LeTrionnaire et al *Dopamine pathway characterization during the reproductive mode switch in the pea aphid* for PCI Entomology

This paper makes a convincing case that dopamine pathway genes in the pea aphid, including those involved in the sclerotization and melanization of cuticle, are down regulated in the head of the sexual-producing morph known as a sexupara compared to the asexual-producing morph known as the virginopara. The paper extends previous work that first identified differences in the expression of genes encoding cuticular proteins and enzymes involved in dopamine synthesis. The fact that samples from L2 and L4 larvae, collected over ten years ago, seem to show a consistent down regulation of both *pale* and *ddc* (two genes encoding enzymes involved in dopamine synthesis) in sexuparae heads (this time using RNAseq as opposed to a cDNA array) is a nice result. They then build on this by examining the expression of other genes whose products functionally interact with dopamine, showing that genes whose products are involved in the connection between dopamine and the cuticle (sclerotization and melanization) are mostly also down regulated in the heads of sexuparae (while the expression of genes whose products are involved in the neurotransmitter function of dopamine are unaffected). By in situ hybridization the authors also show that *pale* and *ddc* are first expressed in the central nervous system during late embryogenesis, and by RT-qPCR show that the differences (sexupara versus virginopara) in the expression of *pale*, *ddc*, and the dopamine-cuticle genes begin during late embryogenesis.

The thrust of the paper, however, is not to describe the sexupara phenotype. Rather, the paper aims to connect these gene expression differences in the heads of sexuparae and virginoparae to the mechanism by which these different morphs produce sexual versus asexual progeny. To this end, three functional approaches are employed, two loss of function and one gain of function: 1) injection of AMPA, a tyrosine hydroxylase inhibitor into virginoparae (loss of function); 2) injection of dopamine into sexuparae (gain of function); and 3) CRISPR-cas9 targeting of *ddc* (loss of function). The first experiment reveals no effect on the reproductive mode of progeny (i.e., all were asexual). This is perhaps not surprising; though the authors do speculate, it is not obvious how modification of the head cuticle might be involved in the loss of the asexual-promoting signal. Without any sort of confirmation that the AMPA injections actually reduce dopamine levels, however, this result is difficult to interpret. The second experiment, which also shows no effect (i.e., no significant increase in the percentage of asexual progeny), similarly lacks confirmation that the dopamine (administered through an artificial diet) is really getting where it needs to go (the head?) or is functional in the sexuparae. Incidentally, since the progeny of sexuparae generally are born in a temporal sequence (sexual females, males, then asexual females), it would also be useful to know where in the sequence asexual females appeared (comparing dopamine-fed sexuparae with controls). If asexual females appear earlier from dopamine-injected sexuparae then this might indicate an effect.

Despite the enormous amount of work involved, the third experiment is also not informative in terms of testing the role of dopamine in the reproductive switch. Since the mutagenesis of *ddc* was attempted in eggs (from which a morph similar to a virginopara in producing asexual progeny normally hatch), I suppose that the authors were testing whether they might produce a sexupara-like hatchling that produced sexual progeny. Unfortunately, the eggs injected with CRISPR-cas9 + *ddc* guide RNA largely failed to develop, precluding any assessment of the reproductive fate of progeny. While the authors concede this, they do suggest that the results demonstrate that *ddc* is involved in cuticle

melanization. This wouldn't be surprising, and many of the eggs injected with CRISPR-cas9 + *ddc* guide RNA do indeed fail to melanize. The authors infer that the failure to melanize is due to loss of *ddc* function, going on to suggest that this failure may also be the source of lethality. This inference is not supported, however, because there is no confirmation that *ddc* has been disrupted (e.g., by sequencing the locus). In addition, dead or developmentally disrupted eggs also fail to melanize, and thus it is not clear to me that the observed phenotype is not simply due to the injections. The authors state that non-injected and water-injected eggs always melanize, but they only show data for non-injected eggs and do not show data for negative controls (e.g., water-injected, or other mock-injected eggs). It is also true that unfertilized eggs fail to melanize, so ensuring that the sexual females have mated with males is another potential issue with this type of experiment.

In sum, the paper does a nice job of extending previous results and supporting the claim that sexuparae down regulate dopamine pathway genes, including those involved in the sclerotization and melanization of cuticle, in their heads. That said, it is difficult to conclude much if anything from the three functional experiments. I wonder if it would make more sense to combine the gene expression data (RNAseq, RT-qPCR, and in situs) with a description of the morphology of sexuparae. Is the head cuticle actually thinner? Is it actually less melanized? If so, these data would nicely compliment the gene expression work.

Potential technical concerns

- The embryonic CNS expression of *ddc* is suggested to be in Group I and IV cells, but as written this comes off as a bit of a guess. Thus it might be useful for the authors to describe more about how they made this determination.
- I was a bit concerned that only one endogenous control, RpL7, was used for the RT-qPCR. This was also the case in Nakabachi et al 2005, however, and it is a ribosomal protein, so perhaps the authors could simply add a sentence reassuring reader that expression of this gene is stable—that is, more than just referring to the gene as "invariant".

Minor suggestions

- Abstract: Consider making it explicit that asexual females are viviparous early in the abstract. As it stands, this is only implicit and reader may have a hard time understanding how the signals of asexual mothers are transduced to embryonic progeny without knowing that embryos are found within asexual mothers.
- Intro: regarding "ending up with the production of clonal oviparous sexual females and males in their offspring", if "clonal" is taken to mean genetically identical, this is strictly true, as males are XO.
- Intro: Not clear how "More precisely, the cuticle of the pea aphid has been described as made of three layers: the outer epicuticule, the inner epicuticule and the procuticle" clarifies role of RR2 proteins. In which layer are the latter found?
- M&M 6: For the AMPA injections, it is mentioned that the progeny of injected mothers was monitored for two generations. Were all progeny examined? If not, for how many days were progeny collected? If the subsequent generation was also examined, which individuals of the initial generation were used to produce the next?
- M & M 6: In the dopamine feeding experiment, perhaps it would help to explain that aphids must be born onto the artificial diet in order to consume it. More specifically, it needs to be explained that in this experiment it is the newly born larvae

(sexuparae) that are feeding on the artificial diet. A naïve reader might assume that mothers fed on the diet and that the idea was to get the dopamine from the digestive tract into the embryos. Also, how long were larvae allowed to feed on artificial diet? It is the progeny of these blue sexuparae that are examined, but how many days of progeny were collected once they started giving birth?

- M & M 8: The phrase "asexual and sexuparae embryos" is confusing because sexuparae are asexual. Consider using "calculated for embryos dissected from virginoparae and sexuparae mothers (three biological replicates) were compared…" or something similar.
- Results (gene expression in heads): Can tyrosine hydroxylase and dopa-decarboxylase *both* be rate limiting?
- Results (gene expression in heads): Were the samples truly *already used* for cDNA arrays? I assume that the L2 and L4 samples were collected at the same time (in the same experiment) but not necessarily already used, correct? Perhaps you could clarify. *[I assume that's why L2 and L4 were used, given that L3 and adults were already used for the microarray study.]*
- Results (gene expression in heads): It is mentioned in M & M that the FDR was calculated for the RNAseq analysis, but this doesn't seem to be reported anywhere in the paper.
- Results (gene expression in embryos): Rather than "the associated p-value (0.09 for *aaNAT*, 0.1 for *black* and 0.06 for *ebony*) were close~~d~~ to the significance threshold of 0.05" perhaps it would be sufficient to point out that the observed differences, while not in all cases statistically significant ($p < 0.05$), were in a consistent direction (down regulation in sexuparae).
- Results (pharma approaches): Instead of "for the control injected with water" I suggest simply "for the control" lest reader think you only injected water, rather than Ringer's.
- Fig 1 caption: Perhaps define "PO" as phenoloxidases? Since it is italicized, "PO" suggests a specific gene, but is that the case?
- Fig 2 caption: Instead of just "statistically analyzed" would it be useful to be more specific as to the test applied? EdgeR?
- Fig 3: Instead of "Maternal Signal?", I suggest "Maternal RNA?" or "Maternally Provided?" instead to avoid any implication that this somewhat mysterious expression is related to the asexual-promoting maternal signal involved in the switch between reproductive modes.
- Fig 3: Panel d is labeled as "control" but the caption suggests that antisense, not sense, probe was used.
- Fig. 5 caption: Name statistical test in caption (currently says "which one…").
- Several places in the manuscript and in figures/figure captions: The term "parthenogenetic individual(s)" or simply "parthenogenetic" is used as a substitute for virginopara(e). This is potentially misleading, since sexuparae are also parthenogenetic. Perhaps just use virginopara(e) instead.

Typos and minor grammar/copy-editing suggestions (red indicates suggested modification)

- Abstract: towards their embryos >> to their embryos
- Abstract: that dopamine pathway >> the dopamine pathway
- Abstract (suggestion): decrease of the autumnal photoperiodic signal >> decrease in photoperiod

- Abstract: a *pale* inhibitor >> an inhibitor of the *pale* product
- Abstract: short-day~~s~~ and long-day~~s~~ conditions
- Abstract: observed a putative effect
- Abstract: knock out the *ddc* gene
- Abstract: mimicked drosophila phenotype >> mimicked the *Drosophila ddc* phenotype
- Abstract (suggestion): photoperiod shortening signal integration prior to the reproductive mode switch >> integrating changes in photoperiod and reproductive mode
- Intro: season variation >> seasonal variation
- Intro: 3-month~~s~~ diapause
- Intro: adaptation to seasonality (?)
- Intro: Since several years >> For several years
- Intro: a large number of cuticular protein mRNAs were down regulated
- Intro: down-regulated >> down regulated (for consistency)
- Intro: as were the cuticular proteins
- Intro (suggestion): to modify artificially internal concentration >> to artificially increase the internal concentration of dopamine
- Intro (suggestion): performed the genome editing of ddc gene >> targeted the ddc gene
- M & M 1: after having been laid
- M & M 1: dopamine hydrochloride feeding experiment (see below) was
- M & M 6: dopamine hydrochloride
- M & M 6: blue-label~~l~~ed larvae
- M & M 6: their progeny (corresponding to G2) were kept on plants until they reached adulthood
- M & M 6: the abdomens of these adults were then dissected
- M & M 7: to amplify these genomic regions
- M & M 8: A p-value less than or equal to 0.05
- M & M 8: **Statistical analyses**
- M & M 8: Dopamine hydrochloride [in two places]
- Throughout manuscript, *Drosophila* is often neither capitalized nor italicized.
- Results (gene expression in heads): **Dopamine pathway genes expression in the heads of sexuparae and parthenogenetic individuals.** >> **Dopamine pathway genes are expressed in the heads of sexuparae and virginoparae.**
- Results (gene expression in heads): dopamine pathway ~~synthesis and~~ signaling
- Results (gene expression in embryos): subjected to photoperiod shortening
- Results (pharma approaches): reared under long day~~s~~ conditions
- Fig 2 caption: long day~~s~~ (LD) and short day~~s~~ (SD)-reared aphids.
- Table 1 caption: various concentrations
- Table 2 caption: dopamine hydrochloride [also in table itself]