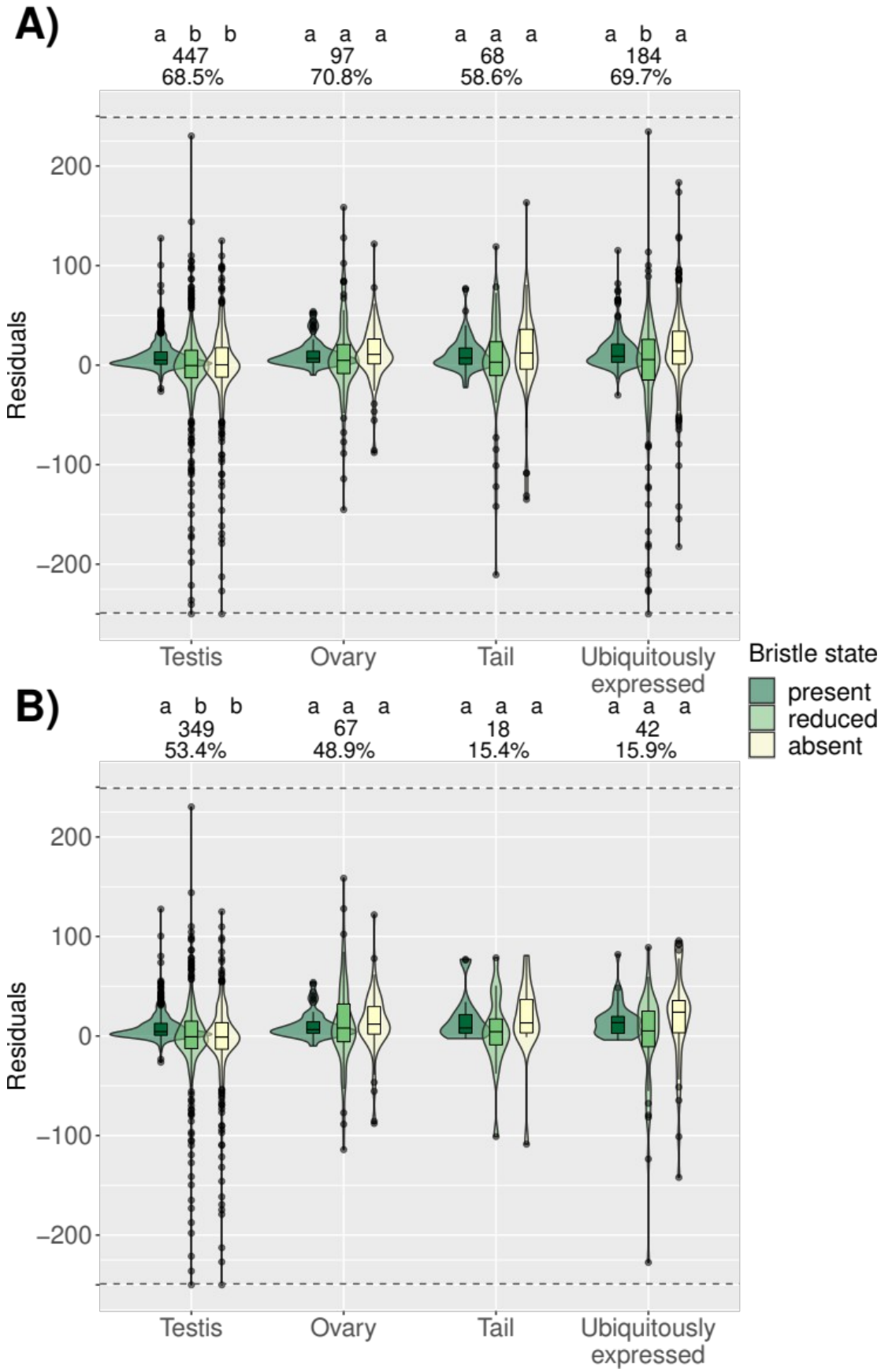
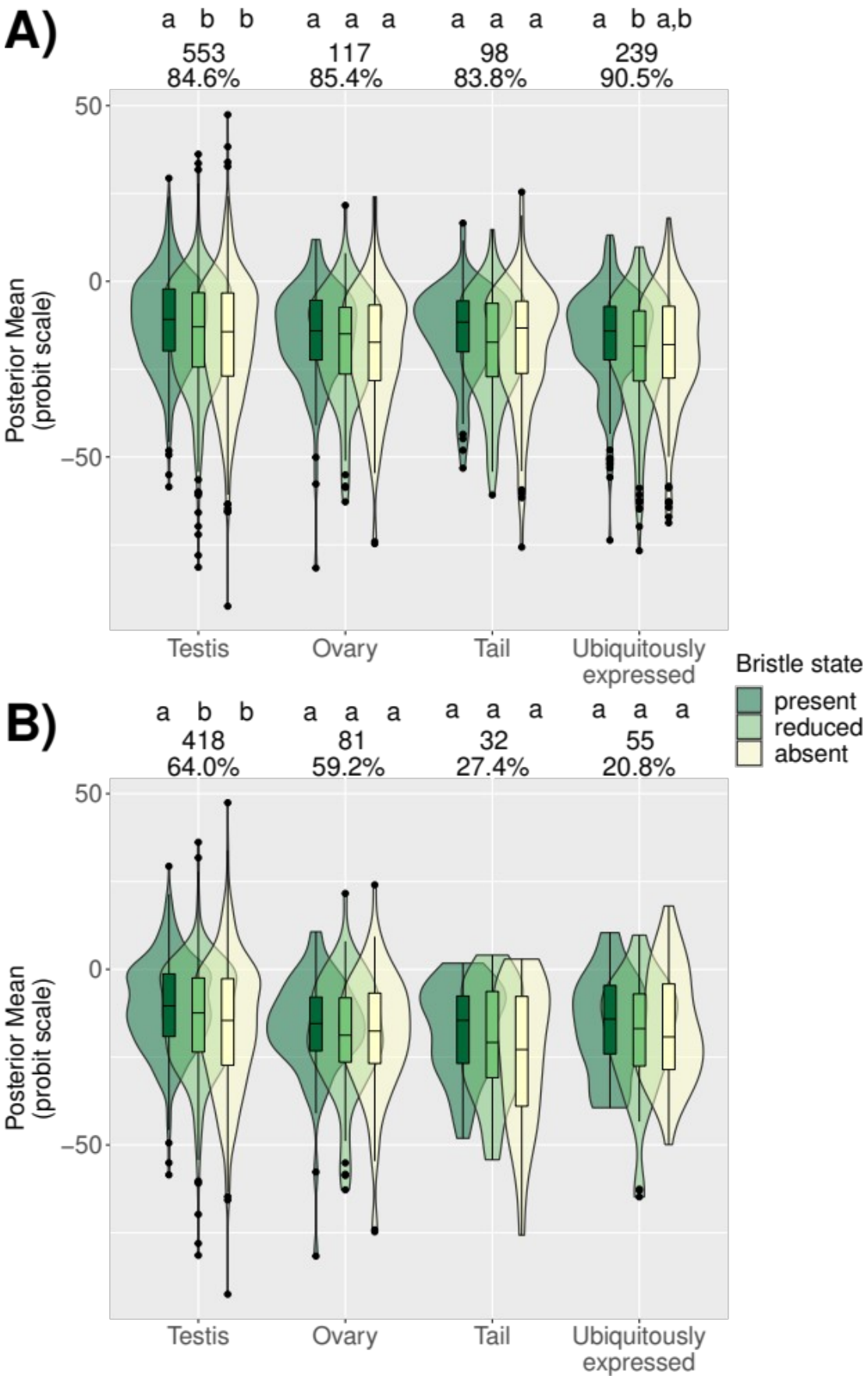


Figure 2. Distributions across orthogroups (OGs) with different functional annotations of the median residuals for species with different bristle states, when fitting similarity decay models for the species with bristles present. **A)** All OGs and **B)** OGs also annotated as having higher

expression in adults compared to hatchlings. Large dots indicate the means of the distributions. Letters above the data for each annotation category give the results of Dwass-Steele-Critchlow-Fligner all-pairs *post-hoc* tests, with different letters denoting significant ($p < 0.05$) differences between bristle states. The inset numbers above each group of boxplots give the number and percent of all OGs in each annotation group for which decay models were fit (see also table S1 – sheet 3). To make the figure clearer, OGs with values < -250 (N = 3) and > 250 (N = 0) have been plotted at y = -250 and y = 250 respectively, and separated from the remaining points by dashed horizontal lines.



630 **Figure 3. A)** Distributions across orthogroups (OGs) with different annotations of the posterior mean estimate of the probability (on the probit scale) of observing a gene for species with different bristle states. **B)** Same as for **A)** but only for OGs annotated as having higher expression in adults

compared to hatchlings in *M. lignano*. Letters above the data for each annotation category give the results of Dwass-Steele-Critchlow-Fligner all-pairs *post-hoc* tests, with different letters denoting
635 significant ($p < 0.05$) differences between bristle states. The inset numbers above each group of boxplots give the number and percent of all OGs in each annotation group included in the analysis models were fit.