

# The adaptive roles of aneuploidy and polyclonality in *Leishmania* in response to environmental stress

Gabriel Heringer Negreira, Robin de Groote, Dorien van Giel, Pieter Monsieurs, Ilse Maes, Geraldine de Muylder, Frederik Van den Broeck, Jean-Claude Dujardin, and Malgorzata Domagalska

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# Review #1

## 1. Evidence, reproducibility and clarity:

### Evidence, reproducibility and clarity (Required)

Negreira et al. have studied aneuploidy in *Leishmania* selected using a "flash selection" with SbIII or miltefosine (MF). They provided evidence for the SbIII arm that a few parasites in the population with a specific genotype were enriched during drug selection, and these selected parasites with continuous drug pressure further present modifications in their ploidy. For MF selection they show a different scenario where first a minor population with a mutation in the MT gene is selected and with further passages with drugs, parasites with changes in ploidy are further enriched.

Here are some comments that hopefully will be helpful for the authors.

The plasticity of the *Leishmania* genome is fascinating. It is remarkable that these parasites can tolerate so many and frequent changes in ploidy. Either these changes are stochastic and serendipitous or as convey by the authors are part of the parasite arsenal to respond to a changing environment. They cleverly used single cell sequencing and bar-coded parasites in this well designed and well conducted study to assess the role of ploidy in parasite biology.

1. Drugs are not inducing any of the changes observed, instead the drugs are selecting for parasites with different genotypes (e.g. polyploidy of chromosome 23 for SbIII or parasites with mutations in MT). This is an important conceptual difference and the authors need to change their text throughout starting at line 28.
2. Line 170. It is probably expected that no cells have increased copy of chromosome 23, 27 and 31 after single cell genomics. None of the first passages of the four SePOP are polyploid for chromosome 27. One possibility is that a subpopulation of cells with increased copy of chr. 23 (because of MRPA?) and 31 (because of ?) are first selected and in subsequent passages cells triploid for 27 are selected. Of note the ploidy of chr. 27 appears to decrease from passage 4 to 5 in SePOP1 which is unusual if the drug pressure is maintained.
3. Line 194. I agree with the concept of the selection of pre-existing aneuploid cells but the additional some changes observed are, in my opinion, just selected because these changes occur continuously.
4. Their barcoded strategy was interesting but it would appear that different lineages are enriched in the 4 SePOP. It would be of interest to test whether those lineages have similar ploidy at the onset. I am unclear of why they have to amplify the barcode prior sequencing. Could they just not get this info from the SePOP data; it is my understanding that the drug selection was done with the barcoded population. This would have facilitated the correlation barcode-specific ploidy.
5. The MF screen was harsh and the parasites selected (derived from few clones within the population when considering the time needed to expand) contained SNPs in MT. Difficult to compare the two screens. Passages with higher MF concentration led to

major changes in ploidy but with few common features between the MePOP lines.  
6. I am not asking for extra work but as a suggestion to help in linking ploidy with phenotype it would have been very interesting to look at 5 passages without drug (SbIII or miltefosine) to see whether a decrease in ploidy is correlated to a decrease in resistance.

**\*\*Minor points\*\***

1. The environment studied (high drug pressure) is unlikely to occur in nature. The authors may wish to comment on how this may translate in the sand fly or in animals.
2. In Fig. S2 MRPA in SePOP1 is a signature of extrachromosomal amplification. Was that studied?
3. For Chromosome 31 in the Sb screen, it would appear that the proximal (left) part is of lower copy number than the distal (right) portion of the chromosome. How could this have happened? Deletion of a portion of chromosome 31 for one allele? This has been described before (Mukherjee et al., 2013) in SbIII resistant lines as one telomeric end of Chr. 31 encodes AQP1, the route of entry of SbIII.

## **2. Significance:**

### **Significance (Required)**

The plasticity of the *Leishmania* genome is fascinating. It is remarkable that these parasites can tolerate so many and frequent changes in ploidy. Either these changes are stochastic and serendipitous or as convey by the authors are part of the parasite arsenal to respond to a changing environment. They cleverly used single cell sequencing and bar-coded parasites in this well designed and well conducted study to assess the role of ploidy in parasite biology.

## **3. How much time do you estimate the authors will need to complete the suggested revisions:**

### **Estimated time to Complete Revisions (Required)**

#### **(Decision Recommendation)**

Between 1 and 3 months

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**Publons); note that the content of your review will not be visible on Web of Science.**

### **Web of Science Reviewer Recognition**

No

## **Review #2**

### **1. Evidence, reproducibility and clarity:**

#### **Evidence, reproducibility and clarity (Required)**

Negreira et al. present a study that aims to understand the early evolution of aneuploidy.

They use *Leishmania*, a protozoan parasite known for its genome plasticity, as model, and two drugs as stress inducers. In this work, they use single-cell genomics and lineage tracing to detect changes in chromosome copy numbers. They conclude that, although parasites tend to have genomes with unusual plasticity, aneuploidy dynamics depend on the stressor more than the organism.

**\*\*Further experiments:\*\***

Lines 121-124: I believe the authors should corroborate the statement that expansion of lineages that were fitter prior to drug exposure is stochastically by doing a statistical test comparing their obtained data and randomly generated simulated values. Given that there is still a considerable proportion of lineages with higher fitness and found in more than one passage, I believe this experiment/test would add strength to the conclusion.

**\*\*Minor issues:\*\***

Fig. 1B: Add label to top horizontal axis, showing frequency of each karyotype.

Lines 92-96: Could the authors postulate how and why pre-existing aneuploid cells seem to be selected upon SbIII exposure?

Fig. 3: Are panels B and C swapped in the figure or the reference swapped in the text?

Fig. 3C seems to refer to the mutation (lines 173-179), whereas Fig. 3B seems to relate to the surviving lineages (lines 183-186).

Lines 94-97: Could the authors comment on the advantages and disadvantages of such an aggressive selection method? I am not surprised with such a drastic decrease in lineage diversity in this context.

Could the authors elaborate on what is different in chromosome 31 that makes it so prone to change?

## **2. Significance:**

### **Significance (Required)**

Aneuploidy can be well-tolerated, beneficial, or deleterious. Particularly, they can confer resistance against environment stresses, including drug pressures. This study aims to understand how aneuploidy arises. The authors approach this question using a model organism, *Leishmania donovani*, and two distinct drugs as environmental stressors. Using single-cell DNA sequencing and lineage tracing, the authors find that the appearance of aneuploidy is dependent on the drug used, which makes it dependent on the environmental stressor, rather than pre-determined. Importantly, they present a new barcoding method that may be useful to the field of experimental genome evolution.

## **3. How much time do you estimate the authors will need to complete the suggested revisions:**

### **Estimated time to Complete Revisions (Required)**

#### **(Decision Recommendation)**

Less than 1 month

**4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at [Web of Science Reviewer Recognition Service](#) (formerly Publons); note that the content of your review will not be visible on Web of Science.**

### **Web of Science Reviewer Recognition**

Yes

## **Review #3**

# 1. Evidence, reproducibility and clarity:

## Evidence, reproducibility and clarity (Required)

This interesting, well written paper uses cutting edge technologies to address the evolutionary dynamics of changes in *Leishmania donovani* genomes in response to high drug pressure. Using single-cell genome sequencing and lineage tracing with a newly adapted cell barcoding system, the authors were able to follow aneuploid changes and lineage selection following exposures to high concentrations of either antimony or miltefosine. The main conclusions drawn from the careful bioinformatic analyses and methodic representation of 864 single cell genomes and 453 different traceable lineages were that for each drug exposure there was polyclonal selection of pre-adapted parasites complemented by de novo adaptations. Consistent changes in aneuploidy were associated with the populations selected by antimony, while miltefosine selected for populations that had a point mutation in a miltefosine transporter gene. These conclusions are well supported by the data.

# 2. Significance:

## Significance (Required)

One general comment is that the contribution of pre-adapted lineages to the emergence of drug resistant populations under conditions of natural exposure is apt to be overstated from the current analysis. As the authors discuss, the *L. donovani* line used is already pre-adapted to resist antimony due, at least in part, to the amplification of the MRP gene on chromosome 23. So it is expected that lineages adapted to strong antimony pressure will pre-exist in this line. It seems possible that the de novo adaptations that were observed, involving further copy number amplification of chromosome 23 and other chromosomes (eg chr 31), might be facilitated by their pre-existing aneuploidies. Thus the evolutionary dynamics observed might be very particular to these sorts of pre-conditioned cells. It should also be discussed that the culture conditions themselves may pre-condition the parasites for antimony resistance (and possibly other drugs). Continuous passage of *L. donovani* in axenic culture produced consistent patterns of aneuploid changes, including amplification of Chr 23 (Barja et al., Nat Ecol evol, 2017). Thus a potential caveat of the use of cultured promastigotes is that their culture adaptations might involve genes on the same chromosomes that confer drug resistance.

For the miltefosine selection, of the 7 lineages surviving in at least one of the MePOP replicates, only lineage 302 is represented more than once. What is the evidence that the adaptive mutations in the other 6 lineages were pre-existing and did not arise de novo?

Figs 3b and 3c are incorrectly referenced in the text.

Discussion p. 8 - "Interestingly, the Gly160Asp mutation also correlated with the frequency of a specific lineage (lineage 27) and appeared in 3 of the 4 MePOPs, indicating that this was a pre-existing mutation found in that lineage." Lineage 302 would appear to be the correct lineage, not 27. Please clarify.

**3. How much time do you estimate the authors will need to complete the suggested revisions:**

**Estimated time to Complete Revisions (Required)**

**(Decision Recommendation)**

Less than 1 month

**4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at [Web of Science Reviewer Recognition Service](#) (formerly Publons); note that the content of your review will not be visible on Web of Science.**

**Web of Science Reviewer Recognition**

Yes

# Full Revision



**Manuscript number:** RC-2022-01811

**Corresponding author(s):** Malgorzata A., Domagalska

*[Please use this template only if the submitted manuscript should be considered by the affiliate journal as a full revision in response to the points raised by the reviewers.]*

*If you wish to submit a preliminary revision with a revision plan, please use our "[Revision Plan](#)" template. **It is important to use the appropriate template to clearly inform the editors of your intentions.***

## 1. General Statements [optional]

*This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.*



## 2. Point-by-point description of the revisions

*This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.*

*Reviewer #1 (Evidence, reproducibility and clarity (Required)):*

*Negreira et al. have studied aneuploidy in Leishmania selected using a "flash selection" with SbIII or miltefosine (MF). They provided evidence for the SbIII arm that a few parasites in the population with a specific genotype were enriched during drug selection, and these selected parasites with continuous drug pressure further present modifications in their ploidy. For MF selection they show a different scenario where first a minor population with a mutation in the MT gene is selected and with further passages with drugs, parasites with changes in ploidy are further enriched.*

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*1. Drugs are not inducing any of the changes observed, instead the drugs are selecting for parasites with different genotypes (e.g. polyploidy of chromosome 23 for SbIII or parasites with mutations in MT). This is an important conceptual difference and the authors need to change their text throughout starting at line 28.*

R: We agree with the reviewer and adapted the text. These changes were introduced as follow:

Line 27 (line 19 in the new version):

**“we revealed that *antimony-induced* aneuploidy changes *under antimony pressure* result from the polyclonal selection of pre-existing karyotypes”**

Line 201 (line 187 in the new version):

**“*This approach revealed that* the flash selection with Sb<sup>III</sup> *induced led to* a fourfold reduction in lineage diversity that stabilized between passages 3 to 4, leaving between 101 to 131 of detectable lineages”**

Line 354 (line 381 in the new version)

**“The flash selection performed with miltefosine revealed a contrasting scenario where aneuploidy remained unchanged even after a stronger bottleneck ~~induced by~~ associated with the drug at passage 1, 25  $\mu$ M and illustrated by the strong decrease in barcode diversity (from 453 to 7 lineages).”**

*2. Line 170. Its is probably expected that no cells have increased copy of chromosome 23, 27 and 31 after single cell genomics. None of the first passages of the four SePOP are polyploid for chromosome 27. One possibility is that a subpopulation of cells with increased copy of chr. 23 (because of MRPA?) and 31 (because of ?) are first selected and in subsequent passages cells triploid for 27 are selected. Of note the ploidy of chr. 27 appears to decrease from passage 4 to 5 in SePOP1 which is unusual if the drug pressure is maintained.*

R: We agree with the reviewer that the aneuploidy changes seen in the SePOP1-4 can be explained by the initial selection of subpopulations of cells with a beneficial pre-existing dosage increase in one or two chromosomes (e.g., chromosome 23 and 31) followed by the selection of additional cumulative modifications emerging in subsequent time points. This conclusion was previously stated throughout the text and is also depicted by the minimum spanning tree in figure 1C, but we made some alterations in the text in order to better state this conclusion:

Line 100 (line 87 in new version):

**“Using single-cell genome sequencing, we could uncover the evolutionary paths that might have led to the emergence of such aneuploidy changes, ~~which involved~~ indicating a process of selection of pre-existing karyotypes complemented by further ~~de-novo cumulative~~ alterations in chromosome copy number along evolution”**

Line 168 (line 156 in new version):

**“However, none of the sequenced promastigotes showed amplification of chromosomes 23, 27 and 31 concomitantly, and no pre-existing karyotype was identified with a pentasomy in chromosome 23 as observed in the SePOP3, suggesting that some of the aneuploidy ~~modifications were generated along adaptation to Sb<sup>III</sup>~~ changes seen in SePOP1-4 happened after initial exposure to Sb<sup>III</sup>.”**

Line 191 (line 177 in new version):

**“Altogether, our single-cell data suggest that (i) aneuploidy changes observed in the Sb<sup>III</sup>-exposed populations are explained by the ~~selection of pre-existing aneuploid cells, complemented by additional somy changes generated de-novo during the experiment and~~ initial selection of subpopulations bearing pre-existing chromosomal amplifications**

**followed by the further selection of cumulative karyotypic modifications emerging in subsequent time points and (ii) that the aneuploidy changes seen in SePOP1-4 would have a polyclonal origin.”**

Regarding the decrease of chr.27 in SePOP1 from passage 4 to 5, we believe this decrease is not very significant as its somy value (2.71) indicates that the majority of cells still display a trisomy for this chromosome. Moreover, this decrease coincides with the moment where a dosage increase (from ~3 to ~10 copies per haploid genome) in the MRPA locus happens exclusively in that population and in that passage (see supplementary figure S2B), which likely has a stronger impact in Sb<sup>III</sup> tolerance compared to the trisomy of chr27.

*3. Lane 194. I agree with the concept of the selection of pre-existing aneuploid cells but the additional somy changes observed are, in my opinion, just selected because these changes occur continuously.*

R: The changes in mentioned above starting at line 191 were also done in response to this comment.

*4. Their barcoded strategy was interesting but it would appear that different lineages are enriched in the 4 SePOP. It would be of interest to test whether those lineages have similar ploidy at the onset. I am unclear of why they have to amplify the barcode prior sequencing. Could they just not get this info from the SePOP data; it is my understanding that the drug selection was done with the barcoded population. This would have facilitated the correlation barcode-specific ploidy.*

R: We agree that it would have been interesting to integrate the single-cell genomics and the barcode data in order to determine if the selected lineages had similar karyotypes at the onset of the experiment. However, although the genome coverage of individual cells in the single-cell genomics method used in our study is enough to determine differences in chromosome copy number, it is not enough to evaluate, at sequence level, individual genomic loci such as the lineage barcodes. This is because the genome coverage per cell is too low (in our case 0,8x) meaning that most genomic loci are mapped by just a single sequence read or not mapped at all (10X Genomics, 2020). Thus, it was not possible to determine the lineage barcode of individual cells from the single-cell data.

Regarding the need for amplifying the barcodes: in contrast to WGS, a targeted amplification of the barcodes enabled us to obtain millions of reads covering the barcodes. This, in turn allowed quantifying accurately the frequency of each barcoded lineage.

This is now mentioned in the text starting in line 514 (548 in the new version):

**“Barcode amplification was done using the same DNA samples used for bulk whole genome sequencing. Targeted amplification of the barcodes is needed as the number of reads containing a lineage barcode (~50 pair end reads per sample on average in our case) in the whole genome sequencing data is insufficient for the determination of the frequency of each barcoded lineage in the parasite pool.”**

*5. The MF screen was harsh and the parasites selected (derived from few clones within the population when considering the time needed to expand) contained SNPs in MT. Difficult to compare the two screens. Passages with higher MF concentration led to major changes in ploidy but with few common features between the MePOP lines.*

R: The screen of the BPK282 strain under Sb<sup>III</sup> or miltefosine pressure provides two contrasting models and this is one of the interests of the present study. The BPK282 strain belongs to a population of *L. donovani* parasites from the lowlands of the Indian subcontinent, where parasites were exposed to strong Sb<sup>III</sup> pressure for decades, even more since these parasites are transmitted from human to human. This population is characterized by strong genomic variations affecting Sb<sup>III</sup> susceptibility, of which the intra-chromosomal amplification of MRPA is a well known driver of Sb<sup>III</sup> pre-adaptation. BPK282 has this intrachromosomal amplification of MRPA and thus it is strongly pre-adapted to Sb<sup>III</sup>. In contrast, at the time of isolation of BPK282, miltefosine was not yet implemented in clinical practice in the Indian sub-continent (ISC). BPK282 is considered highly susceptible to miltefosine and pre-adaptation to this drug was not, until the present study, identified in this strain and in the ISC population it was isolated from. We performed the flash selection with both drugs to investigate if aneuploidy modulations would follow similar patterns in these two contrasting environments, one where the strain is pre-adapted, and another where it is highly susceptible.

We state this starting in line 242 (line 230 in new version):

**“The results described above demonstrated the importance of aneuploidy for parasite adaptation to high Sb<sup>III</sup> pressure together with the polyclonality of corresponding molecular adaptations. We aimed here to verify if the same features would be observed with another anti-leishmania drug, miltefosine. In contrast to Sb<sup>III</sup>, there was – at least before present study – no pre-adaptation known to miltefosine in the BPK282 strain, which is considered very susceptible to the drug (23).”**

We also added two sentences in the discussion reiterating this contrast between Sb<sup>III</sup> and MF in BPK282:

Starting in line 353 (377 in new version):

“Finally, we assessed the role and dynamics of aneuploidy under strong pressure of another drug, miltefosine. **Noteworthy, BPK282 was isolated from the population endemic in the Gangetic plain, before miltefosine was implemented in the region (in sharp contrast to Sb<sup>III</sup>). Hence different results were expected for the scenario of genomic adaptation and clonal dynamics.**”

In addition, we believe that the results of the miltefosine flash selection further corroborate the notion that aneuploidy modulations seen in these drug selection experiments can happen de novo along adaptation to the drug. This was not well stressed in the manuscript and thus we included the following statement during in the discussion:

Starting at line 360 (line 387 in the new version):

**“This demonstrated that the strong bottleneck associated with initial exposure to miltefosine in the first passage did not impair the potential for aneuploidy modulations in later passages, and that these modifications depend on the strength of the stress caused by the drug. These observations are also in agreement with the notion of aneuploidy modulations happening de novo during adaptation to the drug as the aneuploidy profiles seen at passage 9 in the MePOPs exposed to 100  $\mu$ M are also very different from the pre-existing karyotypes identified in the single-cell data of BPK282.”**

*6. I am not asking for extra work but as a suggestion to help in linking ploidy with phenotype it would have been very interesting to look at 5 passages without drug (Sb<sup>III</sup> or miltefosine) to see whether a decrease in ploidy is correlated to a decrease in resistance.*

R: Unfortunately, we do not have access to the selected populations anymore, but we agree that characterizing these selected populations after keeping them for a few passages without drug would further strengthen the understanding of the relationship between aneuploidy modulations and Sb<sup>III</sup> tolerance.

*Minor points*

*1. The environment studied (high drug pressure) is unlikely to occur in nature. The authors may wish to comment on how this may translate in the sand fly or in animals.*

First of all, the population of *L. donovani* from which strain BPK282 originated has been naturally under high drug pressure since decades, given the anthroponotic nature of transmission in the Indian sub-continent and the absence of reported animal reservoir. An additional pressure came from the strong pollution with Arsenic, that is present in the lowlands where BPK282 was isolated (Perry et al., 2011). The same authors showed that chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs (cross resistance) in a mouse model of visceral leishmaniasis and concluded that arsenic contamination in the Gangetic plain may have played a

significant role in the development of *Leishmania* antimonial resistance (Perry et al., 2013). This might explain why antimony resistance drivers like amplification of MRPA were already present in the populations even before antimony was implemented in the region (Imamura et al., 2016).

This is now mentioned in the text starting at line 311 (320 in new version)

**“This pre-adaptation likely comes from the combination of high antimony pressure for decades, highly endemic pollution with arsenic – which can cause cross-resistance to antimonials (33, 34) – and anthroponotic transmission without animal reservoir.”**

Secondly, in current study, we pushed further the parasite and experimentally exposed it to even higher drug pressure. Our flash selection approach was done as a general model to investigate the mechanisms that *Leishmania* exploits in order to adapt to sudden and strong environmental stresses, with a focus on aneuploidy changes. This is stated in the manuscript.

Starting at line 93 (line 79 in the new version):

**“In the present study we aimed to address these questions using a reproducible in vitro evolutionary model to study aneuploidy modulations and karyotype evolution in the context of adaptation to sudden environmental stresses, invoked here by the direct exposure to high concentrations of 2 drugs, trivalent antimonial (Sb<sup>III</sup>) or miltefosine (further called ‘flash selection’).”**

In addition, for miltefosine, the concentrations used in our flash selection are lower than the concentrations found in the blood of treated patients or inside macrophages. Thus, for miltefosine, parasites are likely to be exposed to similar or even higher concentrations than those used in our study. We now highlight this in the discussion of the manuscript:

Starting at line 291 (line 290 in new version):

**“This abrupt change in environments is also a characteristic of drug treatment. In the case of antimonials, measures made in patients treated for visceral leishmaniasis estimate a peak of 10 mg/L or ~82 µM of Sb in the blood after only 2 hours post drug administration (26). For miltefosine, blood concentrations can be as high as 70 µg/ml, or 172 µM after 72h (27). Moreover, bone marrow-derived macrophages exposed to 10 µM of miltefosine in vitro display intracellular concentrations of the drug as high as 323 µM after 72h (28). This illustrates that *Leishmania* parasites are directly exposed to sharp increases in drug concentrations – in the case of miltefosine, even higher than the concentrations used in this study – in patients upon drug administration.”**

With respect to the importance of sand flies or animals in the environmental pressure, (i) animals play a negligible role given that transmission of *L. donovani* in the ISC is anthroponotic, without

animal reservoir and (ii) the sand fly hosts the parasite for a short period of time (max 10 days), during which the parasite is not exposed to drugs.

2. In Fig. S2 MRPA in *SePOP1* is a signature of extrachromosomal amplification. Was that studied?

R: We previously showed that amplification of MRPA in *L. donovani* encountered in the Indian sub-continent was intrachromosomal (Imamura et al., 2016); further amplification of that specific gene could occur by intrachromosomal expansion/contraction or indeed by episomal amplification. However, one of the core messages of present paper is that increased copy of chr23 automatically leads to increased dosage of the intra-chromosomal MRPA amplicon. We adapted the text in order to acknowledge the possibility of episomal amplification:

Starting at line 140 (line 127 in the new version):

**“The BPK282 strain already contains a natural intra-chromosomal amplification of the MRPA gene that may bring a pre-adaptation to Sb<sup>III</sup> (14), and the locus might be subject to further intrachromosomal expansion or, contraction, or episomal amplification.”**

3. For Chromosome 31 in the *Sb* screen, it would appear that the proximal (left) part is of lower copy number than the distal (right) portion of the chromosome. How could this have happened? Deletion of a portion of chromosome 31 for one allele? This has been described before (Mukherjee et al., 2013) in *SbIII* resistant lines as one telomeric end of Chr. 31 encodes AQP1, the route of entry of *SbIII*.

R: The figures 1A and 2F and 3A do not indicate the copy number of intra-chromosomal segments as they reflect a single numeric value representing the sum of each chromosome at different time points (the x axis of the graphs). Thus, there is no information on differences between distal or proximal copy numbers inside a chromosome in those figures. The only figure showing read depth along the chromosome is fig S2B and corresponds to chr23 and not 31. It is indeed possible that there are telomeric deletions affecting AQP1 but this was not the scope of our study, since we were interested in understanding the reasons and possible drivers of increased gene dosage of chr31.

Reviewer #1 (Significance (Required)):

*The plasticity of the Leishmania genome is fascinating. It is remarkable that these parasites can tolerate so many and frequent changes in ploidy. Either these changes are stochastic and serendipitous or as conveyed by the authors are part of the parasite arsenal to respond to a changing*

*environment. They cleverly used single cell sequencing and bar-coded parasites in this well designed and well conducted study to assess the role of ploidy in parasite biology.*

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*Reviewer #2 (Evidence, reproducibility and clarity (Required)):*

*Negreira et al. present a study that aims to understand the early evolution of aneuploidy. They use Leishmania, a protozoon parasite known for its genome plasticity, as model, and two drugs as stress inducers. In this work, they use single-cell genomics and lineage tracing to detect changes in chromosome copy numbers. They conclude that, although parasites tend to have genomes with unusual plasticity, aneuploidy dynamics depend on the stressor more than the organism.*

*Further experiments:*

*1. Lines 121-124: I believe the authors should corroborate the statement that expansion of lineages that were fitter prior to drug exposure is stochastically by doing a statistical test comparing their obtained data and randomly generated simulated values. Given that there is still a considerable proportion of lineages with higher fitness and found in more than one passage, I believe this experiment/test would add strength to the conclusion.*

R: We believe that the stochasticity per se is not the relevant aspect of our results, but the fact that the expansion of different lineages in different populations is followed by the emergence of the same somy changes in a set of chromosomes (23, 27, and 31), thus showing a process of convergent evolution. Therefore, we decided to reduce the emphasis on the stochasticity itself and adapted the text to highlight this process of convergence. This was done in the following parts of the manuscript:

Starting at line 98 (line 84 in new version):

**“we revealed that changes in aneuploidy under Sb<sup>III</sup> pressure have a polyclonal origin, arising from the reproducible survival of a specific subset of lineages, which further expand stochastically differentially between independent replicates but converge to similar aneuploidy modifications”.**

Starting at line 220 (line 205 in new version):

**“Moreover, most of the positively affected lineages were enriched in only one of the SePOPs (Fig. 2C and fig. S3B) (figure 2C and supplementary figure S3B), suggesting that.**



**Altogether, these data indicate that (i) a subset of lineages was fitter to Sb<sup>III</sup> prior the drug exposure and (ii) ~~their~~ the further expansion of these surviving lineages was stochastically driven. ~~divergent between independent replicates.~~**”

Starting at line 344 (line 366 in the new version):

**“From 453 different traceable lineages, 303 consistently disappeared during Sb<sup>III</sup> exposure and 60 showed an increased frequency in at least one replicate. Most of these positively affected lineages were enriched in only one of the SePOP replicates, suggesting (i) higher tolerance to Sb<sup>III</sup> in a subset of lineages that reproducibly survived the flash selection and (ii) further expansion of these surviving lineages being stochastically driven. , including lineages which were dominating the population at the onset of the experiment (figure 2F), thus indicating that these lineages had a fitness disadvantage to Sb<sup>III</sup> compared to the other lineages. Among the surviving lineages, 60 could further expand in at least one of the SePOPs, leading to different clonal compositions in each population. Interestingly, changes in clonal composition in each SePOP coincide with the moments where changes in aneuploidy are observed in these populations, suggesting that these aneuploidy changes are due to the emergence of subsets of fitter lineages. Moreover, the observation that the same set of 3 chromosomes displayed dosage increases in all SePOP despite the fact that different lineages dominated each SePOP points to a process of convergent evolution, which further supports the notion of these chromosomes being under positive selection.”**

*Minor issues:*

2. Fig. 1B: Add label to top horizontal axis, showing frequency of each karyotype.

R: A label stating ‘Number of Cells’ was added at figure 1B.

3. Lines 92-96: Could the authors postulate how and why pre-existing aneuploid cells seem to be selected upon Sb<sup>III</sup> exposure?

R: We believe that some aneuploidy changes, like the dosage increase of chromosome 23 (from 3 to 4 copies) offer an adaptive advantage to the cells bearing it by over-expressing genes related to Sb<sup>III</sup> tolerance. This was discussed in the manuscript.

starting at line 304 (314 in the new text):

**“Chromosome 23 bears the MRPA genes which encode an ABC-thiol transporter involved in the sequestration of Sb-thiol conjugates into intracellular vesicles (28). Amplification of**

MRPA genes through extra-or intra-chromosomal amplification is a well-known driver of experimental Sb<sup>III</sup> resistance. The line here used (BPK282) is remarkably pre-adapted to Sb<sup>III</sup> (18) – like other strains of the Gangetic plain – thanks to a pre-existing intra-chromosomal amplification of MRPA genes encountered in 200 sequenced *L. donovani* isolates of that region (13). The recurrent dosage increase of chromosome 23 observed here under Sb<sup>III</sup> pressure is a rapid way to further amplify the MRPA gene and this mechanism was likely selected instead of further amplifying MRPA genes intra-chromosomally.”

4. Fig. 3: Are panels B and C swapped in the figure or the reference swapped in the text? Fig. 3C seems to refer to the mutation (lines 173-179), whereas Fig. 3B seems to relate to the surviving lineages (lines 183-186).

R: Indeed, figures 3B and 3C were erroneously positioned in the panel. This is now fixed in the new version.

5. Lines 94-97: Could the authors comment on the advantages and disadvantages of such an aggressive selection method? I am not surprised with such a drastic decrease in lineage diversity in this context.

R: We now added a section at the beginning of the discussion commenting this:

starting at line 291 (line 281 in the new version):

**“Historically, adaptation in *Leishmania* was mainly addressed using a ‘gentle’ stepwise approach where parasite populations are exposed to progressively increasing drug concentrations in vitro over the course of months, allowing these populations to adapt to each concentration before proceeding to the next increment (19, 23-25). This approach is useful to reveal mechanisms promoting full resistance against that drug which emerge at the later time points where drug concentration is high, but it precludes the evaluation of mechanisms allowing parasites to cope with sudden and strong environmental changes as initial concentrations are often too permissive. Importantly, in nature, changes in environmental pressures are often abrupt rather than gradual, and therefore, demand for mechanisms which allow parasite populations to quickly adapt to the new environment.”**

And then on line 300 in the new version:

**“In the present study, we investigated the mechanisms governing the early adaptation of *Leishmania* promastigote populations to a direct exposure to high concentrations of two drugs – Sb<sup>III</sup> and miltefosine – as models of sudden environmental stresses.”**

6. Could the authors elaborate on what is different in chromosome 31 that makes it so prone to change?

# Full Revision

R: We improved our discussion about the potential drivers of dosage increases for the other 2 chromosomes (chr 27 and chr 31) which, apart from chr23, are also consistently amplified under SbIII exposure.

Starting at line 320 (line 331 in the new version):

**“Regarding the other 2 chromosomes, chromosome 31 also bears a gene involved in antimony resistance, the sodium stibogluconate resistance protein gene (LdBPK\_310951.1). Interestingly, the ortholog of this gene displayed an increased copy number in *L. braziliensis* promastigotes experimentally selected for antimony resistance in vitro compared to non-selected lines (31). Moreover, this same study found a 50 kb intrachromosomal amplification affecting 23 genes (out of a total of 31 amplified genes) in chromosome 27 in the Sb<sup>III</sup> resistant line, with many of these genes displaying a copy number more than 10 times higher compared to the Sb<sup>III</sup> sensitive line (31). Among these genes, a WW domain/Zinc finger C-x8-C-x5-C-x3-H type - protein gene (LdBPK\_270130.1 ortholog in *L. donovani*) was also the gene with the most upregulated expression compared to the SbIII-sensitive line. Importantly, CCCH type zinc finger proteins are known targets of antimony (32), and therefore, a higher expression of this gene might mitigate its inactivation by the drug.”**

And for chromosome 31, we also discussed further its potential role in general response against drug-induced stresses.

Starting at line 360 (line 394 in new version):

**“At 100  $\mu$ M, aneuploidy changes were specific to each of the 4 MePOP replicates, with the exception of chromosome 31 that consistently showed a higher copy number than the control. The fact that an increase in copy number of chromosome 31 was observed under strong Sb<sup>III</sup> and miltefosine pressure, as well as under pressure of other drugs (24) might indicate that the dosage increase in this chromosome has also a general role against multiple types of stresses. Noteworthy, there are several ABC transporters in that chromosome (ABCC4-7 and ABCD3) which could play a role in drug efflux (36). Moreover, ontology analysis of chromosome 31 in *L. braziliensis* have demonstrated an enrichment of genes involved in iron metabolism which could play a role in general adaptation to oxidative stresses (37), but empirical evidence is still lacking.”**

Reviewer #2 (Significance (Required)):

*Aneuploidy can be well-tolerated, beneficial, or deleterious. Particularly, they can confer resistance against environment stresses, including drug pressures. This study aims to understand how aneuploidy arises. The authors approach this question using a model organism, Leishmania donovani, and two distinct drugs as environmental stressors. Using single-cell DNA sequencing and lineage tracing, the authors find that the appearance of aneuploidy is dependent on the drug used, which makes it dependent on the environmental stressor, rather than pre-determined. Importantly, they present a new barcoding method that may be useful to the field of experimental genome evolution.*

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*Reviewer #3 (Evidence, reproducibility and clarity (Required)):*

*This interesting, well written paper uses cutting edge technologies to address the evolutionary dynamics of changes in Leishmania donovani genomes in response to high drug pressure. Using single-cell genome sequencing and lineage tracing with a newly adapted cell barcoding system, the authors were able to follow aneuploid changes and lineage selection following exposures to high concentrations of either antimony or miltefosine. The main conclusions drawn from the careful bioinformatic analyses and methodic representation of 864 single cell genomes and 453 different traceable lineages were that for each drug exposure there was polyclonal selection of pre-adapted parasites complemented by de novo adaptations. Consistent changes in aneuploidy were associated with the populations selected by antimony, while miltefosine selected for populations that had a point mutation in a miltefosine transporter gene. These conclusions are well supported by the data.*

*Reviewer #3 (Significance (Required)):*

*3 comments, 3 responses*

*Comment 1*

*One general comment is that the contribution of pre-adapted lineages to the emergence of drug resistant populations under conditions of natural exposure is apt to be overstated from the current analysis. As the authors discuss, the L. donovani line used is already pre-adapted to resist antimony due, at least in part, to the amplification of the MRPA gene on chromosome 23. So it is expected that lineages adapted to strong antimony pressure will pre-exist in this line. It seems possible that the de novo adaptations that were observed, involving further copy number amplification of chromosome 23 and other chromosomes (e.g., chr 31), might be facilitated by*

*their pre-existing aneuploides. Thus, the evolutionary dynamics observed might be very particular to these sorts of pre-conditioned cells.*

R: Although BPK282 is indeed pre-adapted to antimony due to an amplification of the MRPA locus, this strain is a clone, so this intra-chromosomal amplification is shared among all cells in the population. Thus, it is probable that this intra-chromosomal amplification alone is not the only reason why some lineages are better adapted to antimony than others, but its combination with variations in aneuploidy affecting chromosome 23. We agree that de novo adaptations were likely facilitated by the presence of pre-existing aneuploidies. This was already commented in answers to comments 2 and 3 of reviewer 1.

## *Comment 2*

*It should also be discussed that the culture conditions themselves may pre-condition the parasites for antimony resistance (and possibly other drugs). Continuous passage of *L. donovani* in axenic culture produced consistent patterns of aneuploid changes, including amplification of Chr 23 (Barja et al., Nat Ecol evol, 2017). Thus a potential caveat of the use of cultured promastigotes is that their culture adaptations might involve genes on the same chromosomes that confer drug resistance.*

R: Indeed, and we are aware of the work of Barja et al 2017. However, the flash selection models characterize a competition assay between (sub)clonal lineages which are exactly in the same environment (lineages within each SePOP were in the same culture flasks). Thus, although culture adaptation might indeed lead to pre-conditioning against SbIII due to amplification of chr23, this pre-conditioning should affect the entire population and does not explain the differences in susceptibility to SbIII between the lineages within each SePOP. Moreover, the controls (maintenance in the same culture medium but without drug pressure) did not show any change in their aneuploidy, while SePOP showed an increase in some of several chromosomes, including chromosome 23 (see fig.1A).

## *Comment 3*

*For the miltefosine selection, of the 7 lineages surviving in at least one of the MePOP replicates, only lineage 302 is represented more than once. What is the evidence that the adaptive mutations in the other 6 lineages were pre-existing and did not arise de novo?*

R: We agree that evidence for pre-existing mutations is only present for lineage 302 and changed that in the text.

At line 29 (line 22 in the new version):

“In the case of miltefosine, early parasite adaptation was associated with independent **pre-existing** point mutations in a miltefosine transporter gene.”

*Figs 3b and 3c are incorrectly referenced in the text.*

R: Fixed in new version.

*Discussion p. 8 - "Interestingly, the Gly160Asp mutation also correlated with the frequency of a specific lineage (lineage 27) and appeared in 3 of the 4 MePOPs, indicating that this was a pre-existing mutation found in that lineage." Lineage 302 would appear to be the correct lineage, not 27. Please clarify.*

R: Indeed, the correct is lineage 302. This has now been fixed in the new version.

**Additional modifications in the manuscript:**

1) The mutation in the LdMT gene affecting the codon of amino acid 1016 was described as a Glutamate to stop codon mutation (Glu1016Stop), while in fact the original amino acid is a Serine (Ser1016Stop). This was corrected in the new version.

## References

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Dear Dr. Domagalska,

Thank you for the submission of your revised manuscript for consideration by EMBO reports. The first version of your manuscript was previously reviewed by three referees for Review Commons, and their detailed reports have been transferred to us along with your revised manuscript. We have now received the full set of comments of the three referees who re-evaluated your work (included below). As you will see, they are all satisfied with the revision, they explain that their previous concerns have been adequately addressed, and they now support publication of your work in EMBO reports.

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Referee #3:

Negreira et al. have provided a revised version of a manuscript that was evaluated by 3 reviewers for 'Review Commons'. I was reviewer #1. The initial comments of the three reviewers were positive and this revised version has improved. No new experiments were requested; thus it is mostly about either improving the style or interpretation or improved linkages with the literature. Overall, I think that the manuscript has improved and the authors responded adequately to the comments. It is really state of the art in terms of technology. I am less convinced than the authors (and some of the other reviewers) about the biological importance of these findings but yet I feel that it is important to report, so the fascinating field of aneuploidy in *Leishmania* can further advance.

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Rev\_Com\_number: RC-2022-01811

New\_manu\_number: EMBOR-2023-57413V1

Corr\_author: Domagalska

Title: The role of aneuploidy and polyclonality in the adaptation of the Protozoan parasite *Leishmania* to high drug pressure

The authors have addressed all minor editorial requests.

Dr. Malgorzata Domagalska  
Institute of Tropical Medicine, Antwerp  
Belgium

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For <b>phase II and III randomized controlled trials</b> , please refer to the <b>CONSORT</b> flow diagram (see link list at top right) and submit the <b>CONSORT</b> checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

#### Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have <b>primary datasets</b> been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were <b>human clinical and genomic datasets</b> deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are <b>computational models</b> that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective <b>data citations</b> in the reference list.	Not Applicable	