

Dear Editor and Reviewers,

First and foremost, we would like to express our gratitude for taking the time to review our manuscript. We appreciate your constructive comments and believe they have significantly enhanced the quality of our work.

Before addressing your comments point by point, we would like to explain two major changes made to the manuscript:

1. **Change of Title:** We have revised the title of the paper from “Social evolution in termites reduces natural selection efficacy” to “The impact of social complexity on the efficacy of natural selection in termites.” This change was made to clearly distinguish our study from a recent publication by Ewart et al. (2024, Proc. R. Soc. B). In addition to confirming Ewart et al's findings on a “termites versus non-termites” comparison but with a larger number of species, our study also explores which factors would explain the variation within termites for the dN/dS ratio. The new title we propose thus seeks to emphasize the second part of our manuscript, focusing on intra-termite variation.
2. **Addition of a Co-Author:** We have added Alice Ha as a co-author, as she conducted the RELAX analysis that was requested by a reviewer. This analysis supports our first conclusions by confirming that the observed increase in dN/dS is primarily due to a relaxation of purifying selection.

Below, you will find our detailed responses to each of the reviewers' comments.

Thank you again for your valuable feedback.

Yours sincerely,

Camille Roux, Alice Ha, Arthur Weyna, Morgan Lodé and Jonathan Romiguier

Reviewer #1

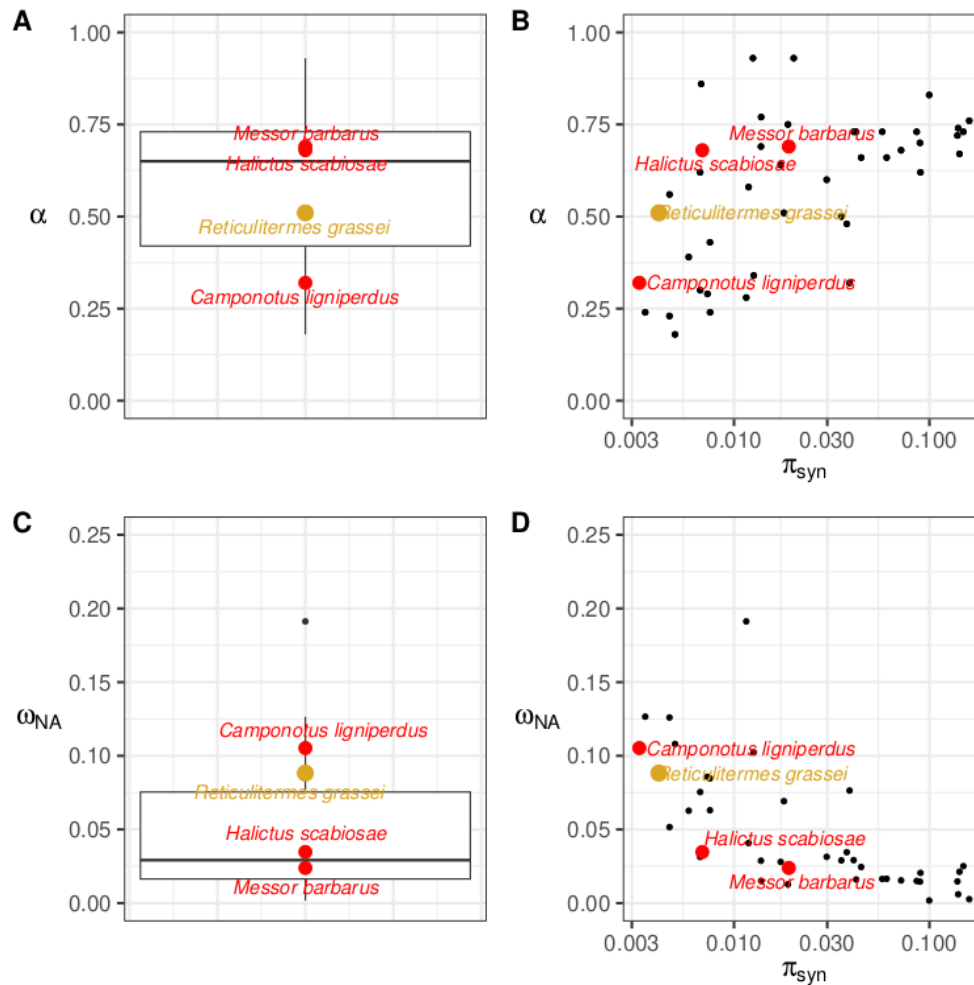
“I have two major comments for the authors. First, this paper is premised on using dN/dS ratios as a proxy for effective population size and as an indicator of the efficacy of natural selection, where higher dN/dS is assumed to result from accumulation of weakly deleterious mutations as a result of low effective population size and reduced selective efficacy. However, elevated dN/dS can, of course, also result from positive selection, which is not mentioned anywhere in this manuscript (discussed in Wertheim et al. 2015). I think that the hypothesis of positive selection leading to eusociality is as plausible as the hypothesis that eusociality increases the strength of genetic drift, and as it currently stands, this paper is not able to distinguish the two hypotheses.”

We are well aware of the existence of positive selection in molecular evolution, but think that it is very unlikely that it may explain genome-wide increase of dN/dS values. First, during ant evolutionary history, even an extreme hotspot of positive selection (i.e. branch leading to Formicoids) does not translate into particularly high genome-wide dN/dS (see table S3 of Romiguier et al 2022 and “absrel analysis” part of the data repository of the paper <https://zenodo.org/records/5705739>). This means that even exceptionally intense positive selection episodes (30-fold more events than all other branches) cannot involve a sufficiently high number of genes for significant increase of genome-wide dN/dS.

Second, a meta-analysis (Rouselle et al. 2020) carried out on a large phylogenetic scale in 44 non-model animal species, including the termite *Reticulitermes grassei* and 3 other eusocial insects (*Camponotus*, *Messor*, and *Halictus*) had previously looked at the relationship between genetic diversity measured at synonymous positions and the effectiveness of natural selection measured by :

- alpha: the proportion of non-synonymous substitution that is adaptive.
- omega_NA: the rate of substitution of non-adaptive mutations at synonymous positions relative to the neutral rate.

These estimates combine information drawn from divergence data, but also from intra-specific molecular diversity.



This study included 4 species of eusocial insects with *Camponotus ligniperdus* (ants), *Halictus scabiosae* (bees), *Messor barbarus* (ants) and *Reticulitermes grassei* (termites).

The conclusions of this article were a general relationship in animals between effective size (approximated by neutral diversity π_{syn}) and selection efficiency, with an increase in alpha and a reduction in omega_NA.

If we go back to the available analyses entitled 'Adaptive Protein Evolution in Animals and the Effective Population Size Hypothesis' (Table S1), we can see that eusocial species (in red in the above figure), including the termite of the genus *Reticulitermes* (in orange), are neither particularly associated with high values of alpha or low values of omega_NA.

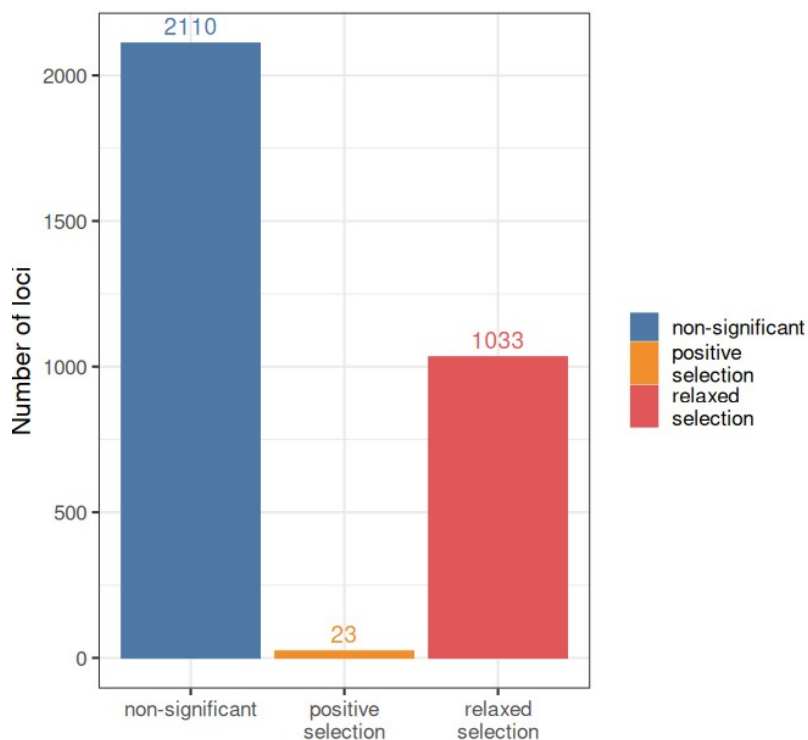
Admittedly, we recognise that *Reticulitermes grassei* may not be representative of the rest of the termites, but for the moment the genomic evidence tends to support the hypothesis of a decrease in the efficiency of natural selection linked to the small population size of eusocial species rather than the hypothesis of a genome-wide accumulation of adaptive substitutions.

“I would encourage the authors to include additional analyses using methods explicitly aimed at measuring the strength of natural selection, distinct from positive selection and in correlation with trait presence, such as RELAX from the HyPhy family of methods (Wertheim et al. 2015)”

We conducted the analysis suggested by the reviewer using the RELAX software. This analysis was performed on 3,166 coding genes from Blattodea data, with termites as the test group and the rest of the tree as the reference group. RELAX identified a change in selection for 1,056 genes after Bonferroni correction, and 2,110 genes for which the selection pressure is not significantly different in termites.

Among the 1,056 genes with a change in selection, there are:

- 23 genes with evidence of positive selection ($k > 1$).
- 1,033 genes with evidence of relaxed purifying selection ($k < 1$).



Thus, there are approximately 45 times more genes in termites with relaxed purifying selection than with positive selection which is in line with the message of our article. This result is also in line with RELAX analyses in Weyna et al. 2021 (10.24072/pcjournal.3), where relaxed selection was responsible of 90% of gene dN/dS increases across Hymenoptera evolution.

“Likewise, as currently phrased the title of the paper is making a strong causal claim that cannot be supported by this approach, and needs to be reworded”

Since the neutral theory of molecular evolution of Kimura, the H_0 hypothesis in molecular evolution is the N_e hypothesis to explain the diversity in the effectiveness of selection, so, we don't think that going in this direction is such a “strong causal claim”.

In social insects, the reduction in N_e is explained by the reduction in the number of reproductive individuals compared with species where a greater number of females are fertile (not just queens), suggesting that the real causal claim that would be strong would be to say that the reduction in N_e would lead to genome-wide positive selection, since this is not supported by any quantitative theory.

We hope that the new RELAX analyses help to clarify that the patterns we observed are due to N_e and less effective purifying selection, as expected by the neutral theory of molecular evolution (Kimura, 1983).

“Eusociality represents the pinnacle of social organization”- what does this mean? I am uncomfortable with how this implies a goal-directed kind of evolution; consider rephrasing or removing.”

We had no intention of suggesting a goal-directed view of evolution, especially when our message highlights that this evolutionary path leads to an increased accumulation of maladaptive substitutions. We removed this term and replaced it by “highest level of social organization”

“Both ants and termites display sharp contrasts in terms of levels of social complexity, ranging from small colonies of hundred individuals with low caste polymorphism, to large colonies of several millions of individuals and extreme caste differentiations.”- haven’t defined caste, so consider adding a definition prior to this sentence or change caste polymorphism/differentiation to simply physical differentiation.”

We added a definition of caste just before this sentence.

“In social insects like ants and termites, a caste is defined as a subset of individuals within a colony that perform specialized roles, often with distinct morphological traits. Both ants and termites display sharp contrasts in terms of levels of social complexity, ranging from small colonies of hundred individuals with low physical differentiation, to large colonies of several millions of individuals and extreme physical differentiations.”

“Hence, the evolution of d_S is solely driven by the mutation rate.” This contradicts your prior statement that synonymous substitutions can be under selection, consider rephrasing to something like “Hence, the evolution of d_S is usually assumed to be solely driven by the mutation rate.”

This is how we refined the sentence:

Hence, in absence of selective pressure on synonymous positions, the evolution of d_S is solely driven by the mutation rate.

“Paragraph starting with “Eusociality in ants, bees, wasps and termites appears to be such a life-history trait by restricting reproduction to few individuals.” Along with the discussion of Weyna and Romiguier 2021, consider also citing Barkdull and Moreau 2023, who found no relationship between worker reproduction and strength of natural selection across ant genomes.”
Done.

“We thus hypothesize further that termite species with the highest levels of social organization might feature the lowest effective population size, in a similar way than in mammals or birds where species with large body size feature lower effective population size than small body size species (Botero-Castro et al., 2017; Romiguier, Ranwez, et al., 2013).” The justification for this hypothesis based on an analogy to body size in birds is not clear; consider expanding your explanation of this analogy and how it justifies your hypothesis.

Thanks for the suggestion. Here, we are implying that large complex superorganisms (high level of eusociality with high colony size, high queen longevity) are analogous in terms of N_e to species with large body size/high longevity. To make it clearer, we modified this part by: “We thus hypothesize further that termite species with the highest levels of social organization might feature the lowest effective population size. This would mean that large superorganisms (i.e. large colonies)

are analogous in terms of N_e with large organisms, reflecting previous works in mammals or birds where species with large body size feature high dN/dS ratio (Botero-Castro et al., 2017; Romiguier, Ranwez, et al., 2013)”

“The authors then aligned the assembled orthologous using MAFFT v7.305”- the assembled orthologous what?

Apologies for the confusion. In French, "orthologous" is commonly used as a noun in this context, but this isn't the case in English. We've clarified the correct usage as "orthologous genes" in the text.

“Each loci which had sequences with...” change loci to locus.

Done

“These estimates were then associated with each terminal branch of the available phylogenies.”

How?

We understand where the confusion arose: we neglected to mention in the text that the MAPNH tool estimates non-synonymous and synonymous substitutions values for each branch. The values used afterwards are specifically the estimated dN/dS from the terminal branches.

We replace “*These estimates were then associated with each terminal branch of the available phylogenies.*” by “*Non-synonymous and synonymous substitution counts are estimated for each branch in the phylogeny.*”

However, for downstream analyses, we use the dN/dS ratio estimated from the terminal branches as representative for each species.”

“Each species was correlated with its social category following Michener’s classification in 1969 Michener (1969) and average individual size.” Where was the size data obtained?

Sources are listed in the Materials and Methods section, lines 153 to 162.

From the sentence starting with “Before carrying out an ANOVA analysis to test an effect of social organization on dN /dS”... to “To identify specific differences between groups, we used a Tukey-HSD (Honest Significant Difference) test, which tests the differences in means between each pair of groups (Table 1).”- these are methodological details which belong in Materials and Methods, not in the results.

We acknowledge that this result is not biologically informative on its own and, taken out of context, appears highly methodological. This part has been moved to Materials and Methods.

Statements like “we observe a notably lower efficiency of natural selection within the clade that includes termites” and “To do this, we take advantage of the information available on adult body length in Blattodea and test its impact on the effectiveness of purifying selection.” How can you disentangle the efficacy of natural selection/strength of drift from the effects of positive selection?

Positive selection becoming genome-wide across an entire clade with a reduced effective population size appears unlikely to us. We now confirm this idea with the RELAX analyses.

“This result converges with what has been observed in another insect clade where eusociality has evolved, the Hymenoptera, whose dN /dS is not affected by body size (Benitiere et al., 2022; Weyna and Romiguier, 2021).” Consider that individual body size in eusocial insects may not be the most appropriate analog to body size in solitary organisms, and that colony size may be a more appropriate metric. In contrast to the Benitiere and Weyna studies you cite here, Rubin 2022 did find a correlation between colony size and rates of molecular evolution in ants and bees. You should discuss this study.

Indeed, the studies by Benitiere et al. and Weyna and Romiguier do not address colony sizes. This gap is addressed by Rubin (2022), whose analysis aligns fully with the *Ne*-hypothesis. We have added this reference.

“As eusociality is a reproductive strategy characterized by parental care shared by many sibling individuals...” This does not sound like parental care- perhaps change to “characterized by care of offspring shared by many siblings”.

Done.

“High parental care has also been identified as the best ecological predictor of low genetic diversity (Romiguier, Lourenco, et al., 2014), which theoretically increases inbreeding and the relative advantages of kin selection (Tabadkani et al., 2012).” This sentence is confusing. Does high parental care increase inbreeding, or does low genetic diversity increase inbreeding? Either way, the casual relationship is not clearly explained. Please rephrase for clarity.

We made an effort to clarify this section.

“Multiple Piece nests are typically larger, but social complexity is at its highest in Separated Piece strategies...” Need a more substantial explanation of the multiple piece nest strategy.

We acknowledge that our initial definitions were quite succinct, and we now provide more detailed explanations of these different nesting strategies within the text to enhance clarity.

“This intricate social structure likely leads to higher dN/dS values due to the formation of larger colonies, which in turn reduces the number of breeding individuals, significantly affecting N_e and thus natural selection efficiency.” Again, this is in contrast to results in ants from Barkdull and Moreau 2023, so it could be interesting to discuss that finding here.

Done.

“Here, we classify the nesting strategies of termites as OP (One Piece), MP (Multiple Pieces), and SP (Separated Pieces) following the categorization in the termite literature (Mizumoto and Bourguignon, 2021).” This sentence feels out of place, since it is the last sentence in your discussion of nesting strategies. Either move this to the beginning of your nesting strategy section, or remove.

Indeed, this was a relic from an earlier version. We sincerely thank the reviewer for noticing it, and we have removed the inconsistency.

“Interestingly, species with true workers display significantly higher dN/dS ratios than those with pseudergates...” This finding also contrasts with Barkdull and Moreau 2023, who found no effect of worker polymorphism on patterns of molecular evolution. Could you explore/discuss why termites might differ from ants in this regard?

We suggest that these differences in results between ants and termites on worker specialization can be explained by differences in turnover between these two groups. In termites, "true workers" are highly represented in one clade which suggests that this trait is highly stable throughout their evolution. In contrast, worker polymorphism may be less stable, as suggested by fig 2 of Barkdull 2023.

We therefore propose that worker polymorphism in ants might not be maintained over long enough periods to have as clear effects as in termites on dN/dS ratio. We discuss this point in the last section of Results and Discussion.

“Such a finding have been suggested in ants, where a correlation between dN/dS ratios and another proxy of social complexity (queen/worker dimorphism) have been reported (Romiguier, Lourenco, et al., 2014). This finding was therefore based on only 7 species.” Again, you should cite other work in ants, including Rubin 2022 and Barkdull and Moreau 2023, which used much

larger datasets than Romiguier 2014, and who found different patterns to those in Romiguier 2014.

Done

“Supplementary analyses on more species, including other eusocial clades such as bees or wasps will be useful to further confirm our conclusion.” Again, this would be a great place to discuss Rubin 2022, which included 11 bee species. You should also cite and discuss Shell et al. 2021, which analyzes 16 bee genomes and finds that eusocial lineages exhibit stronger positive selection, which contrasts your findings in termites. Consider also including work in social shrimp (e.g. Chak et al. 2020), where analysis of mitochondrial genomes suggests a link between sociality and relaxation of selection.

Done.

Figures 1 and 3- rainbow color scales are perceptually misleading (see Borland and Taylor 2007 and Stoelzle and Stein 2021) and cannot be used by people with achromatopsia. Please re-color these figures using a more appropriate color scale.

Done.

Figures 1, 2, and 4: please indicate statistical significance on these plots.

Done.

Reviewer #2

However, alas, a similar study has recently been published, most likely while this manuscript was in its final stages: Ewart et al. "Pervasive relaxed selection in termite genomes." *Proceedings of the Royal Society B* 291.2023 (2024).

The results from the current study support the findings from Ewart et al. while adding additional important findings on the effect of social complexity. I find it is, therefore, important that that paper should be cited and results compared, e.g. that transcriptomic results on larger species set confirm genomic analyses....

It is true that by the time we completed our manuscript, the Ewart article had just been published.

While our study follows a similar approach, Ewart's analysis primarily focuses on a comparison between termites and cockroaches. In contrast, our research extends the comparison beyond cockroaches to include more distant groups like Zoraptera, offering a broader perspective in the first part of our article.

Additionally, the second part of our study delves into the relationship between the effectiveness of purifying selection and various traits across 68 termite species. This is a significant aspect that Ewart et al. do not explore, as their study includes three termite species, which cannot allow them to address this relationship.

However, we acknowledge the precedence of their work and have appropriately cited it in our text.

1. Overall, although I agree that high dNdS is likely caused by low Ne due to social complexity, I would suggest toning down in some places that this is proven here. High dNdS can also be caused by adaptive evolution. There were no formal tests carried out for a relaxation of selection, just dN/dS. But the results do offer strong support.

We included a RELAX analysis that reveals the increase in dN/dS in termites is primarily driven by approximately 30% of genes showing evidence of relaxed purifying selection, while fewer than 1% of the genes are associated with positive selection. We hope this additional analysis strengthens the argument that eusociality significantly reduces the efficiency of purifying selection across a substantial portion of the genome.

2. What is the cause and effect of eusociality and low Ne - again I agree with the main assertions in this manuscript - but there are two examples where it could be argued that a low Ne preceded the evolution of eusociality: in solitary bees and in *Cryptocercus*. Using ants as an example where this was not the case is difficult since it is difficult to find a suitable non-eusocial outgroup for ants. This is discussed to some extent but then on lines 298ff the authors suggest eusociality is associated with decreases in Ne, rather than low Ne. The alternative scenarios and how they can be addressed in the future could be more openly addressed.

This is an extremely important point that we have not emphasized as much as it deserves. Although testing evolutionary causality with such a limited number of empirical examples (solitary bees and *Cryptocercus*) is challenging, it would be remarkable to observe a significant reduction in effective population size in these groups prior to the evolution of eusociality and its impact on molecular evolutionary patterns. We propose applying the approach used in social spiders of the genus *Stegodyphus* to study both social and solitary bees, as well as termites and *Cryptocercus*.

We have added the following small paragraph on these aspects:

Demographic inferences using PSMC-like approaches (Li2011inference, terhorst2017robust, lynch2020inference) in non-social species with high d_N/d_S ratios, such as *Cryptocercus* and solitary bees, as well as in closely related eusocial groups, could provide a clearer understanding of the demographic context in which eusociality evolved.

This would be particularly valuable for pinpointing the timing of Ne variations associated with the emergence of eusociality.

This approach has already been applied to social spiders of the genus *Stegodyphus*, where it revealed a reduction in Ne associated with increasing social complexity (ma2024sociality).

Moreover, it could help determine whether the reduction in Ne preceded the emergence of eusociality in bees (from solitary species) and termites (from *Cryptocercus*), or if it resulted from the evolution toward more complex social structures.

3. Also, please be careful when suggesting that low Ne is confirmed here, e.g. line 298. Theory predicts low Ne - and it certainly makes a lot of sense but the analyses here do not allow any direct inferences on Ne.

These are not major points and just need to be reworded to make it clear what is an interpretation and what is more directly measured by the data. For these reasons, I believe the title needs to be adapted accordingly, e.g. evolutionary rates support or something similar.

We made small adjustments as suggested and change the title by: Molecular evolution patterns support reduced efficacy of natural selection in termites

- line 37: should be "effective population size"

Done

- lines 37-40: citations and examples missing

Done

- line 56: dS is "solely" driven by mutation rate - should be toned down to "mainly" or "predominantly" as other factors such as tRNA abundance, substitution bias play a role.

Done

M&M:

Blattodea dataset: were these alignments always single copy orthologues? And not always across all species?

The analyzed alignments are indeed of orthologous genes present in a single copy. However, the number of sequences varies among species, with some loci missing. In the previous version of the manuscript, we did not specify that we had set a threshold for the minimum number of available sequences per alignment; alignments with fewer than 30 sequences (corresponding to 30 species) were excluded from the analysis.

- Social traits: Please provide a table showing how species were classified by social traits for both data sets

Done (see Tables S1 and S2, along with the supplementary material available on Zenodo).

- line 133: Neo- and Basal Isoptera - please explain the distinction and why it is relevant

We added this precision: "(Neoisoptera which is a clade grouping most families with high level of sociality vs Basal Isoptera)"

- line 134: worker type - please elaborate

We added this precision; ("true workers" than are completely sterile vs "pseudergates" that can become reproductives)

Results & Discussion:

- line 157: "we observe a notable lower efficiency of selection" - this is one example of point 1 above - you observe higher dnds ratios and this can be interpreted as.... There are more examples (e.g. line 167) but I won't list them all

We have sought to qualify our statements by emphasizing that what is primarily observed is a higher average genomic dN/dS, rather than a direct measure of the effectiveness of natural selection. However, the latest analysis using RELAX indicates that there are 40 times more genes under relaxed purifying selection than under positive selection in termites, compared to non-termites.

- line 165 and elsewhere: from here onwards the authors write "Blattodae" instead of "Blattodea"

Corrected

- lines 185ff - refer to figure 2B in the text

Done

- figure 1 caption: utilising "identical" coding genes - what is meant here?

We apologize for the confusion, we meant here "*Utilizing the same set of coding genes*".

- figure 2: what do the two colours mean? Please use a legend. Should there not be two regression lines in plot 2A - one for termites, one for others?

We have added definitions for the colors and included the second regression line.

- Table 1: caption is a bit short and lacking details

We have provided a revised version of Table 1 with more detailed explanations, addressing its previous lack of clarity.

- line 201ff: this is a good point about phylogenetic corrections not being necessary for dnds. But what if the cause of the differences, i.e. Ne, is phylogenetic, for example if a reduction in Ne occurred in the common ancestor of all termites and Cryptocercus? This could be tested by comparing Termites+Cryptocercus to other groups as was done in table 1

In our opinion, Ne (or eusociality) being phylogenetic does not change the fact that several branches with high dN/dS from an eusocial clade are several valid tests for an association between Ne/eusociality with reduced selection efficiency. Reduction in Ne (likely linked to increased

parental care) is indeed likely to have occurred in the common ancestor of termites and *Cryptocercus*, as we suggest in the discussion.

- line 210f: I'm not sure if the dnds of *Cryptocercus wrighti* can be considered "intermediate" - it is quite close to the mean for termites. Please provide a supplementary table of mean dnds values for all investigated species for each data set to make this clearer.

It's true that the term 'intermediate' is imprecise and requires clarification. We use it here to describe *Cryptocercus* because its dN/dS ratio is either very close to the maximum dN/dS ratio observed in non-termites (Evangelista dataset) or consistently higher than the maximum dN/dS in non-termites (Bucek dataset), while still being consistently lower than the minimum dN/dS ratio found in termites.

We present the *min* and *max* dN/dS values for the different groups and datasets in the two tables below.

Evangelista dataset:

Group	Min	Mean	Median	Max
Cryptocercus	0.0905	0.0905	0.0905	0.0905
Non-termite species	0.0554	0.0672	0.0671	0.0919
Termites	0.0968	0.107	0.108	0.115

Bucek dataset:

Family	Min	Mean	Median	Max
Apicotermitinae	0.168	0.179	0.176	0.211
Archotermopsidae	0.145	0.149	0.147	0.155
Foraminitermitinae	0.150	0.150	0.150	0.151
Kalotermitidae	0.140	0.149	0.145	0.163
Macrotermitinae	0.156	0.167	0.164	0.181
Mastotermitidae	0.144	0.144	0.144	0.144
Nasutitermitinae	0.147	0.170	0.162	0.256
Rhinotermitidae_A	0.140	0.140	0.140	0.140
Rhinotermitidae_B	0.132	0.154	0.155	0.190
Rhinotermitidae_C	0.167	0.174	0.174	0.180
Rhinotermitidae_D	0.152	0.161	0.160	0.171
Rhinotermitidae_E	0.161	0.178	0.169	0.224
Sphaotermitinae	0.161	0.161	0.161	0.161
Stylotermitidae	0.135	0.135	0.135	0.135
Syntermitinae	0.156	0.173	0.174	0.188
Termitidae_A	0.160	0.165	0.165	0.171
Termitidae_B	0.168	0.173	0.171	0.185
Termitinae	0.158	0.170	0.171	0.185
Cryptocercus	0.125	0.125	0.125	0.125
Outgroup	0.0925	0.101	0.103	0.106

- line 219: this should be ALLOparental care

Corrected

- Figure 4a: where is the dot for *Cryptocercus* in the left hand boxplot? It should be at around 0.125

We acknowledge that there was a significant confusion when manually completing the table with social traits. The social traits attributed to *Cryptotermes* (one-piece nesters and pseudergates) were mistakenly also assigned to *Cryptocercus* also. We have revised the figures and redone the associated statistical tests. While the test values have changed slightly, the overall interpretations remain the same. We thank the reviewer for identifying this error.

- Social complexity: please also discuss the possible effect of indirect selection due to sterile workers in more socially complex species.

Thanks for this suggestion. We added the following sentences for discussing the result on “true workers” vs “pseudergates”:

One potential effect increasing further dN/dS in "true workers" species is that workers being sterile, worker-specific genes can not be directly selected but only via kin selection. Such an indirect selection is predicted to be reduced compared to direct selection, an idea that has been supported by previous work in the pharaoh ant (Warner et al. 2017).

- line 287: the individual effect of worker type and nesting type is likely impossible to pull apart as they correlate. Therefore, the causes of indirect selection and N_e are also difficult to isolate. Please discuss.

Thanks for this interesting suggestion. We added the following sentence:

To better quantify the effect of indirect selection in termites and disentangle it from the effect of N_e , an interesting perspective would be to replicate our study on queen-biased genes vs worker-biased genes.

- line 298: effective population size is not determined in this study.

We changes effective population size by “significant decreases in terms of natural selection efficiency”.