nature portfolio

Peer Review File

Asexuality shapes traits in a hybrid fish

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Review of Lafond et al.

Thanks for the opportunity to review this manuscript. I found the general topic to be very interesting and the use of geometric morphometrics novel and fresh.
The manuscript contends that uniquely in Chrosomus fishes two types of triploids exist that differ only in the reproductive mode, namely either asexuality or sexuality (and the asexuals are sperm dependent). This system is amazingly interesting and highly unusual.
The underlying study is comparing triploids of both types from the field, and some from common garden conditions. The two types collected from the field differ in body shape, but the ones raised under common garden conditions do not. The authors argue that the two types of triploids live in the same environment in nature, but that is actually quite hard to measure, and I do not find the argument convincing. Maybe the fishes seek out slightly different microhabitats. Furthermore, the argument is significantly weakened because under common garden conditions in the lab, the two types converge on the same phenotype (Fig. 2). The sample size for the common garden fishes is small and unbalanced, making the findings harder to understand. Maybe a larger scale common garden experiment would make the data more conclusive. In Figure 2 there is some differentiation between the two types, but the sample size may just be too small. Other sample sizes are sufficient, and I think the stats in general are fine.

The discussion is quite speculative and is not well connected to the introduction.

Throughout, there are small issues with the English, but nothing that could not be fixed by careful copy editing. This of course should not factor into my recommendation.

Reviewer #2:
Remarks to the Author:
Referee Opinion for Manuscript: "Asexuality shapes animal traits"
Title and Authors:

- Title: Asexuality shapes animal traits
- Authors: Joëlle Lafond, Christelle Leung, Bernard Angers

I had the pleasure of reading many previous articles by these authors about their fascinating fish hybrid system. Indeed, the Chrosomus eos-neogaeus system is a very interesting model for understanding the correlates and causes of the switch from sexual to asexual reproduction. This manuscript investigates the morphological differences between “ sexually and asexually” reproducing all-female triploid hybrid fish using geometric morphometric analyses (I will explain below, why I am using “”). The authors demonstrate that reproductive strategies are associated with

distinct shape differences in the fish, which could impact their ecological niches and fitness. This is indeed an important and innovative finding.

General Comments: The study addresses a significant question in evolutionary biology regarding the impact of reproductive strategies on animal morphology. This question has rarely been addressed before and is therefore innovative. However, there are several points and clarifications that need to be addressed to improve the manuscript's clarity and scientific rigor and I see a potential problem in how the data are interpreted, which may also require some change.

I would first like to emphasize the Strong Points of the submitted manuscript:
• Genotype-phenotype connection: I would like to applaud the authors on performing a high-quality analysis of phenotypic traits in connection to genetic analyses. This complex approach is rarely seen but very welcome.

• Innovative hypothesis: as already stated, the tested hypothesis is interesting and innovative.

• Diverse Lineages and Environments: Authors used two lineages from two distinct environments for each compared form, providing a comprehensive dataset, which neatly excludes the possibility that observed differences between, what they call sexual and asexual, strains are merely due to lineage-specific effect. The study design is therefore excellent in this respect.

• Selective Mortality Exclusion: They neatly excluded the effect of selective mortality, enhancing the validity of their results.

• Robust Methods: I haven’t noticed any problems in technical usage of methods and statistical data evaluation.

However, there are also some drawbacks connected to this study and the way how the results are interpreted and put to the context of existing literature:

The manuscript is sometimes a bit difficult to navigate due to oddly separated chapters. For instance, the text starts with “Main” (Main what?) rather than “Introduction” (line 54). The Results chapter is not clearly distinguished from the Introduction and at places, the interpretation within this Results section appears more like a discussion and may be shifted there.

In particular, I found the distinction between “sex” and “asexuality” overly simplified and not clearly described. Indeed, there are various ways, how to look at these reproductive categories and opinions differ. But if one wishes to use these as alternatives, it is necessary to provide clear definition how these are understood for the purpose of present paper. For instance, without such a definition, the first paragraph is hard to understand. E.g., what is meant by reappearance of asexuality, in what terms is it “more flexible”, etc?

What is more important, I had the impression that the dichotomy between sexual and asexual reproductive modes is too much enforced throughout the Introduction. Interestingly, it is

sometimes invoking in the text, that asexuality is due to mitotic gametogenesis, which is not true, especially in vertebrates, where it has been shown in various fish, amphibian and reptilian taxa that asexuality is mostly achieved by changes to oogonial formation before meiosis, which otherwise proceeds normally (lines 55-65). I might have misunderstood the text properly here. However, theories have even been proposed (Moritz et al. 1989 evol.ecol. of unisexual vertebrates; Janko et al. 2018 Mol.Ecol; Stock et al. 2021 Phil.Trans.Royal.Soc, Carman et al. 1997) that sex and asexuality, at least in hybrids, in fact form a continuum which is embedded within the speciation process itself. If so, it may rely on accumulation of subtle incompatibilities leading to changes in specific points of gametogenic process, while maintaining the rest of meiotic machinery relatively untouched.

I had the impression that the system studied should be described in greater detail. It is particularly important that the mechanisms of gamete formation in 2n and both forms of 3n hybrids are made clearer. Why some triploids are called meiotic and others ameiotic. Is there an information available that they really don't employ meiosis? What methods have been used to demonstrate this? If existence of meiosis may not be excluded, I would suggest using different naming here. This is important to explain since both types are described as involving exclusion of haplome prior to meiosis (lines 101-105, within the figure 1 caption).

Hypothesis Complexity: The hypothesis that the production of reduced vs. unreduced gametes impacts their size and hence altered investment into reproductive traits is interesting and worth of testing. However, as presented here, it appears to me as a bit oversimplified, and I would suggest to include more detailed discussion. It is the case that some asexual hybrids, like gynogenetic fish and parthenogenetic lizards undergo several types of gametogenesis simultaneously, while only one type of their oocytes is able to overcome the meiotic checkpoints. E.g. Alves et al. 2001 Genetica, Dedukh et al. 2021 Int.J.Mol.Sci, Arakelyan et al. 2023 Curr.Zool., Marta et al. 2023 eLife, showed that majority of gonial cells in various asexual hybrids fail to progress through meiosis, which may impacting may phenotypic traits significantly (lines 271-277).

In these cases, great proportion of oogonia actually fails to progress through meiosis while only small portion actually undergoes the successful endoduplication (in case of clonality) or genome exclusion in case of meiotic hybridogenesis. This massive failure, which clearly differs among lineages and forms may impact on overall phenotype too, and is not necessarily connected to the sex-asex dichotomy.

Hybrid State Consideration: The study does not fully consider the hybrid state of the fish studied as it did not include both parental species into analyses. This was not a major problem in comparing both forms of triploids, which indeed seem to combine similar genomes in same dosages and the comparison is therefore justified. However, combining triploids with diploid hybrids and only one parent (C. eos) without including the other parental species limits the ability to differentiate the impact of hybrid admixture, additivity, and transgressivity (lines 227-230).
Data Interpretation and Epigenetic Factors studied previously:
While the authors neatly show that distinct reproductive strategies employed by triploids may be manifested at morphological differences, which is a very interesting discovery, they do not

elaborate on their recent findings published in Molecular Ecology, that the switch from one reproductive mode to another in these triploids depends on epigenetic imprinting. Given they proposed in that paper that such epigenetic differences depend on maternal origin, couldn't this partially explain the observed differences also in phenotype (lines 271-277)?

The authors state: "Taken together, these results indicate that morphological differences are the consequence of altered gametogenesis in the asex hybrids.' I would counter-argue that observations may also be interpreted as if these differences show that morphological and gametogenetic differences just covary. This may mean that either one may cause the other, as suggested by the author here, or that they both reflect some more fundamental differences, like in epigenetic landscape, as suggested by authors' previous publication in Molecular Ecology. Surprisingly, this is not mentioned here.

Conclusion: In summary, the manuscript presents a novel hypothesis relevant to very active research field (evolution of reproductive modes) and hence provides valuable insights into the morphological consequences of reproductive strategies. However, several areas need improvement. The text should be more clearly structured, with a more comprehensive debate on the continuum between sex and asexuality. Additionally, ideally, both parental species should be used for comparison. If this is not possible, the overall comparison between sexual and asexual strains should be toned down or better explained (this does not concern the main part of the MS devoted to differences among triploids).

Overall Recommendation:
• I consider the present MS as an interesting advance in our understanding the evolution of non-sexual reproductive modes and recommend its publication after Major Revision.

I do not require anonymity,
Sincerely, Karel Janko (janko@iapg.cas.cz)

Reviewer #3:
Remarks to the Author:
The study entitled "Asexuality shapes animal traits" uses a geometric morphometric approach to test whether the reproductive strategy influences morphology in two groups of all-female hybrid fish. The authors hypothesized that body shape would differ significantly between sexual and asexual hybrids. The results of the study support this theory. Eye position and the top margin of the opercle are located more dorsally while the pectoral fin and the lower margin of the opercle are located more ventrally in asexual individuals than in sexual individuals. Due to these shape differences, particularly in the opercle, the authors conclude that sexual and asexual females might occupy alternative ecological niches which could reduce competition between both groups of hybrids.

In my opinion, the manuscript is well-written and deals with an intriguing phenomenon that could

be of great interest to a wide variety of readers: the possible influence of reproductive strategy on overall body morphology. However, there are a few issues that I believe need to be addressed before the manuscript can be considered for publication. These primarily refer to the figures, the geometric morphometric analysis and the conclusions the authors draw from the results.

Major Comments

• Lines 32 - 37: This may be a minor comment, but I find the first introductory sentences of the summary to be quite general and uninformative. As a result, the reader might have a difficult time following the transition to your study. Thus, I think it would be beneficial for the reader if the summary followed the path of the Introduction section more closely, by briefly explaining sexuality, asexuality, and the paradox of sex.

• Figure 2: Although I think that this figure is generally well-designed, I believe there is still room for improvement. For instance, I would recommend replacing the illustration a with one of the photographs you used for your GM approach (for example, the ones in Fig. 3). This would help the reader to follow your landmark placement. In addition, I think the PCA plots b and c would benefit from visualizing shape differences by placing convex hulls around each group's morphospace and by including pictograms of extreme shapes along both axes.

• Line 133: The amount of variance in Principal Component 1 seems unusually low to me. I would generally expect the variance of PC1 to be above 50%. Do you have an explanation for this little information content of PC1? In case you do, please include this explanation in the results section.

• Line 134 - 142: Please provide detailed descriptions of the 9 landmark locations you selected, either here, or in the methods section of your manuscript. Without any descriptions, it is difficult to evaluate if your landmark selection is solid.

• Lines 191 – 193: As mentioned in the comment above, I am not able to fully assess your landmark selection since detailed descriptions of the positions are lacking. However, from the brief descriptions of 4 landmarks here and the locations of the dots in figure 2, I assume that you mainly used type II and III landmarks instead of using type I landmarks (see Curran, 2018 for a detailed definition) for your GM approach. For instance, the center of the eye is not a reliable and repeatable landmark, since it can be very challenging to hit the center precisely. I would therefore suggest to carefully review the landmark selection and to replace certain landmarks in case they fall into type III.

• Line 280: You claim here that asexually reproducing females have a larger operculum than sexually reproducing females. Although I agree that this is likely, I do not think that your data justifies this statement, since you only quantified the shape but not the size of the operculum. However, this issue could be easily addressed by conducting linear measurements of the operculum based on the photographs you have used for your GM approach. Additionally, you mention that operculum shape is "known to be ecologically relevant in trophic and habitat specialization of fishes". Although this is definitely the case, you only refer to a difference in size instead of a difference in shape here. I would recommend elaborating on the identified shape

differences here and what they may indicate in an ecological context.

• Lines 294 – 296: In my opinion, the identified difference in operculum size alone is insufficient to justify the statement that “asexually reproducing hybrids may actually have a lesser impact on sexual females than previously thought, by occupying a somewhat different ecological niche than the sexual species”. I would recommend including other morphological features to bolster this conclusion. This might even be feasible without conducting any additional measurements. For example, you also identified a significant difference in eye position and pectoral fin position in your analyses. Both traits have been shown to be ecologically relevant in fishes as well. Thus, I would suggest incorporating these results in your discussion to support the conclusion of alternative ecological niches in asexual and sexual females.

Minor comments

• Line 55: From my point of view, it could be confusing for the reader to use “sex” as an abbreviation for sexuality. Thus, I would recommend to either use a different abbreviation or to not abbreviate it at all. Especially since you do not abbreviate it in some cases anyway like in line 67, for instance.

• Line 86: Please delete the word “from” here.

• Line 134: I would recommend replacing “Landmarks placement” with “Locations of landmarks” or similar.

• Figures 2-3: I think it would be beneficial for visualization, if the landmarks were in the same color and size in both figures.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Thanks for the opportunity to review this manuscript. I found the general topic to be very interesting and the use of geometric morphometrics novel and fresh.

The manuscript contends that uniquely in Chrosomus fishes two types of triploids exist that differ only in the reproductive mode, namely either asexuality or sexuality (and the asexuals are sperm dependent). This system is amazingly interesting and highly unusual.

The underlying study is comparing triploids of both types from the field, and some from common garden conditions. The two types collected from the field differ in body shape, but the ones raised under common garden conditions do not. The authors argue that the two types of triploids live in the same environment in nature, but that is actually quite hard to measure, and I do not find the argument convincing. Maybe the fishes seek out slightly different microhabitats. Furthermore, the argument is significantly weakened because under common garden conditions in the lab, the two types converge on the same phenotype (Fig. 2). The sample size for the common garden fishes is small and unbalanced, making the findings harder to understand. Maybe a larger scale common garden experiment would make the data more conclusive. In Figure 2 there is some differentiation between the two types, but the sample size may just be too small. Other sample sizes are sufficient, and I think the stats in general are fine.

The discussion is quite speculative and is not well connected to the introduction.

Throughout, there are small issues with the English, but nothing that could not be fixed by careful copy editing. This of course should not factor into my recommendation.

RESPONSE FROM THE AUTHORS TO REVIEWER 1

We thank Reviewer #1 for the relevant suggestions that helped to improve the manuscript. Responses from the authors are in blue.

1. Fish environments might actually differ slightly in nature (Introduction).
   We do agree with Reviewer #1 that in their natural environment, the two types of triploids may use different ecological niches. We thus modify the related sentence to specify that they rather share the same reproduction niche (page 5-6, line 122-124):

   "They reproduce at the same time and location[30], and spermatozoids from males of a close species (often *C. eos*) are required for either cytosolic components (asexual triploids) or as additional genetic material (sexual triploids)."

2. Under common garden conditions in the lab, the two types converge on the same phenotype (Results, Fig.2).
   We think that there is a misunderstanding of the result here, as two types of triploids (according to their reproductive modes) are actually morphologically different, which shows in both the clustering and RDA analyses. There are, however, two lineages used, which displayed the same morphological shape in common garden, so maybe there was some confusion regarding that point.

3. Common garden fish sample size is too low and unbalanced to support results (Material and Methods).
   Unfortunately, it was not possible to assess individual reproductive mode, ploidy and lineage at the larval stage without sacrificing the individuals, so we started the common garden experiment with a total of 62 individuals, originated from two distinct geographical locations, a relatively high number of individuals, considering the logistic constraints. Individual biotypes were assessed only at the end of the experiment and resulted in 13 asexual and 8 sexual triploids, and 27 sexual and 14 asexual diploids. While we acknowledge the smaller sample size compared to the triploids from natural environments, the results were very conclusive for both the clustering and RDA for the comparison of the different mode of reproduction. While a new common garden experiment would definitely strengthen this result, it is unfortunately not something doable in a short period of time.

4. Discussion should be less speculative and more connected to the introduction (Discussion).
   The discussion was modified to reflect this comment, as well as some comments/suggestions of the following reviewer, Dr. Janko.

5. English revision is needed.
   English underwent revision with professional corrector software for improved readability and elimination of grammatical errors.

Reviewer #2 (Remarks to the Author):

Referee Opinion for Manuscript: "Asexuality shapes animal traits"

Title and Authors:

- Title: Asexuality shapes animal traits
- Authors: Joëlle Lafond, Christelle Leung, Bernard Angers

I had the pleasure of reading many previous articles by these authors about their fascinating fish hybrid system. Indeed, the Chrosomus eos-neogaeus system is a very interesting model for understanding the correlates and causes of the switch from sexual to asexual reproduction. This manuscript investigates the morphological differences between "sexually and asexually" reproducing all-female triploid hybrid fish using geometric morphometric analyses (I will explain below, why I am using ""). The authors demonstrate that reproductive strategies are associated with distinct shape differences in the fish, which could impact their ecological niches and fitness. This is indeed an important and innovative finding.

General Comments: The study addresses a significant question in evolutionary biology regarding the impact of reproductive strategies on animal morphology. This question has rarely been addressed before and is therefore innovative. However, there are several points and clarifications that need to be addressed to improve the manuscript's clarity and scientific rigor and I see a potential problem in how the data are interpreted, which may also require some change.

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- Innovative hypothesis: as already stated, the tested hypothesis is interesting and innovative.
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- Selective Mortality Exclusion: They neatly excluded the effect of selective mortality, enhancing the validity of their results.
- Robust Methods: I haven't noticed any problems in technical usage of methods and statistical data evaluation.

However, there are also some drawbacks connected to this study and the way how the results are interpreted and put to the context of existing literature:

The manuscript is sometimes a bit difficult to navigate due to oddly separated chapters. For instance, the text starts with "Main" (Main what?) rather than "Introduction" (line 54). The Results chapter is not clearly distinguished from the Introduction and at places, the interpretation within this Results section appears more like a discussion and may be shifted there.

In particular, I found the distinction between “sex” and “asexuality” overly simplified and not clearly described. Indeed, there are various ways, how to look at these reproductive categories and opinions differ. But if one wishes to use these as alternatives, it is necessary to provide clear definition how these are understood for the purpose of present paper. For instance, without such a definition, the first paragraph is hard to understand. E.g., what is meant by reappearance of asexuality, in what terms is it “more flexible”, etc?

What is more important, I had the impression that the dichotomy between sexual and asexual reproductive modes is too much enforced throughout the Introduction. Interestingly, it is sometimes invoking in the text, that asexuality is due to mitotic gametogenesis, which is not true, especially in vertebrates, where it has been shown in various fish, amphibian and reptilian taxa that asexuality is mostly achieved by changes to oogonial formation before meiosis, which otherwise proceeds normally (lines 55-65). I might have misunderstood the text properly here. However, theories have even been proposed (Moritz et al. 1989 evol.ecol. of unisexual vertebrates; Janko et al. 2018 Mol.Ecol; Stock et al. 2021 Phil.Trans.Royal.Soc, Carman et al. 1997) that sex and asexuality, at least in hybrids, in fact form a continuum which is embedded within the speciation process itself. If so, it may rely on accumulation of subtle incompatibilities leading to changes in specific points of gametogenic process, while maintaining the rest of meiotic machinery relatively untouched.

I had the impression that the system studied should be described in greater detail. It is particularly important that the mechanisms of gamete formation in 2n and both forms of 3n hybrids are made clearer. Why some triploids are called meiotic and others ameiotic. Is there an information available that they really don’t employ meiosis? What methods have been used to demonstrate this? If existence of meiosis may not be excluded, I would suggest using different naming here. This is important to explain since both types are described as involving exclusion of haplome prior to meiosis (lines 101-105, within the figure 1 caption).

Hypothesis Complexity: The hypothesis that the production of reduced vs. unreduced gametes impacts their size and hence altered investment into reproductive traits is interesting and worth of testing. However, as presented here, it appears to me as a bit oversimplified, and I would suggest to include more detailed discussion. It is the case that some asexual hybrids, like gynogenetic fish and parthenogenetic lizards undergo several types of gametogenesis simultaneously, while only one type of their oocytes is able to overcome the meiotic checkpoints. E.g. Alves et al. 2001 Genetica, Dedukh et al. 2021 Int.J.Mol.Sci, Arakelyan et al. 2023 Curr.Zool., Marta et al. 2023 eLife, showed that majority of gonial cells in various asexual hybrids fail to progress through meiosis, which may impacting may phenotypic traits significantly (lines 271-277). In these cases, great proportion of oogonia actually fails to progress through meiosis while only small portion actually undergoes the successful endoduplication (in case of clonality) or genome exclusion in case of meiotic hybridogenesis. This massive failure, which clearly differs among lineages and forms may impact on overall phenotype too, and is not necessarily connected to the sex-asex dichotomy.

Hybrid State Consideration: The study does not fully consider the hybrid state of the fish studied as it did not include both parental species into analyses. This was not a major problem in comparing both forms of triploids, which indeed seem to combine similar genomes in same dosages and the comparison is therefore justified. However, combining triploids with diploid hybrids and only one parent (*C. eos*) without including the other parental species limits the ability to differentiate the impact of hybrid admixture, additivity, and transgressivity (lines 227-230).

Data Interpretation and Epigenetic Factors studied previously:

While the authors neatly show that distinct reproductive strategies employed by triploids may be manifested at morphological differences, which is a very interesting discovery, they do not elaborate on their recent findings published in Molecular Ecology, that the switch from one reproductive mode to another in these triploids depends on epigenetic imprinting. Given they proposed in that paper that such epigenetic differences depend on maternal origin, couldn't this partially explain the observed differences also in phenotype (lines 271-277)?

The authors state: "Taken together, these results indicate that morphological differences are the consequence of altered gametogenesis in the asex hybrids.' I would counter-argue that observations may also be interpreted as if these differences show that morphological and gametogenetic differences just covary. This may mean that either one may cause the other, as suggested by the author here, or that they both reflect some more fundamental differences, like in epigenetic landscape, as suggested by authors' previous publication in Molecular Ecology. Surprisingly, this is not mentioned here.

Conclusion: In summary, the manuscript presents a novel hypothesis relevant to very active research field (evolution of reproductive modes) and hence provides valuable insights into the morphological consequences of reproductive strategies. However, several areas need improvement. The text should be more clearly structured, with a more comprehensive debate on the continuum between sex and asexuality. Additionally, ideally, both parental species should be used for comparison. If this is not possible, the overall comparison between sexual and asexual strains should be toned down or better explained (this does not concern the main part of the MS devoted to differences among triploids).

Overall Recommendation:

- I consider the present MS as an interesting advance in our understanding the evolution of non-sexual reproductive modes and recommend its publication after Major Revision.

Remarks on code availability:

I haven't found any issues and the codes were properly and clearly formatted.

I do not require anonymity,

Sincerely, Karel Janko (janko@iapg.cas.cz)

RESPONSE FROM THE AUTHORS TO REVIEWER 2 (Karel Janko)

We heartfully thank Dr. Janko for the review of this paper, having provided deeply interesting and relevant suggestions, leading to a strong improvement of the paper's quality. As such, we have applied Dr. Janko's recommendations. Here are the descriptions of each response/addition/modification:

1. The manuscript is sometimes a bit difficult to navigate due to oddly separated chapters. For instance, the text starts with "Main" (Main what?) rather than "Introduction" (line 54). The Results chapter is not clearly distinguished from the Introduction and at places, the interpretation within this Results section appears more like a discussion and may be shifted there.
   We have modified the first title by "Introduction" as it is standard for Nature Communications, and we, furthermore, added a "Results" title to separate this section from others. We then changed the actual titles to subtitles for this section.

2. In particular, I found the distinction between "sex" and "asexuality" overly simplified and not clearly described. Indeed, there are various ways, how to look at these reproductive categories and opinions differ. But if one wishes to use these as alternatives, it is necessary to provide clear definition how these are understood for the purpose of present paper. For instance, without such a definition, the first paragraph is hard to understand. E.g., what is meant by reappearance of asexuality, in what terms is it "more flexible", etc?

   We acknowledge that distinction between sexual and asexual reproduction could not be simplified through a dichotomy. In that light, we remodeled the first paragraph for a more in depth definition, and explain better the reappearance of asexuality in species, focusing specifically in hybrid organisms. Here's how it goes now (page 3, lines 54-65):

   "Sexual reproduction is characterized by the production of genetically diversified individuals, resulting from genetic shuffling that occurs during meiosis through recombination, ploidy reduction, and effective fusion of two different pronuclei[1]. This mode of reproduction has been a successful adaptation already present in the last eukaryotic common ancestor[2] and provided populations with an efficient mechanism to cope with fluctuating environments[3,4]. At the opposite, many organisms with fully sexual ancestors showed the reappearance of asexual reproduction as either a facultative or obligatory process[5]. This mode of reproduction is achieved by a wide variety of processes across taxa, ranging from mitotic divisions (as in fission, fragmentation, budding) to different modified meiotic pathways (as in parthenogenesis or gynogenesis)[6,7]. This flexibility of processes is especially obvious in many hybrid complexes, where the accumulation of subtle incompatibilities between parental haplomes can cause aberrant gametogenesis, leading to a continuum of forms between sexual and asexual organisms[5,8]."

3. What is more important, I had the impression that the dichotomy between sexual and asexual reproductive modes is too much enforced throughout the Introduction. Interestingly, it is sometimes invoking in the text, that asexuality is due to mitotic gametogenesis, which is not true, especially in vertebrates, where it has been shown in various fish, amphibian and reptilian taxa that asexuality is mostly achieved by changes to oogonial formation before meiosis, which otherwise proceeds normally (lines 55-65). I might have misunderstood the text properly here. However, theories have even been proposed (Moritz et al. 1989 evol.ecol. of unisexual vertebrates; Janko et al. 2018 Mol.Ecol; Stock et al. 2021 Phil.Trans.Royal.Soc, Carman et al. 1997) that sex and asexuality, at least in hybrids, in fact form a continuum which is embedded within the speciation process itself. If so, it may rely on accumulation of subtle incompatibilities leading to

changes in specific points of gametogenic process, while maintaining the rest of meiotic machinery relatively untouched.

We didn't intend to say that asexuality was due to mitotic gametogenesis, rather than different processes including for example mitotic gametogenesis or different modified meiotic pathways. We have rewritten this section for more clarity, as shown in the precedent comment (page 3, lines 54-65):

"Sexual reproduction is characterized by the production of genetically diversified individuals, resulting from genetic shuffling that occurs during meiosis through recombination, ploidy reduction, and effective fusion of two different pronuclei[1]. This mode of reproduction has been a successful adaptation already present in the last eukaryotic common ancestor[2] and provided populations with an efficient mechanism to cope with fluctuating environments[3,4]. At the opposite, many organisms with fully sexual ancestors showed the reappearance of asexual reproduction as either a facultative or obligatory process[5]. This mode of reproduction is achieved by a wide variety of processes across taxa, ranging from mitotic divisions (as in fission, fragmentation, budding) to different modified meiotic pathways (as in parthenogenesis or gynogenesis)[6,7]. This flexibility of processes is especially obvious in many hybrid complexes, where the accumulation of subtle incompatibilities between parental haplomes can cause aberrant gametogenesis, leading to a continuum of forms between sexual and asexual organisms[5,8]."

4. I had the impression that the system studied should be described in greater detail. It is particularly important that the mechanisms of gamete formation in 2n and both forms of 3n hybrids are made clearer. Why some triploids are called meiotic and others ameiotic. Is there an information available that they really don't employ meiosis? What methods have been used to demonstrate this? If existence of meiosis may not be excluded, I would suggest using different naming here. This is important to explain since both types are described as involving exclusion of haplome prior to meiosis (lines 101-105, within the figure 1 caption). Exact mechanisms leading to the formation of each type of gametes are still unclear, but hybrid reproduction mode was clarified using cross experiment. Briefly, the detection of *C. eos* as the progeny of triploid hybrids confirmed the occurrence of a pathway similar to meiotic hybridogenesis but only for half of the triploids. The presence of tetraploid offspring for all these females revealed the formation of unreduced triploid eggs as a probable failure of meiotic hybridogenesis. This reproductive pathway is thus expected to normally reject the *C. neogaeus* haplome somewhere in the process of producing the ova, though its failure to do so was documented. The remaining female triploids produced diploid and triploid hybrids. The triploids excluded the haplome received from paternal leakage (a specific *C. eos* haplome) and produced eggs with the diploid hybrid genome through an ameiotic hybridogenesis. Both types of hybridogenesis occurred in a mutually exclusive manner.

As these processes were described in (Lafond 2019, Journal of Heredity), we add this in lines 109-111 (page 5):

"See [31] for a more extensive description of the complex and [30] for a more detailed description of the gametogeneses employed by its members."

5. Hypothesis Complexity: The hypothesis that the production of reduced vs. unreduced gametes impacts their size and hence altered investment into reproductive traits is interesting and worth of testing. However, as presented here, it appears to me as a bit oversimplified, and I would suggest to include more detailed discussion. It is the case that some asexual hybrids, like gynogenetic fish and parthenogenetic lizards undergo several types of gametogenesis simultaneously, while only one type of their oocytes is able to overcome the meiotic checkpoints. E.g. Alves et al. 2001 Genetica, Dedukh et al. 2021 Int.J.Mol.Sci, Arakelyan et al. 2023 Curr.Zool., Marta et al. 2023 eLife, showed that majority of gonial cells in various asexual hybrids fail to progress through meiosis, which may impacting may phenotypic traits significantly (lines 271-277). In these cases, great proportion of oogonia actually fails to progress through meiosis while only small portion actually undergoes the successful endoduplication (in case of clonality) or genome exclusion in case of meiotic hybridogenesis. This massive failure, which clearly differs among lineages and forms may impact on overall phenotype too, and is not necessarily connected to the sex-asex dichotomy. We thank Reviewer #2 for this hypothesis that we find interesting. We have added this hypothesis to the discussion. This part of the discussion changed to this (page 15, lines 303-314):

"In order to produce clonal eggs, modification of gametogenesis is required, often including mechanisms that allow duplication of the genome of the reproductive cells prior to meiosis (premeiotic genome endoreplication)[37]. The resulting unreduced (or partially reduced) oocytes are then larger and less abundant than those of sexually reproducing females31. This fundamental difference with sexual organisms is expected to lead to a trade-off between either less abundant, larger eggs, or more abundant, smaller eggs. The consequence of such a dichotomy in gametogenesis may lead to a domino effect, influenced by hormones and gene expression, to the observed variation in development. Furthermore, different gametogenic processes can lead to a different number of surviving ova, as observed in the hybrids of Squalius[38], Cobitis[37,39] and Darevskia[40] complexes. Different reproduction modes could thus lead to difference in the number of gametes growing to a mature stage, and thus to similar consequences as exposed before."

6. Hybrid State Consideration: The study does not fully consider the hybrid state of the fish studied as it did not include both parental species into analyses. This was not a major problem in comparing both forms of triploids, which indeed seem to combine similar genomes in same dosages and the comparison is therefore justified. However, combining triploids with diploid hybrids and only one parent (*C. eos*) without including the other parental species limits the ability to differentiate the impact of hybrid admixture, additivity, and transgressivity (lines 227-230).

Again, we acknowledge the importance of the hybrid effect on individual morphology. However, it was not possible for us to add the other parental species, due to its scarcity in our study area (Vergilino et al. (2016), BMC Evolutionary Biology), and even its absence in the two sampled sites of this current study. This would introduce an additional environmental effect on traits that are known to be highly plastic (Leung et al. (2018), PeerJ).

As the main objective of the current study was to assess the morphological differences between sexually and asexually reproducing forms, comparison of both forms of triploids are conclusive, as recognized by the reviewer. The addition of the diploid biotypes allowed to disentangle additional confounding factors (page 12, lines 248-251), but restricted to females harboring at least one *C. eos* haplome. Therefore, we toned down our conclusions, by adding a paragraph in the discussion accordingly (page 14-15, lines 293-303):

"While morphological differences between asexual and sexual individuals are expected to result from specific epigenetic profiles and gene expression, the interactions between C. eos and C. neogaeus genomes in a hybrid genomic context result in admixture, additivity, and transgressivity[33,36]. The comparison made

with diploid sexual C. eos and asexual hybrids of the same fish complex strongly supported the effect of the reproduction mode on the head morphology, as trait differences are not unique to triploid hybrids, but are found globally as an inherent shape of reproductive strategy in females harboring at least one C. eos haplome. It would be relevant to parallelize the morphological differences observed here with a system of females harboring at least one C. neogaeus haplome (C. neogaeus and C. neogaeus × eos-neogaeus triploids)."

Data Interpretation and Epigenetic Factors studied previously:

1. While the authors neatly show that distinct reproductive strategies employed by triploids may be manifested at morphological differences, which is a very interesting discovery, they do not elaborate on their recent findings published in Molecular Ecology, that the switch from one reproductive mode to another in these triploids depends on epigenetic imprinting. Given they proposed in that paper that such epigenetic differences depend on maternal origin, couldn't this partially explain the observed differences also in phenotype (lines 271-277)?
   We actually initially left that part out not to burden the reader, but adding them will certainly improve the interpretation of the results. Here's our changes, in the results & discussion (page 12, lines 240-245 & page 14, lines 285-290):

   "Maternal effects are also known to determine the emergence of either sexual or asexual triploids in this fish complex[31]. Maternal ploidy, either diploid or triploid, leads respectively to asexual and sexual triploid females. If maternal ploidy has an influence on the morphology of the progeny, diploid hybrids, which can also arise from a diploid or triploid mother[31], would be expected to split in two groups clustering with sexual and asexual triploids. Adding diploid hybrids to the study then also allows the discrimination of these effects."

   "The absence of two distinct groups of diploid hybrids also ruled out maternal effects as responsible for these morphological differences. Therefore, while maternal effects are responsible for the different reproductive forms in triploids[31], they do not seem to directly influence their respective morphology. Taken together, these results indicate that the morphological differences are correlated to the altered gametogenesis in the asexual hybrids."

2. The authors state: "Taken together, these results indicate that morphological differences are the consequence of altered gametogenesis in the asex hybrids.' I would counter-argue that observations may also be interpreted as if these differences show that morphological and gametogenetic differences just covary. This may mean that either one may cause the other, as suggested by the author here, or that they both reflect some more fundamental differences, like in epigenetic landscape, as suggested by authors' previous publication in Molecular Ecology. Surprisingly, this is not mentioned here.
   That's a very fair point. We corrected this part in the following way (page 14, lines 288-290):

   "Taken together, these results indicate that the morphological differences are correlated to the altered gametogenesis in the asexual hybrids."

3. Conclusion: In summary, the manuscript presents a novel hypothesis relevant to very active research field (evolution of reproductive modes) and hence provides valuable insights into the morphological consequences of reproductive strategies. However, several areas need improvement. The text should be more clearly structured, with a more comprehensive debate on the continuum between sex and asexuality.

Additionally, ideally, both parental species should be used for comparison. If this is not possible, the overall comparison between sexual and asexual strains should be toned down or better explained (this does not concern the main part of the MS devoted to differences among triploids).
All of these points were assessed in the precedent question.

Reviewer #3 (Remarks to the Author):

The study entitled “Asexuality shapes animal traits” uses a geometric morphometric approach to test whether the reproductive strategy influences morphology in two groups of all-female hybrid fish. The authors hypothesized that body shape would differ significantly between sexual and asexual hybrids. The results of the study support this theory. Eye position and the top margin of the opercle are located more dorsally while the pectoral fin and the lower margin of the opercle are located more ventrally in asexual individuals than in sexual individuals. Due to these shape differences, particularly in the opercle, the authors conclude that sexual and asexual females might occupy alternative ecological niches which could reduce competition between both groups of hybrids.

In my opinion, the manuscript is well-written and deals with an intriguing phenomenon that could be of great interest to a wide variety of readers: the possible influence of reproductive strategy on overall body morphology. However, there are a few issues that I believe need to be addressed before the manuscript can be considered for publication. These primarily refer to the figures, the geometric morphometric analysis and the conclusions the authors draw from the results.

Major Comments

- Lines 32 - 37: This may be a minor comment, but I find the first introductory sentences of the summary to be quite general and uninformative. As a result, the reader might have a difficult time following the transition to your study. Thus, I think it would be beneficial for the reader if the summary followed the path of the Introduction section more closely, by briefly explaining sexuality, asexuality, and the paradox of sex.

- Figure 2: Although I think that this figure is generally well-designed, I believe there is still room for improvement. For instance, I would recommend replacing the illustration a with one of the photographs you used for your GM approach (for example, the ones in Fig. 3). This would help the reader to follow your landmark placement. In addition, I think the PCA plots b and c would benefit from visualizing shape differences by placing convex hulls around each group’s morphospace and by including pictograms of extreme shapes along both axes.

- Line 133: The amount of variance in Principal Component 1 seems unusually low to me. I would generally expect the variance of PC1 to be above 50%. Do you have an explanation for this little information content of PC1? In case you do, please include this explanation in the results section.

- Line 134 - 142: Please provide detailed descriptions of the 9 landmark locations you selected, either here, or in the methods section of your manuscript. Without any descriptions, it is difficult to evaluate if your landmark selection is solid.

- Lines 191 – 193: As mentioned in the comment above, I am not able to fully assess your landmark selection since detailed descriptions of the positions are lacking. However, from the brief descriptions of 4 landmarks here and the locations of the dots in figure 2, I assume that you mainly used type II and III landmarks instead of using type I landmarks (see Curran, 2018 for a detailed definition) for your GM approach. For instance, the center of the eye is

not a reliable and repeatable landmark, since it can be very challenging to hit the center precisely. I would therefore suggest to carefully review the landmark selection and to replace certain landmarks in case they fall into type III.

- Line 280: You claim here that asexually reproducing females have a larger operculum than sexually reproducing females. Although I agree that this is likely, I do not think that your data justifies this statement, since you only quantified the shape but not the size of the operculum. However, this issue could be easily addressed by conducting linear measurements of the operculum based on the photographs you have used for your GM approach. Additionally, you mention that operculum shape is "known to be ecologically relevant in trophic and habitat specialization of fishes". Although this is definitely the case, you only refer to a difference in size instead of a difference in shape here. I would recommend elaborating on the identified shape differences here and what they may indicate in an ecological context.

- Lines 294 – 296: In my opinion, the identified difference in operculum size alone is insufficient to justify the statement that "asexually reproducing hybrids may actually have a lesser impact on sexual females than previously thought, by occupying a somewhat different ecological niche than the sexual species". I would recommend including other morphological features to bolster this conclusion. This might even be feasible without conducting any additional measurements. For example, you also identified a significant difference in eye position and pectoral fin position in your analyses. Both traits have been shown to be ecologically relevant in fishes as well. Thus, I would suggest incorporating these results in your discussion to support the conclusion of alternative ecological niches in asexual and sexual females.

Minor comments

- Line 55: From my point of view, it could be confusing for the reader to use "sex" as an abbreviation for sexuality. Thus, I would recommend to either use a different abbreviation or to not abbreviate it at all. Especially since you do not abbreviate it in some cases anyway like in line 67, for instance.

- Line 86: Please delete the word "from" here.

- Line 134: I would recommend replacing "Landmarks placement" with "Locations of landmarks" or similar.

- Figures 2-3: I think it would be beneficial for visualization, if the landmarks were in the same color and size in both figures.

RESPONSE FROM THE AUTHORS TO REVIEWER 3

We thank the reviewer for their time and relevant suggestions! Below are the point-by-point responses:

Major Comments

1. Lines 32 - 37: This may be a minor comment, but I find the first introductory sentences of the summary to be quite general and uninformative. As a result, the reader might have a difficult time following the transition to your study. Thus, I think it would be beneficial for the reader if the summary followed the path of the Introduction section more closely, by briefly explaining sexuality, asexuality, and the paradox of sex.
   The summary was modified according to Nature Communications' guide and is now more direct.

2. Figure 2: Although I think that this figure is generally well-designed, I believe there is still room for improvement. For instance, I would recommend replacing the illustration a with one of the photographs you used for your GM approach (for example, the ones in Fig. 3). This would help the reader to follow your landmark placement. In addition, I think the PCA plots b and c would benefit from visualizing shape differences by placing convex hulls around each group's morphospace and by including pictograms of extreme shapes along both axes.
   We changed figure 2a to a picture, and we added a figure in the supplementary material containing the convex hulls and extreme shapes on the PCA (Supplementary Figure 1). We also reference to it in Figure 2's legend (page 7, lines 155-156):

   "Convex hulls and extreme shapes of the PCA are shown in Supplementary Figure 1."

3. Line 133: The amount of variance in Principal Component 1 seems unusually low to me. I would generally expect the variance of PC1 to be above 50%. Do you have an explanation for this little information content of PC1? In case you do, please include this explanation in the results section.
   Variation contained within the axes of a PCA varies greatly depending on the number and influence of potential variables explaining such variation. If the data has many relevant variables with similar contribution, the first component (PC1) won't hold over 50% of the variance. This can be seen when comparing broken stick analyses, where sometimes the first variable explain enough variation to be considered alone (over 50% often), while in some other studies, the first 5 or more variables should be considered (where they often all explain somewhere between 10-25%) (See for instance Ueno (2020, Somatosensory & Motor Research) Figure 2, where the max PC is a bit higher than 30%, & Chand (2019, Principal Component Analysis, in Quantitative methods for social sciences) Figure 3, where the max PC is close to 60%).

   A PC1 under a threshold of 50% is not alarming - it just means that there are more than a few potential variables that explain a fair share of the variation. We do not think an explanation is necessary to add in the results section.

4. Line 134 - 142: Please provide detailed descriptions of the 9 landmark locations you selected, either here, or in the methods section of your manuscript. Without any descriptions, it is difficult to evaluate if your landmark selection is solid.
   This is a good point. The descriptions were added in the methods as follows (page 19, lines 386-392):

"Position of the landmarks is the following: 1) Eye center, identified at the intersection of the maximum dorsal and ventral diameters of the eye, 2) Top, posterior part of the Nostril 3) Intersection of the upper lip and snout, 4) Intersection of the upper and lower lips, 5) Most dorsal mouth corner, 6) Intersection of the most dorsal pelvic spin with the body, 7) Dorsal start of the operculum, 8) Vertical projection of the mouth corner on the ventral margin (described in the study as "chin"), and 9) Horizontal projection of the pectoral fin origin on the operculum (described in the study as "bottom of the operculum")."

5. Lines 191 – 193: As mentioned in the comment above, I am not able to fully assess your landmark selection since detailed descriptions of the positions are lacking. However, from the brief descriptions of 4 landmarks here and the locations of the dots in figure 2, I assume that you mainly used type II and III landmarks instead of using type I landmarks (see Curran, 2018 for a detailed definition) for your GM approach. For instance, the center of the eye is not a reliable and repeatable landmark, since it can be very challenging to hit the center precisely. I would therefore suggest to carefully review the landmark selection and to replace certain landmarks in case they fall into type III.
   The strength of these landmarks has already been assessed (Leung et al. (2018), PeerJ). Of our landmarks, only 2 are of type III (the chin & the lower operculum), which were also used to delimit the semi-landmarks. The others are either type I or II, the first one being prevalent. The center of the eye was detected by measurements (we digitally drew a "cross" by combining straight lines for the height and width of the eye, with a 90 degrees angle - the landmark was put at the intersection of those two lines), so it is expected to be reliable. Finally, we have tested the error rate due to landmark input, as described in the method, and it resulted to be very low (landmark placement brings less than 4% of the total variation), so we don't expect landmarks positioning to be an issue here.

6. Line 280: You claim here that asexually reproducing females have a larger operculum than sexually reproducing females. Although I agree that this is likely, I do not think that your data justifies this statement, since you only quantified the shape but not the size of the operculum. However, this issue could be easily addressed by conducting linear measurements of the operculum based on the photographs you have used for your GM approach. Additionally, you mention that operculum shape is "known to be ecologically relevant in trophic and habitat specialization of fishes". Although this is definitely the case, you only refer to a difference in size instead of a difference in shape here. I would recommend elaborating on the identified shape differences here and what they may indicate in an ecological context.
   Thank you for this comment. We indeed meant shape of the operculum, and not the size. We have clarified this point and add the ecological relevant of eye and pectoral fin position too (page 15, lines 315-324):

   "Morphological differences between sexual and asexual individuals mostly affected operculum shape, and eyes and pectoral fin position. Asexually reproducing females showed a proportionally larger operculum than their sexually reproducing counterparts (with the landmarks of the top and bottom of the operculum being positioned respectively more dorsally and ventrally compared to the other group), a more dorsal position of the center of the eye, and a more ventral position of the pectoral fin. Shape of the operculum is known to be ecologically relevant in trophic and habitat specialization of fishes, affecting for example their attractiveness to mates, swimming performance, niche adaptations, detection by prey, and susceptibility to predation[41-43]. Similarly, eye and pectoral fin positions could act on size/distance perception[44], and thus on diverse interactions with the environment, and swimming speed and feeding habits[41], respectively."

7. Lines 294 – 296: In my opinion, the identified difference in operculum size alone is insufficient to justify the statement that "asexually reproducing hybrids may actually have a lesser impact on sexual females than

previously thought, by occupying a somewhat different ecological niche than the sexual species". I would recommend including other morphological features to bolster this conclusion. This might even be feasible without conducting any additional measurements. For example, you also identified a significant difference in eye position and pectoral fin position in your analyses. Both traits have been shown to be ecologically relevant in fishes as well. Thus, I would suggest incorporating these results in your discussion to support the conclusion of alternative ecological niches in asexual and sexual females.
We added a small part for this, as seen in point #6.

Minor comments

1. Line 55: From my point of view, it could be confusing for the reader to use "sex" as an abbreviation for sexuality. Thus, I would recommend to either use a different abbreviation or to not abbreviate it at all. Especially since you do not abbreviate it in some cases anyway like in line 67, for instance.
   It was changed.
2. Line 86: Please delete the word "from" here.
   Done.
3. Line 134: I would recommend replacing "Landmarks placement" with "Locations of landmarks" or similar.
   Done.
4. Figures 2-3: I think it would be beneficial for visualization, if the landmarks were in the same color and size in both figures.
   We modified the figures as suggested, by homogenizing them and removing landmarks points where they were not necessary (Figure 3).

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have put together a very careful and considerate reply to the reviews. The manuscript reads well and the underlying study is highly interesting, as is the Chrosomus system in general. While I think the points made by the reviewers were very well addressed and the resulting version of the manuscript is improved, my two main misgivings are not resolved. The sample size is still very small and I just can't see what the small differences between the two types of triploids ultimately mean. I am not saying everything has to be adaptive, but it would be nice to see a hypothesis.

Reviewer #3:
None

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have put together a very careful and considerate reply to the reviews. The manuscript reads well and the underlying study is highly interesting, as is the Chrosomus system in general. While I think the points made by the reviewers were very well addressed and the resulting version of the manuscript is improved, my two main misgivings are not resolved. The sample size is still very small and I just can't see what the small differences between the two types of triploids ultimately mean. I am not saying everything has to be adaptive, but it would be nice to see a hypothesis.

RESPONSE FROM THE AUTHORS

We thank Reviewer #1 and the Editor for the relevant suggestions that helped to improve the manuscript. Responses from the authors are in blue.

We confirm that the low sample size in common garden was properly caveated in throughout our paper (Results: page 7, lines 157-158; Discussion: page 11, lines 233-234).

Results:

"All individuals reared in controlled conditions were successfully assigned to their reproductive strategies using the k-means clustering analysis (A-11 n=12; B-01 n=9) and a clear difference in the shape of sexual and asexual individuals for both lineages can be observed along the first axis of the PCA (Figure 2c), though sample size was smaller compared to naturally reared individuals."

&

Discussion:

"Notwithstanding a smaller sample size in common garden compared to naturally reared individuals, we ruled out differential survival as a cause of shape differences between sexual and asexual organisms, since individuals reared in a common garden show the same patterns."

Furthermore, justification of the landmarks choice was added in the Methods section (page 15, lines 335-337).

"Landmarks were chosen for their adequacy to describe both the trophic niche (mouth shape) and the individual interaction with the environment (eye and pectoral fin position)30, and were based on similar study cases[30,50,51]."