Review of Trionnaire et al. 2020

This is a well-written and well-reasoned article testing the role of dopamine pathway genes in the regulation of the reproductive mode in aphids in response to changes in photoperiod. The authors perform a series of experiments where they examine levels of gene expression of several genes in of the dopamine pathway in aphids at different stages of their life cycle and after a change in photoperiod, and then perform manipulative experiments (injection of dopamine antagonist and dopamine itself), feeding experiments, as well as CRISPR-Cas9 against one of the pathways genes*, ddc*, to test the causal role of dopamine and *ddc* in the reproductive mode transition. The conclusion is that there is currently not enough evidence to implicate this pathway in the reproductive mode switch. I agree with the conclusions of the manuscript and I think the manuscript, needs only some minor revisions (described below).

I have, however, one major comment for the authors to consider in future work. The author's long-term goal is to "test at which generation (grandmother or daughter) and at which developmental stage (adult, larvae, embryos) the effect of photoperiod shortening occurs". However, the authors do not perform an experiment that directly addresses this question. Instead they measure how photoperiod alters the expression of several genes of the dopamine pathway, a candidate pathway potentially downstream of the photoperiodic cue, across several generations and tissues. An experiment that would actually address the question above would be a photoperiod-shift experiment, where aphids are reared at a particular photoperiod for most of their lives, except for a particular interval of time, when they are shifted to a different photoperiod. This experiment would address what the authors want to know. Once they know the sensitive stage for sensing photoperiod, then they can investigate what hormones and genes are being differentially expressed at that stage. Currently, it is very unclear what the differences in gene expression observed actually mean because they may be happening at non-relevant (non-sensitive) stages of development for purposes of reproductive mode switch. In addition, I believe that dopamine levels in the hemolymph or heads of these aphids have previously been measured. These data should be presented in the introduction of the paper. It is especially important to report levels of dopamine especially at the time in development when the dopamine drug antagonists were injected. Is it known whether the form where the drug was injected does have higher levels of dopamine than the alternative form at that time? If this baseline is not known, and if dopamine levels at this stage are already low, it would not be surprising that the drug injections actually produced no shift in the aphid form observed, as was the case here. For tips on how to pin this system down, especially regarding photoperiod shift experiments, I recommend the authors read Monteiro et al. 2015 (Plos Genetics). Periods of environmental and hormonal sensitivity can be quite short (in the case of eyespot size regulation in butterflies this period is a mere 48hrs), so I think it is important to pin this period down first, and measure levels of candidate hormones at this stage, before pursuing additional experiments on RNA expression differences across life stages.

<u>Minor comments:</u>
In the discussion the authors mention that "Our results also indicate that photoperiod shortening is associated with a reduction in dopamine synthesis", but I believe what their results show is a reduction in levels of the enzymes involved in dopamine synthesis, not dopamine directly.

In the *ddc* CRISPR experiment can the authors comment on whether the retention of green color in the eggs is due to the chorion not being pigmented, due to the cuticle of the embryo inside not being pigmented, or both? May I also suggest that if the injections are done later in development or using lower concentrations of the reagents it may be possible to observe embryos that survive because the number of modified cells is fewer.

In the abstract perhaps it is better to specify that the heads that are looked at are larval heads, and perhaps the order of heads and embryos should be reversed if the embryos are sampled before the larval heads.

In the RT-PCR methods section "The 6/7 most developed embryos were isolated on ice from 25 adult G0 aphids, pooled into liquid nitrogen" it is not clear whether 6/7 embryos were collected **per each** of the 25 G0 aphids? Please clarify.

In section 4. RNA-seq data can you please clarify whether the L2 and L4 larvae indicated here would correspond (in terms of the generation) to the G1 larvae described previously? "In a previous study, we compared the transcriptomes of virginoparae (under long days or LD) and sexuparae (under short days or SD) head samples at two stages of larval development (L2 and L4) using a custom-made cDNA microarray"

Clarification is also needed in the whole-mount hybridization section: "Ovaries containing the ovarioles of developing embryos were dissected from virginoparae adult individuals". Are these G1 adult individuals? May I suggest that the authors include a schematic, with time depicted on the x-axis, of the different generations and tissues from where the data was collected to make reading of the manuscript easier and less confusing? This is a fairly complex system to keep track.

In the results section (and again in the Discussion) the authors mention that one of the genes involved in synaptic function, *prt*, is down-regulated in SD conditions, but two other genes, *vat1* and *vmat*, are not. This does imply that photoperid shortening signals are regulating genes associated with domanine synaptic function, unlike what was stated in the last sentence of that paragraph (and in the discussion). If these three genes are involved in regulating one pathway, having one of the genes in the pathway be affected by an environmental cue would be sufficient to impact the output of the entire pathway. Environmental regulation of all elements of a pathway is presumably not required to lead to the evolution of a plastic response.