Lines 1-2: I think the title misses a verb to be grammatically correct. For instance, "**Assessing the** breeding phenology of a threatened frog species using eDNA and automatic acoustic monitoring". Also, the word "breeding" might be left out because you assess the timing when the hibernation of the species ends, not specifically when breeding commences. Letting go of the word breeding does, in my view, not result in a reduction of strength of the title. You could also, as you did in the first subtitle of your discussion, use the title "Assessing spring emergence of a threatened frog species using eDNA and automatic acoustic monitoring".

Lines 15-16: Please specify what kind of phenological mismatches, and between what actors they act. Between predator and prey?

Line 18: Please add 'the' before phenology. Also, I would state that phenology monitoring is not so much challenging because of their cryptic nature, as most frog species are audibly very apparent, but in stead because of their variable timing of breeding. Making this nuance might be worthwhile. I think you should clearly make the difference between assessing the timing on emerging from hibernation, and the start of breeding, as the former, opposed to the latter, is difficult to monitor. That is also why I would suggest to leave "breeding" out of the title, and just mention "phenology", instead of "breeding phenology".

Lines 20-22: If I understand correctly, your goal is threefold: (1) assess the phenology of this frog species, (2) assess the sensitivity of your eDNA assay by comparing it with automatic acoustic recorders, (3) and evaluate three methods to reduce PCR inhibition in eDNA samples. Please mention this more explicitly in the methods section of the abstract.

Lines 21-22: I am not familiar with the species, but I would think that boreal chorus frog is the most commonly used name for Pseudacris maculata. Also, please mention the scientific name before speaking of a population (i.e. a boreal chorus frog (Pseudacris maculata) population).

Lines 23-24: Please rephrase, because for a reader it is at this stage of the MS not clear that the marsh is about to melt. This can be solved by simply adding "still": "… was still largely frozen".

Lines 24-25: Please specify that the calling of the very first male, and attaining higher chorus activity was determined with the acoustic recorders, as this is not clear now.

Line 26: maybe change "locale" to "site", throughout the ms?

Lines 25-27: Please specify that you are comparing the eDNA signal with the acoustic recorders. Also, is "… were evident in the chorus…" a correct sentence? "…as more males participated in the chorus…" might be an alternative. Lastly, please rephrase the last part in order to reduce the certainty of your findings. Your findings only a suggest that eDNA is a reasonable proxy for calling assemblage size, but more explicit testing is needed to verify this. However, you really did not test this, and your research set-up did not allow to test this, so I would suggest focusing your study on detecting the species, not quantifying it.

Lines 29-31: This text segment is lost in your results section, and should mention it in the methods section. Actually, this is a third goal of your paper, i.e. to test methods reducing inhibition, and you can introduce this goal by stating that you expect high levels of inhibition in these kind of samples. See for instance Harper et al. (2019) in Hydrobiologia (https://doi.org/10.1007/s10750-018-3750-5) or Everts et al. (2021) in Scientific Reports (https://doi.org/10.1038/s41598-021-90771-w). So I would suggest that the authors restructure their paper, by adding the segment of assessing methods to reduce PCR-inhibition as a separate research goal.

Lines 27-29: I think that the English language in the last part of the sentence can be improved: "…limiting detection **probability** and quantification **accuracy**…", could be an improvement.

Lines 33-35: To be honest, I think your findings indicate the opposite. Frog eDNA was only detected 6 days after the first call was detected with your acoustic monitoring. Moreover, acoustic monitoring has the advantage of covering a larger area of your marsh system, while eDNA detections were found to be very spatially structured. Moreover, acoustic monitoring is passive and cheap, while eDNA monitoring is expensive and intensive. I think that when a conservation manager want to assess the breeding phenology of a particular species, he/she would prefer installing a few acoustic detectors rather than sampling eDNA. I would encourage the authors to change their conclusions. Nonetheless, this work is invaluable as they developed and rigorously validated a new eDNA assay that can discriminate between mitotypes, and is actually quite sensitive when compared to the acoustic data. As a result, their assay can be used to detect this species in the landscape. A more general conclusion might indeed be that for other amphibian species that do not call like the one studied here, eDNA might indeed be a good tool to assess its phenology. However, this nuance was not entirely clear for me.

Lines 35-37: Perhaps changing "emphasize" to "demonstrate" might be a good idea, as you clearly show what PCR-inhibiting compounds do with ddPCR reactions.

Line 45: I would suggest explicitly stating "spawning of fish", as you also specified "chorusing in frogs".

Line 45: At this point, I suggest including an explanation about how phenological shifts are bad (as you did in lines 55-61). The sentence about such shifts is followed by the statement that amphibians are heavily threatened, but from the text it is not clear how shifting phenologies are related to the threatening of species. So I would suggest to first discuss the consequences of phenological shifts in general, after which you proceed by zooming in on amphibian species

Lines 48-50: I think there are some language issues in this sentence that can be improved.

Lines 60-61: Suggestion: "… with reduced fitness as a consequence."

Lines 68 – 71: I think it would be a good idea to combine these 2 sentences. For instance: "… of only 23.2% (Jo et al. 2020), while passive acoustic monitoring…"

Lines 72 – 77: An additional and highly relevant reference and statement that you can use here is that eDNA can also be used to pinpoint breeding sites in a landscape, as was shown by Everts et al. (2022) in Environmental DNA (https://doi.org/10.1002/edn3.301) for the invasive American bullfrog.

Lines 77-80: I think this sentence can be improved in terms of English language, as it does not read fluently at the moment. Also, "sampling strategies", instead of "sampling", please specify what kind of analysis, and please reflect on whether it is grammatically correct to say "species have low abundance".

Lines 80-82: I think you should not yet formulate your research objectives at this point.

Lines 83-85: Again, I am not familiar with the species, but why are you describing 2 species here, as you only investigated one (i.e. Pseudacris maculata)? In the abstract you also state "trilling chorus frog population". I think the manuscript would improve greatly if the authors were consistent with the naming of the species.

Lines 89-90: do they typically call while being submerged in the water? Or do they sit on floating or emergent vegetation? This is important for your discussion section later on, explaining why you detected eDNA a few days after the first male called. Males of the American bullfrog, for instance, typically call on the shoreline of ponds, and therefore a calling male does not necessarily mean that eDNA can be detected in the water.

Lines 98-99: please make a statement about the period of larval development before metamorphosis.

Lines 100-111: I would suggest a restructuring of the introduction: Paragraph 1 (lines 40-61) is okay as it is right now. Following paragraph 1, I would suggest introducing your study species and describing the problematics of shifted phenologies for this particular species. Next, proceed with stating that monitoring breeding phenology is challenging, because of a number of reasons you already mentioned, after which you can shift towards the topic of acoustic and eDNA-based monitoring. At the end, you can state the problems of eDNA-based monitoring, and as such, you can swiftly transition to your research goals.
Also, you link climate change to changes in hydroperiod, and not to changes in phenology. Can you please specify how a climate change-induced shorting or lengthening of hydroperiod can affect phenology?

Lines 112-115: Please specify where this wetland is situated. Also, I would add a third goal in which you investigate the efficiency of different methods to reduce ddPCR inhibition. Finally please take a look at the order of your research questions, they are not aligned with how there are provided in the results section. I think your first aim should be assess the phenology, and that is based on your acoustic data. Your second aim is to develop and validate an eDNA assay for this species, and investigate its sensitivity by comparing it to the acoustic data. Your last aim is to assess which inhibition mitigation method works best for this study system.

Lines 121-123: If I understand correctly, you mean that the chorus frog population was genetically determined to be *P maculata*, while it is considered by conservationists as *P. triseriata*? If so, please rephrase, because stating that a population possess a particular genome is very strange to me.

Lines 125 – 128: you can rephrase this sentence so that you can avoid repeating "at an hourly interval" twice.

Lines 140 – 141: what do you mean with "…the third (C) as supplementary as needed. Do you mean "… if needed"?

Lines 200-201: Typo: "… when Round Field Marsh begun to thaw…", omit the 'was'.

Lines 235-236: Maybe it is a good idea to specify that these positive controls and NTC's and field and laboratory blancs are used to detect false negatives and positives, respectively, as PeerJ readers might not be that familiar with eDNA methodologies.

Lines 242-244: what do you mean with setting the fluorescence manually? Was this detection threshold sample dependent? This threshold is supposed to be set and thus identical for all samples. At what amplitude it should be set can be determined based on the mean amplitude of negative and positive droplets in non-inhibited samples. Please use one threshold for all samples, and specify at what amplitude it was set.

Lines 250-253: please combined the two sentences, because this paragraph starts very random by stating that this turtle does not inhabit this swamp.

Lines 261: You can improve the transitioning to the topic of inhibition coping by simply starting the first sentence with: "As PCR-inhibiting compounds are expected to be abundant in the sampled water bodies (Harper et al. 2019), we tested three inhibitor mitigation methods…"

Lines 294-297: Regarding p-values: when they drop lower than 0.001, write $p < 0.001$ might be visually more appealing. Also, with calling activity you mean number of calling male individuals? Or number of calls in general, independent on the number of males? Please specify. Nonetheless, very interesting findings!

Lines 307-310: you can avoid repetition of "in one replicate" here.

Lines 323 – 324: Please repeat that this is because you had PCR amplification of 1 droplet in your NTC.

Lines 342-343: Please add that you are talking about an identical sample.

Lines 343-345: "detections" instead of "detection"

Lines 346-348: As an important part of your study is assessing strategies to counter PCR inhibition, I would suggest to not shunt this figure to the supplementary of the article.

Lines 366-369: Please add that you also comprehensively validated this assay, next to just developing it.

Lines 383 – 399: I miss a part discussing your findings in terms of phenology. Is the observed phenology in line of the expectations?

Lines 388 – 391: I think it is a bit far fetched to state that your data suggests a link between eDNA concentration and calling assemblage size. There were only 2 eDNA samples that were positive at the end of the eDNA sampling period in locale 3, while for 2 positive eDNA samples were retrieved from locale 5, but no chorus frog eDNA was detected in the two sampling days in between. I would suggest focusing this paper on detection, not quantification of abundance, as your experimental setup really does not allow for the latter.

Lines 394-397: I totally agree with this statement, but this sentence could be improved in terms of English.

Lines 397-399: … or frog species like the African clawed frog, producing calls that are barely perceivable with the unaided ear, or for other amphibian species such as newts that do not call at all.

Lines 400: Maybe it is better to talk about microhabitat use in stead of spatial distributions, because the latter implies a larger scale than one particular marsh.

Please 404. I would state that the acoustic monitoring could not help in determining whether these spatial eDNA patterns were due to microhabitat use or different physicochemical parameters affecting eDNA persistence. Do you expect one of the other being more likely? If so, you can elaborate briefly on that.

Lines 410 – 412: "… but increased to almost 50% on April 12th when many males…"

Lines 412-414: "… might be important factors underlying the lower detection rates earlier in the breeding season.", or something like that, to strengthen the link with previous sentences.

Lines 416 – 418: temporal variation in what?

Lines 418-420: I am not a fan of the sentence "led to reduced positive detections", I would suggest changing it to "reduced detection probabilities", or something like this.

Lines 463 – 464: please change sentence to something like " In this study, we did not investigate the source of inhibition nor the underlying mechanisms affecting eDNA detection probabilities." Also, please change ddPCR studies to eDNA studies in lentic systems, because this has in my view nothing to with the technique, but rather with your study system.

Lines 466-467: I am not a native English speaker so please forgive me if I am wrong, but is it correct to say "save for L8"? Is "with the only exception being L8" a better way?

Lines 467 – 468: but also eDNA particles, as you have mentioned in lines 419 - 420

Lines 471 – 473: If you are looking for a study that did use IPC's in an aqueous environmental DNA and amphibian context, see Everts et al. (2021) in Scientific Reports: https://doi.org/10.1038/s41598-021-90771-w

Line 478: why only the latter three? If you want to avoid this question, remove the first two options in the previous sentence.

Line 479 Please make the link between collapse of droplets and reduced detection probabilities. For instance: "… collapse of droplets, and thus the chance of detecting the species at low abundances, was still evident".

Lines 482 – 483: I am not a fan of "worsened the eDNA signals". Please be more objective and specific: in terms of what were the eDNA signals "worsened"?.

Lines 492 – 494: "… ddPCR eDNA" is not correctly formulated. ddPCR is an amplification technique, and eDNA is strictly speaking DNA in the environment. So to correctly formulate this, I would say "…eDNA-based species detection using ddPCR…"

Lines 495-498: the way this sentence is now constructed implies that PCR inhibition is particular to ddPCR, while the opposite is true: ddPCR is better in contexts were inhibiting compounds are abundant. Please rephrase.

Fig. 1: Really cool and clear photo of your study site, but please provide an inset map that shows the location of your study area on a region scale. Also, can you turn the map so that the northside faces upward? I was confused reading your discussion section about the spatial patterns since north was not facing up.

Fig. 4: I think it would be a good idea to have a figure that combines the detections of both acoustic and eDNA methods. This can be done by, for instance, adding a bold boundary to the rectangles if the acoustic sensor detected calling males or not for that particular day (detector A for locales 8,9, detector B for locales 3,6,7, and detector C for locales 1,2,45). Or if the authors would like to keep this figure strictly reserved for eDNA results, an extra figure would seem to me like a good idea.

Fig. 5: I would like to see some adaptions to this figure as well. So if I understood it correctly, the purpose of this figure is to showcase how PCR inhibition looks like in a ddPCR plot? First of all, I would suggest using only samples that were positive or inconclusive. If I look at figure 4, you have both kind of samples both highly and modestly inhibited. Second, please use locale 1, locale 2,… as you do throughout the text, in the figures as well, instead of the code names 10L9, 12L4. In that way, the figure is kept simple and readable, independent of the caption. Third, please specify that the three ddPCR panels in each row and right column of the figure are the three technical replicates ran

on each sample, and that the pink horizontal line is the detection threshold. In any case, I think it is more interesting to show ddPCR plots before and after PCR-inhibiting-compounds-mitigations are carried out, as this is actually one of the research questions, while just showing how PCR inhibition looks like in a ddPCR plot is not as interesting, at least not for me.