Dopamine pathway characterization during the reproductive mode switch in the pea aphid

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Abstract

Aphids are major pests of most of the crops worldwide. Such a success is largely explained by the remarkable plasticity of their reproductive mode. They reproduce efficiently by parthenogenesis during spring and summer generating important damage on crops. At the end of the summer, asexual females perceive the photoperiod shortening and transduce this signal towards their embryos that change their reproductive fate to produce sexual individuals: males and oviparous females. After mating, those sexual oviparous females lay cold-resistant eggs. Previous studies based on large-scale transcriptomic analyses suggested that dopamine pathway might be a key player in the integration of decrease of the autumnal photoperiodic signal to promote the switch of embryonic germline fate. In this study, we investigated the role of dopamine pathway in the photoperiodic response of the pea aphid *Acyrthosiphon pisum*. We first analysed the level of expression of ten genes of this pathway in heads and embryos of aphids reared under long-days (asexual producers) or short-days (sexual producers). We then performed *in situ* hybridization experiments to localize in embryos the *ddc* and *pale* transcripts that are coding for two rate-limiting enzymes in dopamine synthesis. We then used pharmacological approaches to inject aphids with dopamine or a *pale* inhibitor to mimic short days and long days conditions and observe a putative effect on the distribution of their offspring. Altogether, our results indicate that photoperiod shortening is associated with a reduction in dopamine synthesis that might affect cuticle sclerotization process rather than neuro-transmission. Using CRISPR-Cas9 mutagenesis, we also tried to knock out *ddc* gene in eggs produced after the mating of sexual individuals. We could observe strong melanization defaults in *ddc* mutated eggs, which confidently mimicked drosophila phenotype. Nevertheless, such a lethal phenotype did not allow us to investigate the precise role of *ddc* in photoperiod shortening signal integration prior to the reproductive mode switch.

Key-words : *Acyrthosiphon pisum*, photoperiodic response, dopamine pathway, spatio-temporal characterization, CRISPR-Cas9.

Introduction

Aphids are hemipteran insects that adapt their reproductive mode to season variation. They are capable of alternating between asexual and sexual reproduction during their annual life cycle. In spring and summer, they reproduce by viviparous parthenogenesis: each asexual female can produce nearly a hundred of clonal progeny. When autumn starts, asexual females perceive the shortening of the photoperiod, which triggers a switch of the germline fate of their embryos ending up with the production of clonal oviparous sexual females and males in their offspring. After mating with males, oviparous sexual females lay fertilized eggs that enter an obligate 3-months diapause over winter before hatching in early spring. All the individual hatching from those eggs are new genetic individuals with the viviparous parthenogenetic female phenotype. Thus, it is at least four different clonal morphs that are produced within the annual life cycle: males, oviparous sexual females, parthenogenetic viviparous

females producing a progeny of parthenogenetic viviparous females (under long days, dubbed "virginoparae"), and parthenogenetic viviparous females producing a progeny of males and oviparous sexual females (under short days, dubbed "sexuparae"). This peculiar adaptation to season for poikilothermous animals involves several trans-generational complex physiological and molecular mechanisms: sensing of the photoperiod, neuro-endocrine transduction of the signal to the ovaries and orientation of developmental programs for embryos (reviewed in Le Trionnaire *et al*., 2013 and Ogawa and Miura, 2014). Development of embryos in viviparous parthenogenetic aphid ovaries is continuous: each ovariole contains several developing embryos at different stages of development. Viviparity in aphids thus implies a telescoping of generations: grandmothers contain daughter embryos that already enclose (at least for the most developed) the germ cells of the granddaughters. Therefore, sensing of photoperiod by the adult females needs a continuous transduction of the signal towards the embryos, across three generations. It has been strongly suggested that embryos can directly sense the changes in photoperiod (Lees, 1964 and Le Trionnaire *et al*., 2009), which reduces (at least under controlled conditions) to two generations the transduction effect. It is thus interesting to test at which generation (grandmother or daughter) and at which developmental stage (adult, larvae, embryos) the effect of photoperiod shortening occurs. Since several years, our and other groups are involved in deciphering these mechanisms (see Le Trionnaire *et al*., 2013 for a review). Transcriptomic analyses allowed the identification of the active pathways that differ between aphids reared under short or long photoperiod. Most of the analyses were performed on the pea aphid (*Acyrthosiphon pisum*) which was the first aphid species to present a large set of available genomic resources (IAGC, 2010 and Legeai *et al*., 2010). One of the unexpected observations derived from those transcriptomic analyses was a wide regulation of the expression of cuticular protein genes: a large number of cuticular protein mRNAs was down regulated in the heads of pea aphid sexuparae females under short days (Le Trionnaire *et al*., 2009 and Cortes *et al*., 2008). Most of these cuticular proteins belong to the RR2 family, known to be involved (at least in *Tribolium castaneum* and other insects, Arakane *et al*., 2009) in the sclerotization of the cuticle, a process that contributes to cuticle structure stabilization (Andersen, 2010). 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 (Brey *et al*., 1985). Sclerotization involves different molecules, including dopamine. We previously demonstrated that two genes involved in dopamine synthesis (*pale* and *ddc*) were also down-regulated in heads of sexuparae under short days, as for the cuticular proteins (Gallot *et al*., 2010). At that stage, we made several hypotheses on the putative roles of dopamine in the regulation of different downstream pathways such as i) synaptic function, ii) sclerotization of the cuticle, and iii) melanization of the cuticle (**Figure 1**), involving for each pathway various proteins and genes. Dopamine is formed after two enzymatic reactions that first convert L-tyrosine into L-DOPA (by a tyrosine hydroxylase encoded by the *pale* gene in *Drosophila melanogaster*) and second, convert L-DOPA into dopamine (by a DOPA decarboxylase encoded by the *ddc* gene in *D. melanogaster*). Dopamine can be transported in synaptic vesicles by different proteins such as the vesicular monoamine transporters *Vmat*, the vesicle amine transporter *vat1* or the monoamine transmembrane transporter *prt*. For cuticle sclerotization, two acyldopamines (N-β-alanine-dopamine (NBAD) and N-acetyldopamine (NADA)) are formed and incorporated into the cuticular matrix (Andersen, 2010). This involves several enzymes such as an arylalkylamine N-acetyltransferase (encoded by the *aaNAT* gene - also known as *Dat* in *D. melanogaster*) that converts dopamine into NADA, an aspartate 1-decarboxylase (the *black* gene in *D. melanogaster*) that converts aspartate into a β-alanine and the *ebony* enzyme that finally links β-alanine with dopamine to form NBAD. The consequence is the formation of a hard layer of cuticle often at the outer part of the exoskeleton, covering internal softer cuticle layers. Dopamine can also be oxidized by laccases (multicopper oxidases) and/or phenoloxidases (PO) to form the dopamine melanin involved in

cuticle pigmentation, in conjunction in some instances with structural proteins such as the Yellow protein (Arakane *et al*., 2010).

In this paper, we went further into the description of the dopamine pathway regulation of the pea aphid phenotypic plasticity of the reproductive mode. We localized *pale* and *ddc* transcripts in embryos, we analyzed the expression of most of the genes involved in the dopamine-derived pathways in embryos and heads of larvae under short and long days and we attempted to modify artificially internal concentration of dopamine to test the effect on the phenotypic plasticity of the reproductive mode. We finally performed the genome editing of *ddc* gene in fertilized eggs with CRISPR-Cas9 in order to investigate its precise role in the photoperiodic response of aphids.

Materials and Methods

1. Aphid rearing and production of sexuparae and virginoparae embryos

Stocks of parthenogenetic individuals of *Acyrthosiphon pisum* strain LSR1 (IAGC, 2010) are maintained on broadbean (*Vicia fabae*) plants in growth chambers at 18°C under long days conditions (16h of light, 8h of night), at low density (5 individuals per plant) to prevent the induction of winged morphs. From this stock, parthenogenetic L3 individuals were collected to produce in two different batches future sexuparae (under short days) or virginoparae (under long days). Future sexuparae are produced on broadbean at 18°C when placed at 12h of light (12h of night) while virginoparae are produced when maintained at 18°C under 16h of light (8h of night). Those individuals - when they become adult - represent generation 0 (G0). The embryos contained in those G0 are either future virginoparae (under long days) or sexuparae (under short days) and after been laid on the plant until they reach adulthood, they correspond to generation 1 (G1). G1 virginoparae lay down virginoparae females at generation 2 (G2), while G1 sexuparae lay down sexual individuals (oviparous sexual females first and then males) at G2. In our conditions, it has to be noted that LSR1 do not perform well for feeding on artificial medium. For this reason, dopamine hydrochloride feeding experiment (see below) were performed on clone P123 (formerly named P212, Jaquiéry *et al*., 2018) of *A. pisum*, in the same conditions as described above.

2. Dopamine pathway gene annotation and PCR primers definition

In order to find pea aphid homologues for drosophila genes functionally characterized for their involvement in dopamine pathway (as described in **Figure 1**), the amino acid sequences of *pale*, *ddc*, *vat1*, *prt*, *vamt*, *aaNAT*, *black*, *ebony*, *laccase* and *yellow* genes were retrieved from Flybase. A Blastp analysis was then performed on AphidBase (https://bipaa.genouest.org/is/aphidbase/) on the NCBI v2.1 annotation of *Acrythosiphon pisum* genome in order to find the closest homologues for these genes. These genes were then manually annotated to discriminate exon and intron sequences. Coding sequences for these 10 genes are available in **Table S1**. Primers for quantitative RT-PCR were then searched on these sequences using the Primer 3 software (http://primer3.ut.ee/) with default parameters and a maximum size of 150 nt for amplicon length. Primer sequences are listed in **Table S2**.

3. Quantitative RT-PCR analyzes

For Quantitative RT-PCR analysis of dopamine pathway gene expression levels, both virginoparae and sexuparae embryos (see above) were dissected from G0. The 6/7 most developed embryos were isolated on ice from 25 adult G0 aphids, pooled into liquid nitrogen and stored at -80°C before RNA extraction. A total of approximately 180 embryos were collected in both conditions (short and long days). Three biological replicates were performed. Total RNAs were extracted using the RNeasy Plant Mini kit

(Qiagen) according to manufacturer's instructions. The optional DNase treatment was carried out with the RNase-Free DNase Set (Qiagen). RNA quality was checked and quantified by spectrophotometry (Nanodrop Technologies). Before reverse transcription, a second round of DNase digestion was added using the RQ1 RNase-free DNAse (Promega), in order to remove any putative residual DNA. One microgram of total RNA was used for cDNA synthesis using the Superscript III Reverse Transcriptase (Invitrogen) and a poly-T oligonucleotide primer (Promega) following the manufacturer's instructions. The cDNAs were used for the quantitative PCR assay on a LightCycler 480 Real-Time PCR System using the SYBR Green I Master mix (Roche) according to the manufacturer's instructions. A standard curve was performed for each gene (*pale*, *ddc*, *vat1*, *Vmat*, *prt*, *laccase*, *yellow*, *black*, *ebony*, *aaNAT*, and *RpL7* used as a reference gene) using serial dilutions of cDNA products in order to assess PCR primers efficiency. A dissociation curve was produced at the end of each run in order to check for non-specific amplifications. Each Q-RT-PCR was performed on three technical replicates. Thus, for each condition, data were obtained from three biological replicates with three technical replicates for a total of nine measurements per condition and gene. Relative quantification was performed using the standard curve method with normalization to the *A. pisum* ribosomal protein L7 transcript (*RpL7*, Nakabachi *et al*., 2005). Absolute measures for each of the ten target genes (averaged among three replicates) were divided by the absolute measure of *RpL7* transcript.

4. RNA-seq data

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 (Le Trionnaire *et al*., 2009). We recently used the exact same RNA samples (two biological replicates per time point) to perform RNA-seq analyses, for eight datasets (two for L2-LD, two for L2-SD, two for L4-LD and two for L4-SD). Raw reads from RNA-seq data were mapped onto the NCBI v2.1 annotation of *Acrythosiphon pisum* using STAR with default parameters (Dobin *et al*., 2015). Reads were then counted by genes using FeatureCounts (Liao *et al*., 2014) with default parameters. Counts normalisation and differential expression analyses have been performed using the scripts described in Law *et al*. (2016), which is based on EdgeR (Robinson *et al*., 2010). Genes with an adjusted p-value <0.05 were considered as differentially expressed between LD and SD conditions (Le Trionnaire et al., personal communication). From these data, the expression values and the FDR associated with the LD/SD comparisons were retrieved for the ten pea aphid homologues of drosophila genes involved in the dopamine pathway (see above). Raw data are available on Sequence Read Archive (SRA) from NCBI under the SRP201439 accession number.

5. mRNA *in situ* hybridization

Riboprobe synthesis. Templates for riboprobes synthesis were amplified by RT-PCR, cloned and then transcribed into RNA. For this, total RNAs were extracted from adult parthenogenetic females (virginoparae) using the RNeasy Plant Mini kit (Qiagen). A DNase digestion step was carried out using RQ1 RNase-free DNAse (Promega) in order to remove any residual DNA. One microgram of total RNA was reversed transcribed with AMV Reverse Transcriptase (Promega) and a poly-T oligonucleotide primer (Promega) following the manufacturer's instructions. The cDNA produced was used as a template for PCR amplification with specific primers for the two genes *pale* and *ddc*. Sequences of gene-specific primers and length of probes are given in **Table S3**. Amplified fragments were then cloned into StrataClone PCR Cloning Vector pSC-A-amp/kan (Stratagene) and sequenced in order to check for the identity and orientation of the inserted PCR fragment. Insert containing the RNA polymerase promoters were obtained from the recombinant plasmids by PCR with universal primers. These PCR products (at

least 500 ng per probe) were used as a template for synthesis of sense and antisense riboprobes using digoxigenin-labelled dNTPs (Dig RNA Labelling Mix (Roche)) and the appropriate RNA polymerase T7, T3 or SP6 (Roche). After synthesis, DNA was removed with RQ1 RNase-free DNAse treatment (Promega) and labelled riboprobes were purified with the RNeasy MinElute Cleanup kit (Qiagen). Riboprobe quality and quantity was checked on an agarose gel containing SybrSafe (Invitrogen) and quantified with Nanodrop (ThermoFicherScientific).

Whole-mount *in situ* hybridization on aphid ovaries. We adapted the *in situ* hybridization protocol previously described in Gallot *et al.* (2012). Ovaries containing the ovarioles of developing embryos were dissected from virginoparae adult individuals. For this, caudas were removed with clamps and ovaries chains were slightly disrupted from conjunctive tissues under a glass microscope slide within fixation solution (4% paraformaldehyde in PBS buffer). Dissected ovarioles were incubated with the probes of interest: 630 ng/ml for *ddc* and 1000 ng/ml for *pale* in the same conditions as described in Gallot *et al.* (2012). Detection was performed with anti-DIG-alkaline phosphatase (AP) Fab fragments (Roche) diluted 1:2000 in blocking solution. Signal was revealed with 4 µL of NitroBlue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) Stock Solution (Roche)/ml of AP reaction buffer. Ovarioles were then observed under a microscope Nikon 90i connected to a Nikon type DS-Ri1 camera to allow images capture. Controls were performed using the corresponding sense probes.

6. Pharmacological experiments

AMPT injection experiments and offspring analysis. The injection assay was performed on LSR1 strain at the L3 and adult stage of G0 virginoparae (under LD). The tyrosine hydroxylase inhibitor (alpha-methyl-para-tyrosine: AMPT) was directly injected into the thorax from the dorsal side of the aphid using the Nanoject II injector (Drummond). 3.5-inches glass capillaries were used to inject 13.8 nl of AMPT diluted in Ringer's solution for the two first quantity (0.08 µg and 0.16 µg), and 50 nl for 1 µg and 1.6 µg. The control consisted in injecting the same volumes of Ringer's solution in aphids. After injection, aphids were transferred onto *Vicia fabae* plants in a growth chamber at 18°C and under LD conditions (16h of light). Mortality and reproductive phenotype of the progeny of each injected aphid was recorded for the following two generations.

Dopamine hydrochloride feeding. Aphids (P123 strain) were moved from LD (16h of light) to SD (12h) conditions at L3 stage. Once they reached adulthood, individuals were placed on the Ap3 artificial diet (3 individual per cell, 2 cells by condition) as described by Febvay *et al.* (1992) with the replacement of the 3.75 mM of β-alanyltyrosine by a supplement of 3.75 mM of phenylalanine. Exactly 250 µl of diet was added by cell, complemented with 0 (control), 200, 600 or 2000 µg of dopamine hydrochlorid. A droplet of Coomassie Blue was added to label the aphids that did feed on the medium (Tagu *et al.*, 2014). After 48h feeding, adults had laid down L1 larvae, corresponding to G1 sexuparae. Four larvae per condition were collected and placed onto broad bean plants. Only blue labelled larvae were kept. Once adults, their progeny (corresponding to G2) was kept on plants until they reach adulthood. The abdomen of these adults was then dissected to observe the presence of eggs (oviparae) or embryos (virginoparae) and infer their reductive phenotype. Males could also be observed and did not need to be dissected because of their very different morphology when compared to females.

7. CRIPSR-Cas9 editing of *ddc* gene

In order to analyze the function of *ddc* gene in the context of aphid reproductive mode switch, we aimed at generating stable mutant lineages for this gene. The step-by-step protocol for CRISPR-Cas9

mutagenesis in the pea aphid is detailed in Le Trionnaire *et al*. (2019). Briefly, we designed two single guide RNAs (sg1 and sg2) predicted to target the fifth exon of *ddc* gene using the CRISPOR software (**Figure 6a**). We then performed *in vitro* cleavage assays to confirm the *in vitro* efficiency of these guide RNAs to cut - when complexed to Cas9 - a PCR product from *ddc* genomic sequence boarding the fifth exon (**Figure 6b**). The primers used to amplify these genomic region are the following: F-ACTTTGGTGGCGTTGTTG and R-GGACGGAGGCACAGACTAAG. We then induced the production of sexual females from L9Ms10 clone and males from L7Tp23 clone to produce fertilized eggs and inject them with a sg1/sg2/Cas9 mix and waited until melanisation before placing them at 4°C for 85 days for obligate diapause.

8. Statistical analyzes

Q-RT-PCR analyzes. For each of the ten genes involved in the dopamine pathway, the normalized (to *RpL7* invariant gene) expression values (the average of the three technical replicates) calculated in asexual and sexuparae embryos for the three biological replicates were compared using a one-way ANOVA. A p-value of at least 0.05 was applied to identify differentially expressed genes between the two conditions.

Dopamine hydrochlorid feeding experiment. This experiment was performed to estimate the effect of the feeding of sexuparae aphids with various concentrations of dopamine hydrochlorid on the composition of their offspring (number of sexual males, males and asexual females produced). The numbers of the different types of morphs produced by control and treated aphids were compared with a one-way ANOVA. A p-value of at least 0.05 was applied to identify significant differences between treatments.

Results

Dopamine pathway genes expression in the heads of sexuparae and parthenogenetic individuals. In the past, our group demonstrated a decrease of the steady-state level of the *pale* and *ddc* mRNAs in the heads of pea aphid sexuparae under short days (SD) when compared with parthenogenetic aphids reared under long days (LD). This down regulation was detected at the third larval stage (L3) of sexuparae (Gallot *et al*., 2010). These two genes encode rate-limiting enzymes that finally allow the formation of dopamine from L-tyrosine. In order to test whether the various downstream genes of dopamine pathway synthesis and signaling (**Figure 1**) were regulated, we used transcriptomic data generated in aphid heads from LD (parthenogenetic) and SD (sexuparae) reared aphids at L2 and L4 larval stages. We performed RNA-seq on samples already used for cDNA arrays (Le Trionnaire *et al*. 2009) and extracted the expression information specifically for the dopamine pathway genes (**Figure 2**). Statistical analyses revealed that *ddc* and *pale* transcript were significantly down regulated under SD conditions at L2 and L4 stages thus confirming the trend observed at L3 stage. Regarding the genes involved in the synaptic release of dopamine, only *prt* transcript was down regulated at L4 stage under SD conditions while *vat1* and *vmat* were not regulated by photoperiod shortening. All the genes involved in the use of dopamine derived molecules as an uptake for cuticle melanization and sclerotization were regulated between LD and SD conditions. More precisely, *aaNAT*, *black*, *ebony* and *yellow* were significantly down-regulated under SD conditions at L2 and L4 stages while *laccase* transcript was up-regulated at L2 stage and down-regulated at L4 stage. These data thus indicate that in sexuparae heads,

photoperiod shortening signal regulates genes involved in cuticle sclerotisation and melanisation but do not seems to have an impact on the expression of genes associated with dopamine synaptic function.

Localization of *pale* and *ddc* transcripts in parthenogenetic embryos. In order to test whether *pale* and *ddc* are also expressed prenatally, we performed *in situ* localization of the pea aphid *pale* and *ddc* mRNAs in the embryos (and ovarioles) of adult aphids, just before larvae birth. *pale* mRNAs were detected in one early stage of embryogenesis (stage 1, as defined by Miura *et al*., 2003), in a very restricted zone (**Figure 3**). This pattern was reproducible, but difficult to interpret. As it was not detectable in subsequent early stages, we make the hypothesis that it might correspond to a residual signal of maternal *pale* mRNAs. *pale* mRNAs were also detected at later embryogenesis stages (from stage 13 to 17/18), at the anterior part of the embryo, probably in the protocerebrum, and more particularly in discrete pairs of cells that might correspond to neurosecretory cells. We could however not clearly assign the specific type of neurosecretory cells they could correspond to (Blackman, 1987). No labeling was detected in the downstream ganglion chain, but a lateral labeling was detectable within unknown structures that might be mushroom bodies (Kollmann *et al*., 2011). *ddc* mRNAs were detected in the latest stages (from stages 16 to 18) of embryogenesis (**Figure 4**). Labeled cells were located within the anterior part of the embryo, in neuronal cells of the protocerebrum, probably in neurosecretory cells of Group I and Group IV (Blackman, 1987). Labeled cells were also detectable posteriorly, along the ganglion chain of the nervous system. Thus, *pale* and *ddc* genes are both expressed in the embryonic central nervous system but have distinct spatial patterns of expression. As expected, no labeled structures were detected in control samples hybridized with sense probes (**Figures 3 and 4**).

Dopamine pathway genes expression in sexuparae and parthenogenetic embryos. We checked whether the dopamine pathway genes were prenatally regulated by photoperiod in embryos (**Figure 1**). We compared by qRT-PCR the expression of the corresponding mRNAs between late stages (see methods) of sexuparae and virginoparae embryos (**Figure 5**). *vat1*, *vmat* and *prt* which are genes involved in the synaptic release of dopamine were consistently not regulated (with a p-value close to 1) between the two types of embryos. Then *ddc* and *pale* appeared to be more expressed in parthenogenetic embryos compared with sexuaparae embryos, but because of an important variability between replicates, these differences were not significant. Regarding the genes involved in cuticle melanization and sclerotization, *laccase* and *yellow* were significantly down-regulated in sexuparae embryos. *aaNAT*, *black* and *ebony* also appeared to be more expressed in parthenogenetic embryos. Although these differences were not statistically significant, the associated p-value (0.09 for *aaNAT*, 0.1 for *black* and 0.06 for *ebony*) were closed to the significance threshold of 0.05. These analyses show that cuticle-related genes (especially Laccase and Yellow) are already regulated in embryos submitted to photoperiod shortening while the expression of the others are probably in the process of being modified by this signal.

Pharmacological approaches to investigate the role of dopamine in the photoperiodic response. We attempted to modify artificially the internal concentration of dopamine in the pea aphid in order to check any effect on the production of alternative phenotypes when submitted to changes in photoperiod. In a first experiment, we injected AMPT (a tyrosine hydroxylase enzyme inhibitor) in virginoparae aphids (reared under long days conditions) to putatively decrease the concentration of dopamine, trying to mimic the effect of short days, and recorded their progeny. We tested several AMPT concentrations as well as two developmental stages for injection (L3 and adults) (**Table 1**). Survival of L3-injected individual was low (less than 50%). Survival of injected adults was higher: low concentrations (0.08

and 0.16 µg) allowed a 100% survival (as for the control injected with water) while concentrations of 1 µg or 1.6 µg decreased the viability to 60%, or killed all the injected insects, respectively. The progeny of these survivor-injected insects for these different conditions was recorded. Progeny of virginoparae of pea aphid under long days is expected to be 100% of parthenogenetic daughters: this was observed for the control injected with water. None of the treated individuals differed from the control: all the progeny were parthenogenetic. The injection of AMPT had thus no effect on the progeny distribution in our experimental conditions.

In a second experiment, pea aphid sexuparae larval females reared under short days were fed on artificial medium complemented with dopamine hydrochloride, in order to try to increase the internal dopamine concentration potentially mimicking the effect of long days (**Table 2**). Despite the variability of fecundity between samples, there was no significant difference between treatments and control. The progeny of these sexuparae was recorded after feeding. The expected distribution of progeny morphs was given by the controls fed with artificial medium only. As usual, oviparous females are first born, followed by males and by parthenogenetic females for the latest embryos. In the dopamine hydrochloride fed individuals, the distribution had a tendency (not statistically significant) to produce more males and less females (oviparous and parthenogenetic) at all the three tested concentrations. However, the number of treated insects was low. We thus did not observe in our experimental conditions a strong effect of an increase in dopamine concentration on the distribution of sexual and asexual progeny under a short photoperiod regime.

CRISPR-Cas9 mutagenesis of *ddc* gene in fertilized eggs.

Based on a recent protocol of CRISPR-Cas9 mutagenesis developed in the pea aphid *(Le Trionnaire et al*., 2019), we aimed at generating mutant lineages for *ddc* gene. We injected fertilized eggs with two single guide RNAs designed to target the fourth exon and some recombinant Cas9 protein (**Figure 6a**). These guides were validated *in vitro* prior to injection in order to maximise the possibility to generate genome editing events (**Figure 6b**). Finally, we injected 851 fertilized eggs less than 4 hours after being laid on the plant by the sexual females. Among them, 470 (55%) of the eggs were damaged by the micro-injection procedure, a percentage closed to what we observed in previous experiments. Among the remaining eggs, 84 (10%) completed melanisation, indicating their survival. Surprisingly, 297 eggs (35%) appeared to be intact (not damaged by the injection) but did not complete melanisation (**Figure 6c**). Indeed, melanisation in aphid eggs is associated with a gradual transition from green to black colour and usually lasts for a maximum of 5 days, which corresponds to the early steps of embryogenesis until melanine synthesis is operative. The colour defaults we could observed ranged from dark eggs with few patches of green, to green eggs with patches of black and finally eggs that remained almost entirely green (**Figure 6d**). As these colour patterns are usually never observed in non-injected or water-injected eggs, these observations are likely to correspond to a modified melanisation phenotype for eggs edited at *ddc* genomic location. Conversely, injected eggs that completed melanisation were probably not edited. This genome editing experiment suggests that *ddc* gene in the pea aphid is involved in cuticle melanisation. Nevertheless, this phenotype is lethal since eggs with incomplete melanisation did not hatch after the three months diapause. Consequently, those eggs did not hatch and we were unable to test the photoperiodic response after *ddc* directed mutation.

Discussion

Dopamine is a central regulator of various pathways in insects, including synaptic function and cuticle structure. Previous works proposed the hypothesis that dopamine could be involved in the regulation of the plasticity of the reproductive mode in aphids (Le Trionnaire *et al*., 2009 and Gallot *et al*., 2010). This was based on the differential expression between short days and long days conditions of genes involved in dopamine synthesis as well as dopamine conjugation with other components to form cuticle polymers. Dopamine pathway (or more generally catecholamine pathways) has been recently demonstrated to regulate the phase polyphenism in locust, another case of discrete phenotypic plasticity where solitary and gregarious morphs alternate upon population density changes (reviewed in Wang and Kang, 2014). More precisely, it was shown that three genes involved in dopamine synthesis and synaptic release (*pale*, *henna* and *vat1*) were differentially expressed between the alternative morphs of *Locusta migratoria* (Ma *et al*., 2011). Artificial modification of dopamine concentration clearly demonstrated the role of this pathway in the control of phase polyphenism. The switch of reproductive mode in aphids being another striking case of insect polyphenism, we made the hypothesis that dopamine is also involved in the regulation of this process. We combined spatio-temporal characterization of gene expression, pharmacological as well as genome editing approaches to test this hypothesis.

In this work, we analysed the expression within *A. pisum* embryos and heads of larvae of most of the genes involved in dopamine-related pathways. When comparing RNA-seq expression levels of dopamine pathways genes between the heads of long days and short days-reared aphids, it clearly appeared that the key enzymes involved in dopamine synthesis (*pale* and *ddc*) and the genes involved in cuticle structure changes (*aaNAT*, *black*, *ebony*, *laccase* and *yellow*) were down-regulated in sexuparae individuals. On the contrary, genes involved in dopamine release in synapses (*vat1*, *vmat* and *prt*) were not regulated. These data thus confirm previous observations that photoperiod shortening in sexual producers' individuals probably results in cuticle structure modifications triggered by a potential decrease in dopamine synthesis levels (Le Trionnaire *et al*., 2009). We then wanted to test whether these changes were initiated prenatally as a result of an early perception of photoperiod shortening in embryos. We compared the expression levels of these genes in virginoparae embryos (reared under long days) and sexuparae embryos (reared under short days). Some of these genes – including *pale*, *ddc*, *aaNTA*, *black* and *ebony* - showed a tendency of being more expressed (although not significant) in asexual embryos while some others were significantly up-regulated (e.g *laccase* and *yellow*) in comparison with sexuparae embryos. Again, no differences in expression could be observed for genes involved in dopamine release in synapse. These expression data thus suggest that the putative modifications of dopamine concentration and cuticle structure are likely to occur prenatally, or are in the process of being regulated, as an early response to the external stimulus (the shortening of the photoperiod) responsible for the plasticity.

Transcripts coding for key dopamine synthesis enzymes (*pale* and *ddc*) are expressed and specifically localized in embryos, more precisely in paired structures and cells in the central nervous system and associated neuronal ganglions. However, due to the lack of a precise description of the anatomy of aphid embryonic central nervous system, it is difficult to identify with accuracy the cell types expressing those transcripts. We detected a *ddc* mRNA signal in cells that could correspond to neurosecretory cells described in adult aphids (Blackman, 1987). These labelled cells putatively belonging to Group I and Group IV of neurosecretory cells consist in five neurons located in the *pars intercerebralis* of the protocerebrum (Group I), and one to two cells at the posterior part of the protocerebrum (Group IV).

Interestingly, Group I cells have been showed to be involved in the photoperiodic response in the aphid *Megoura viciae* (Steel and Lees, 1977). Specific lesions of these cells abolished the response to the photoperiod. However, the nature of the material secreted by these cells is not known. They resemble neurosecretory cells of other insects such as *D. melanogaster* known to produce insulin peptides (Cao and Brown, 2001). There is thus no clear evidence that such cells might synthesize dopamine and more detailed analyses of the localization of *ddc* mRNAs in the central nervous system of aphid embryos are required.

Altogether, the non-regulation (in our experimental conditions) of the genes involved in dopamine transport within synaptic vesicles suggests a limited role of dopamine neurotransmission regulation during alternation of the reproductive mode. However, the down-regulation of dopamine synthesis observed in the embryos seems to be more correlated with cuticle structure modifications. The hypothesis we made is that the head cuticle of sexuparae is less sclerotized (Gallot *et al*., 2010, this study) and less melanized (this study) than in virginoparae. Whether this fact is a cause or a consequence of the production of alternative reproductive morphs in aphids in not solved yet. In one hand, cuticle structure could modify brain light perception by the sexuparae, but whether or not this has a direct incidence on the plasticity is not known. On the other hand, sexuparae have a very similar morphology to the virginoparae, but to our knowledge, no deep scanning of possible differences has been performed (except for the type of embryos). Cuticle structure could be one of these general phenotypical differences.

In order to go further in the understanding of dopamine regulation for the production of alternative reproductive phenotypes in the pea aphid, we attempted to manipulate artificially dopamine concentration using pharmacological approaches. As dopamine synthesis is down regulated in short days-reared aphids, we tried to artificially decrease this concentration in long days-reared aphids to test whether we could mimic the shortening the photoperiod by injecting AMPT, a tyrosine hydroxylase enzyme inhibitor. None of the tests we performed modified the progeny of the injected aphids; we thus could not confirm or deny this hypothesis. We also tested the reverse option by trying to increase artificially the concentration of dopamine in short days-reared insects in order to mimic the effect of long days. Data are not statistically relevant, but there is a tendency of a modification of the progeny distribution of treated sexuparae compared with untreated ones. Especially they seem to produce more males and less females compared to the non-treated sexuparae. Nevertheless, it is difficult to conclude on a strong effect of an increase of dopamine concentration on a change in sexual and asexual morphs proportions in our experimental conditions.

More clear-cut conclusion on the role of dopamine in the phenotypic plasticity of the reproductive mode should be afforded by gene knock-down or knock-out experiments. We did not succeed to knock-down the *ddc* transcript in the pea aphid by RNAi (data not shown), probably because of the specific expression of these genes in the brain and central nervous system which are tissues/organs difficult to target by RNAi in aphids. We then attempted to edit the *ddc* gene with CRISPR-Cas9 by injecting fertilized eggs with some Cas9 protein together with a pair of single guide RNAs designed to target one specific exon of the *ddc* gene. Surprisingly, the mortality of injected eggs was particularly high in comparison with previous experiments of gene editing on another candidate (Le Trionnaire *et al*., 2019). Indeed, only a few eggs reached complete melanization while the remaining proportion of the eggs (that were not damaged by the injection procedure) displayed various patterns of melanization, from nearly absent to almost complete. It is known in drosophila that *ddc* (alongside with *pale*) is required for

melanin synthesis (True *et al*., 1999). Mutants for *ddc* gene display some body color defects, especially a clearer color due to the absence of melanin. The phenotype observed in aphid eggs thus resembles the drosophila phenotype. This indicate that CRISPR-Cas9 system generated knock-out of the *ddc* gene in eggs that show an altered melanin synthesis in various locations. It is likely that these eggs harbor distinct somatic mutations in various embryonic tissues responsible for a mosaicism materialized by the variety of color patterns observed. Since the addition of melanin at the surface of the egg is necessary to protect the embryo against winter environmental conditions this explains why these eggs could not hatch after diapause and why this mutation had a lethal effect in our conditions. This experiment thus revealed that *ddc* gene was involved in melanization in aphids. Nevertheless, as we needed to inject early-fertilized eggs to get a successful genome editing, the lethal effect of the *ddc* knockout did not allow us to investigate its function in the photoperiodic response.

In conclusion, further functional analyses are required to dissect precisely the specific role of dopamine pathway in the regulation of reproductive polyphenism in aphids. Nevertheless, our study already demonstrated that various dopamine pathways genes were regulated prenatally between long-days and short-days reared embryos. This trend was confirmed in larvae where several of these genes were differentially expressed in the head between asexual and sexuparae (sexual-producers) individuals. This suggests that the setting of the genetic programs involved in the production of seasonal alternative reproductive morphs in the pea aphid takes place early in a long trans-generational process, which is correlated with the development and differentiation of neuronal structures and cells within the embryos. Our results also indicate that photoperiod shortening is associated with a reduction in dopamine synthesis that might affect cuticle structure rather than neuro-transmission. The melanization defaults observed for *ddc* mutated eggs also confirm that dopamine has a major role in aphid cuticle composition and synthesis. Altogether, this work suggests that dopamine pathway mainly affects cuticle sclerotization and melanization during the reproductive mode switch in response to photoperiod in aphids.

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Tables and Figures

Table 1 - Injection of alpha-methyl-para-tyrosine (AMPT) to parthenogenetic aphids. Parthenogenetic aphids were micro-injected with a solution of AMPT at various concentration. The offspring of all surviving aphids was then recorded in terms of production of sexual or asexual individuals.

Injection stage	Quantity of AMPT	Injected aphids (Nb)	Surviving aphids (Nb)	Offspring analysis (%)		
				Asexual females	*Sexual females*	*Males*
L3	0.08 µg	11	4	100	0	0
L3	0.16 µg	11	0	100	0	0
Control	H2O	11	4	100	0	0
Adult	0.08 µg	12	12	100	0	0
Adult	0.16 µg	12	12	100	0	0
Adult	1 µg	23	8	100	0	0
Adult	1.6 µg	6	0	100	0	0
Control	H2O	8	7	100	0	0

Table 2 - Feeding of sexuparae individuals with dopamine hydrochlorid. Short-days reared larvae (sexuparae) were fed on artificial medium complemented with various concentrations of dopamine hydrochloride. The offspring of all surviving aphids was then recorded in terms of production of sexual or asexual individuals. In the bottom lines are indicated the *p-value* of the one-way ANOVA performed to compare the numbers of sexual females, males and asexual females produced between the control and treated aphids. NS: Not Significant.

Dopamine hydrochlorid quantity	Treated aphids (Nb)	Fecundity (mean)	Offspring analysis (%)		
			Oviparous females	*Males*	*Parthenogenetic females*
Control	5	**53.8**	30	12	11.8
200 µg	4	**32**	20.75	20.5	8.25
600 µg	4	**59.2**	28.5	17	14.25
2000 µg	4	**49.7**	24	17.25	8.75
			NS (0.41)	NS (0.37)	NS (0.51)

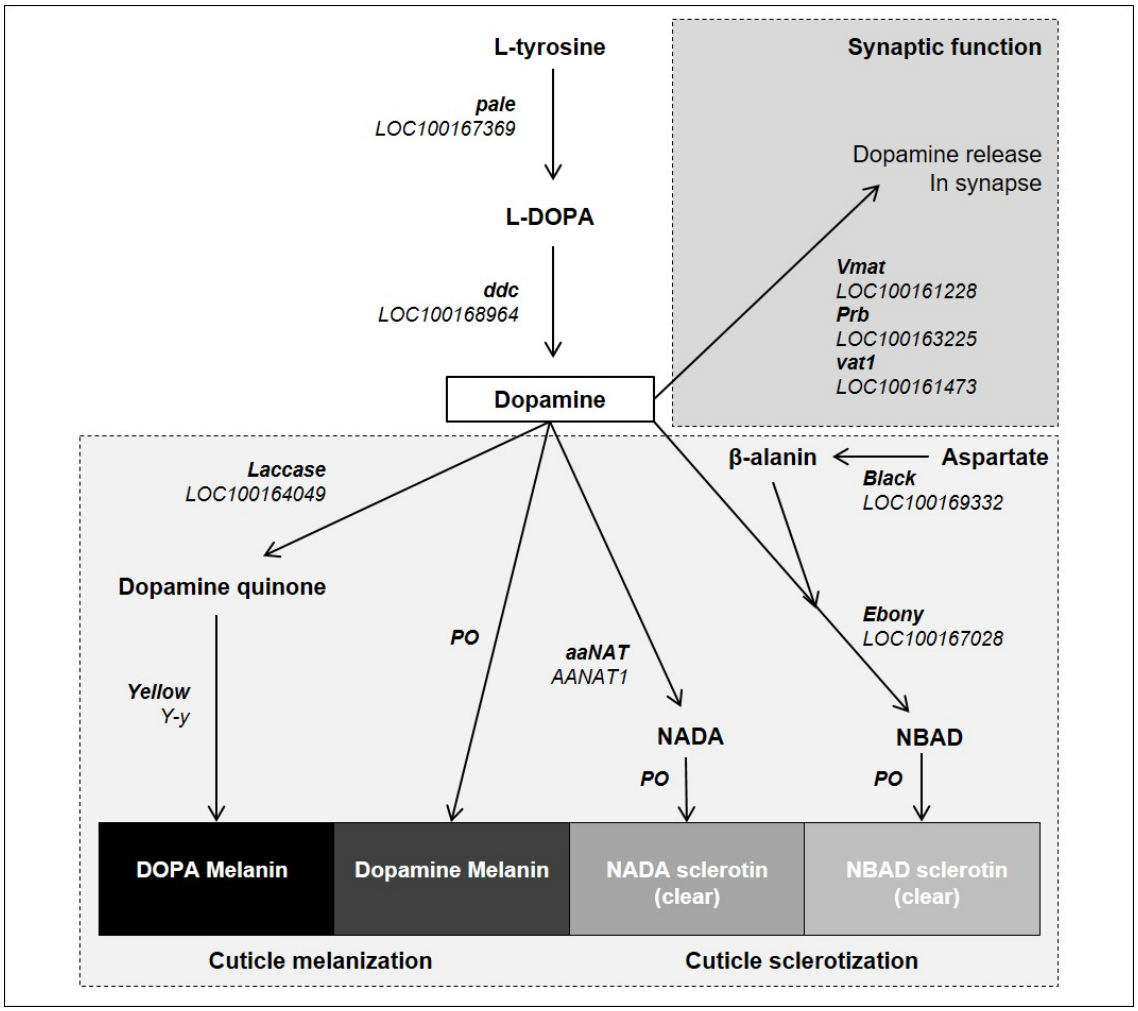


Figure 1 – Dopamine biosynthesis and mode of action pathway. L-tyrosine is first hydroxylised by *pale* (tyrosine hydroxylase) into L-DOPA which is then subsequently decarboxylised by *ddc* (dopa-decarboxylase) to produce dopamine. Dopamine can then directly be released within synapses and act as a neurotransmitter. Vesicle amine transporters such as *Vmat*, *prb* and *vat1* are likely to play a key role in this process. Dopamine can also be used as a structural component of the cuticle: either as DOPA melanin (black cuticle) when processed by *laccase* and *yellow* enzymes, or as NADA sclerotin (clear cuticle) through the action of *aaNAT* enzyme, or as NBAD sclerotin (clear cuticle) when conjugated with β-alanin through the activity of *ebony* and *black* (responsible for metabolization of aspartate into β-alanin) enzymes. In bold are indicated the names of the key enzymes functionally characterized in drosophila; just behind are indicated in italic type the name of the closest pea aphid homologue from the latest release of the pea aphid genome (v2.1).

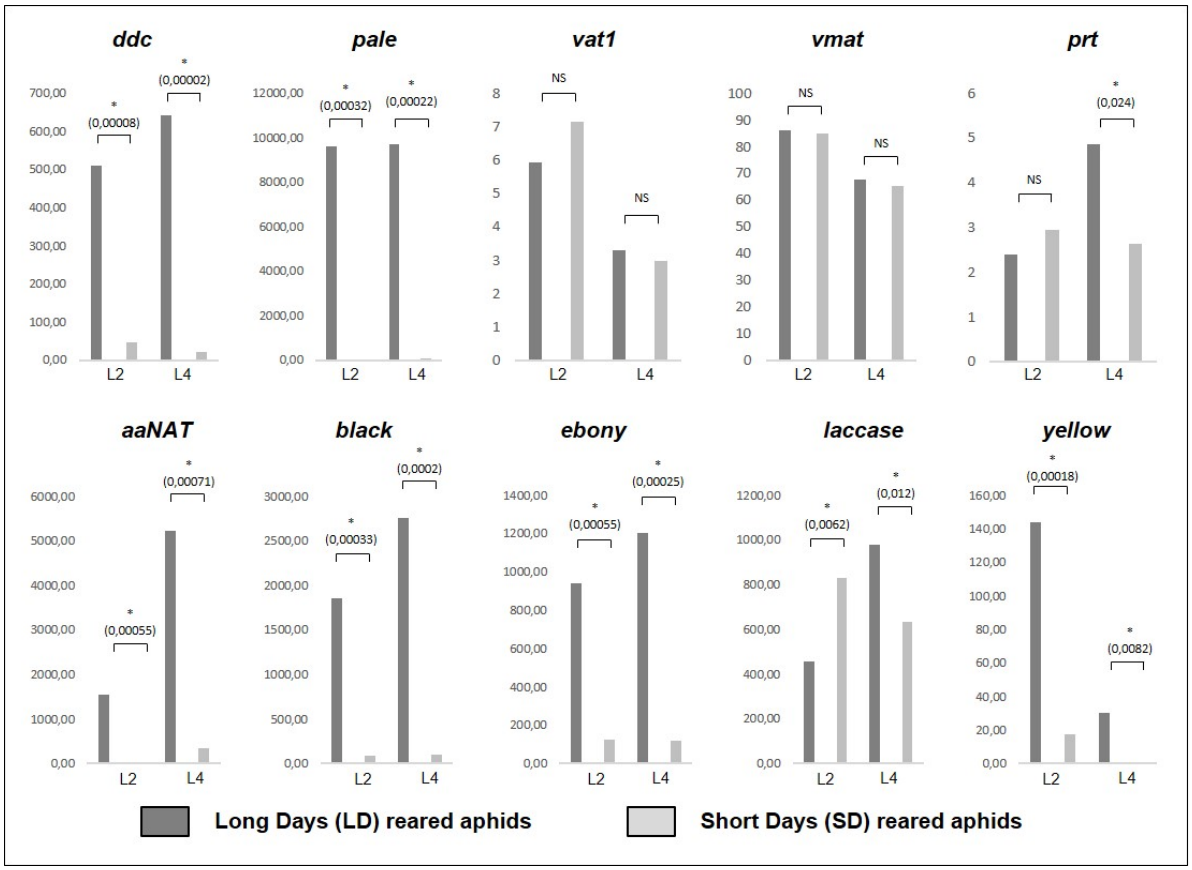


Figure 2 – Expression levels of dopamine pathway genes in L2 and L4 larvae heads of Long days (LD) and Short days-reared aphids. Based on a previous microarray analysis of the transcriptomic response in the heads of aphids when submitted to photoperiod shortening (Le Trionnaire et al., 2009), the same RNA samples were used for RNA-sequencing. The level of expression (expressed as RPKM) of dopamine pathway genes was extracted from this dataset and statistically analyzed to identify differentially expressed genes between the two photoperiod conditions (the p-value of the test is indicated between brackets). *: Significant, NS: Non-Significant.

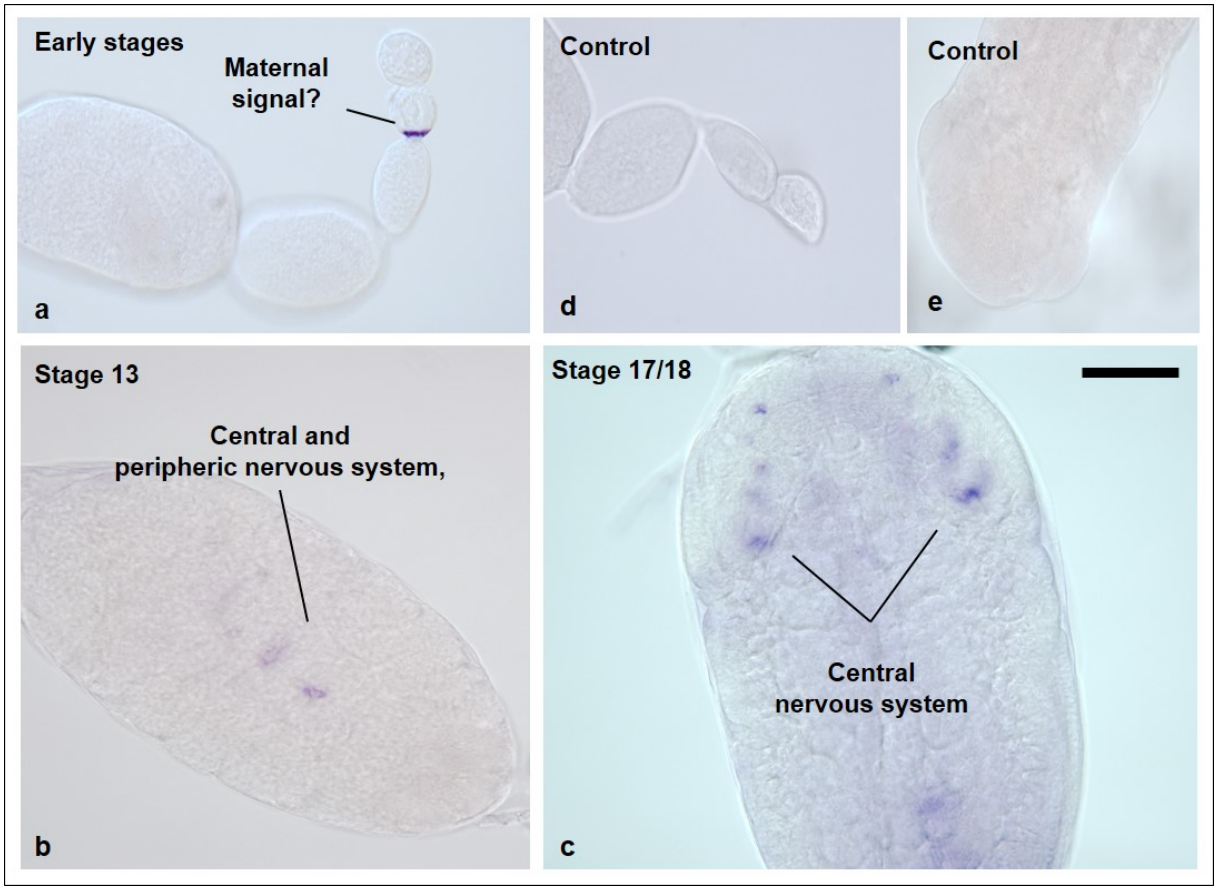


Figure 3 – *In situ* hybridization of *pale* transcripts within parthenogenetic embryos. Ovaries were dissected from parthenogenetic morphs and hybridized with antisense (a, b, c and d) or sense (e) probe.

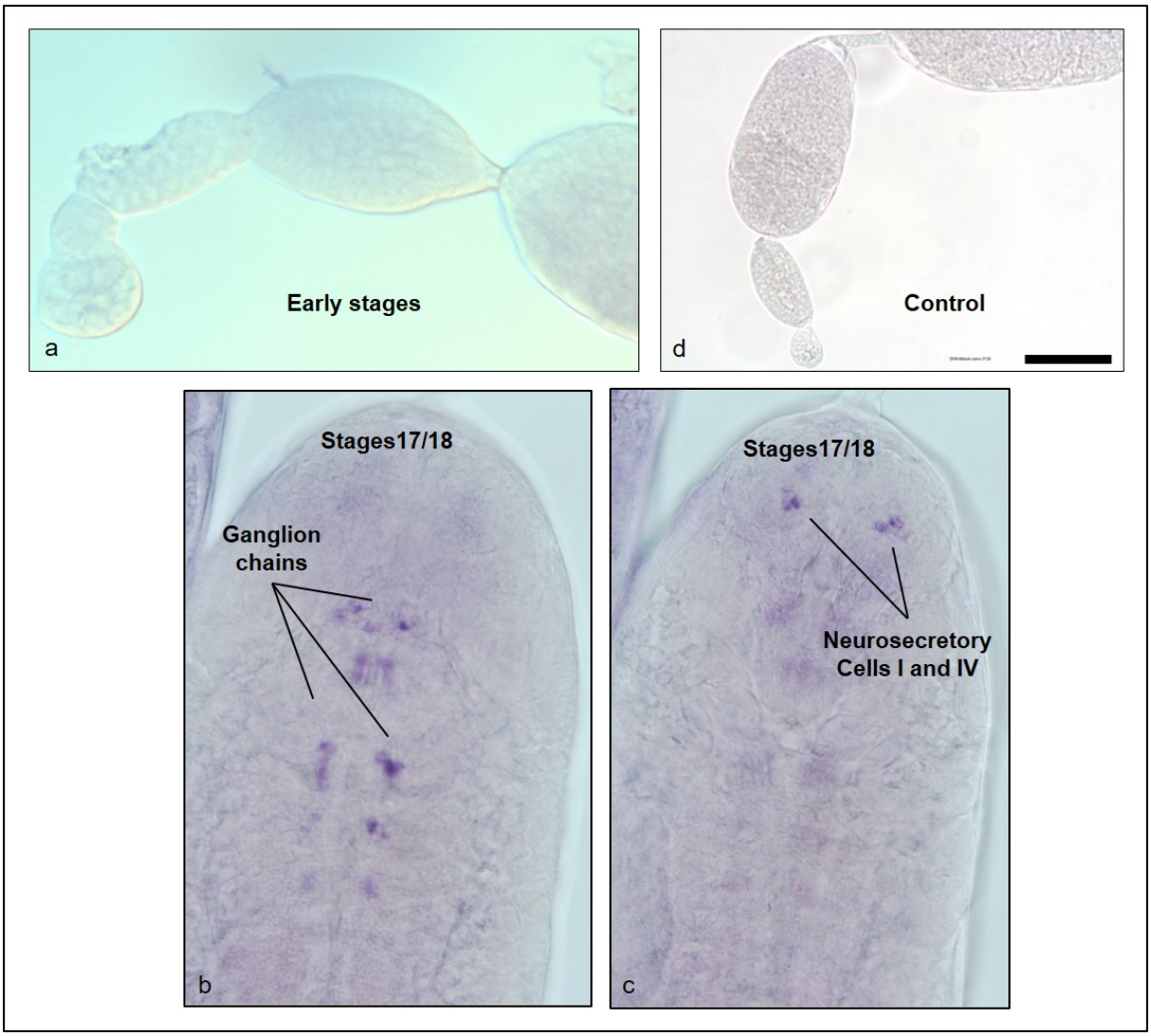


Figure 4 – *In situ* hybridization of *ddc* transcripts within parthenogenetic embryos. Ovaries were dissected from parthenogenetic morphs and hybridized with antisense (a, b and c) or sense (d) probe.

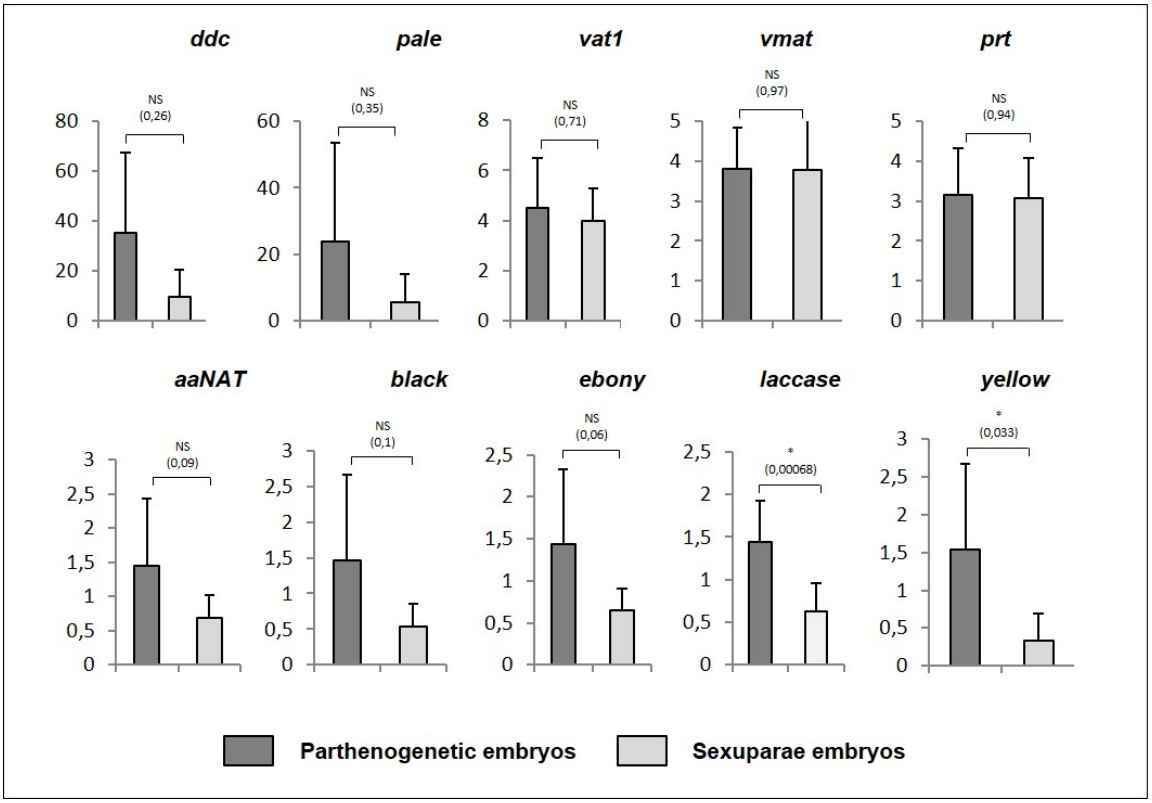
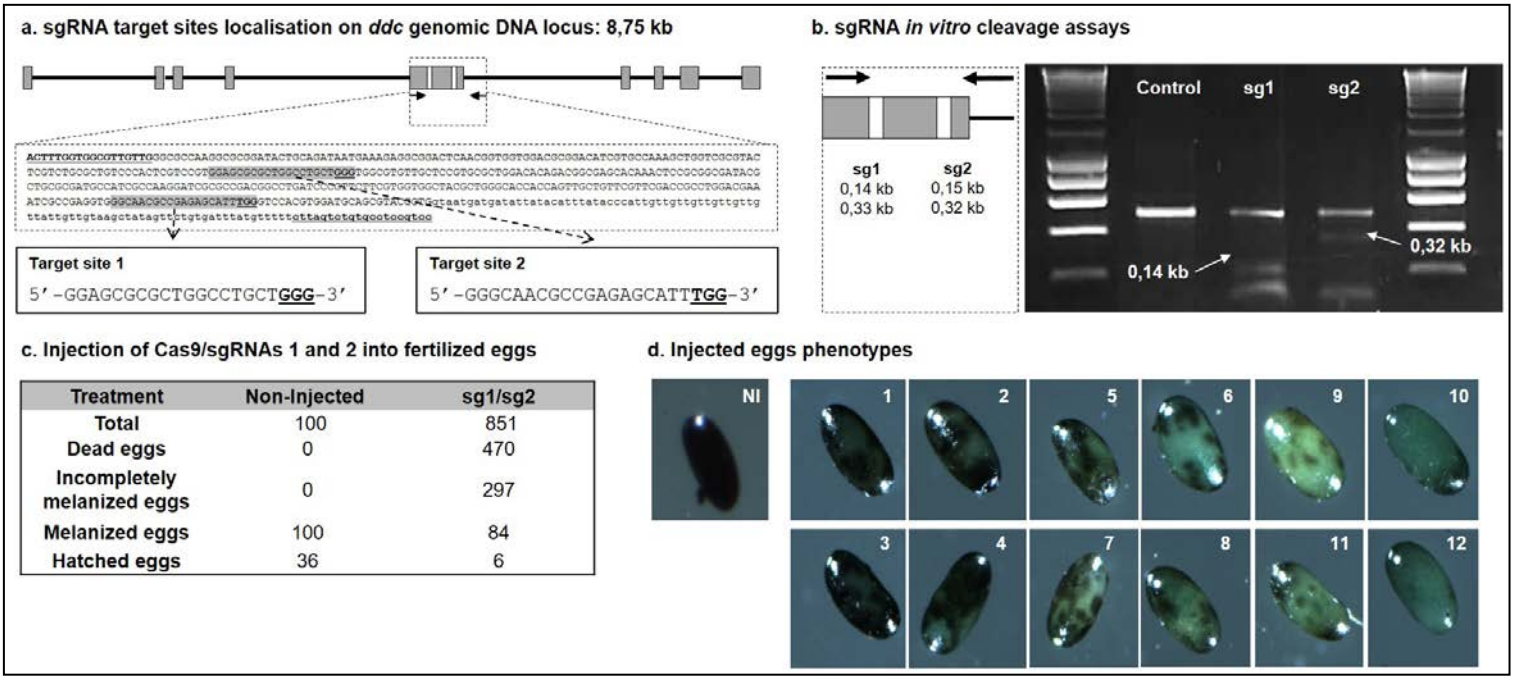


Figure 5 – Expression levels of dopamine pathway genes in parthenogenetic and sexuparae embryos. Most developed embryos from adult aphids placed under long days (parthenogenetic embryos) and short days (sexuparae embryos) were collected for the quantification of the level of expression of dopamine pathway genes. Relative quantification was performed using RpL7 as an invariant gene. Statistical test (which one…) was performed to estimate if the genes were significantly regulated between the two conditions (the p-value of the test is indicated between brackets). *: Significant, NS: Non-Significant.



Treatment	Non-Injected	sg1/sg2
Total	100	851
Dead eggs	0	470
Incompletely melanized eggs	0	297
Melanized eggs	100	84
Hatched eggs	36	6

Figure 6 – CRISPR-Cas9 editing of *ddc* gene and phenotypic defaults observed in injected fertilized eggs. a) The *ddc* coding sequence comprises 9 exons that are spread over a 8,75 kb genomic DNA region. Single guide RNAs were defined to target two regions (target site 1 and 2) within the 5th exon of the gene. On the 5th exon sequence are underlined the Forward and Reverse primers used to amplify the corresponding genomic DNA. **b)** *In vitro* cleavage assay were performed by mixing each sgRNA, the cas9 protein and the PCR product of the 5th exon. The sizes of the expected cleavage products are indicated with white arrows. **c)** This table compiles the results from the micro-injection within fertilized eggs of a sg1/sg2/Cas9 mix in terms of dead eggs, melanised eggs but also eggs with incomplete melanisation. Post-diapause hatching rates are also indicated, including the non-injected control eggs. **d)** A few days after injection, a large proportion of eggs failed to complete melanisation as it can be observed for some non-injected (NI) eggs. Defaults ranged from: i) a nearly complete melanisation (1, 2, 3 and 4), ii) a patchy distribution of dark and green (un melanised part of the eggs) spots (5, 6, 7 and 8) and iii) eggs that barely started melanisation to remain principally green (9, 10, 11 and 12).

Supplementary Tables

Table S1. Coding sequences of dopamine pathway genes charaterized in this study.

>ACYPI008168-RA|LOC100167369|**pale**
ATGGCAGTAGCAGCAGCCCAAAAGAGCCGAGAGATGTTCGCCATCAACAAGTCATATAGCATCGAGAACGGATATCCAGCGAGGAGACGTTCTCTCGTAG
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> ACYPI009626| LOC100168964|**ddc**
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> ACYPI062462| LOC100161473|**vat1**
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>ACYPI004325| LOC100163225|**prt**
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> ACYPI002468| LOC100161228|**Vmat**
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> ACYPI002543| AANAT1|**aaNAT**
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> ACYPI009960| LOC100169332|**black**
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GTTCAGATTTGTAA

> ACYPI007852| LOC100167028|**ebony**
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> ACYPI005091| LOC100164049|**laccase**
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TCTGGGTTTGGAAgtATTTCTGAATCGTCGCACGGTAATTTGCCGCCTTTAGGATATTCATCGCTAGACTTCGACAAGAGAGGTCTCGACGAATTGCGTA
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CCAATTCGACACCAACAGTGGCACGCATTTCTGGCACGCCCATTCCGGACTTCAAAAAATTGATGGTATCTACGGTAGTATTGTTGTACGACAACCACCA
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>ACYPI003242| Y-y|**yellow**
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ACAGTTTGTGCAGGCAATATTCCTTATATTCCACAGGAACAATCAAATTATGTCACATCTAAGGTTTATTAA

Table S2 - Primers used for Q-RT-PCR.

Primer name	Gene ID	Primer sequence	Amplicon length
***pale*-1F**	LOC100167369	CTCGGTTTAGCGTCTCTTGG	303
***pale*-1R**		CATGGATGAAACCCATTTCC	
***ddc*-1F**	LOC100168964	AATTGGTGTCGCAAACCTTC	309
***ddc*-1R**		CCGGCTTCATACACTTCGTT	
***Rpl7*-F**	RpL7	GCGCGCCGAGGCTTAT	81
***Rpl7*-R**		CCGGATTTCTTTGCATTTCTTG	
***vat1*-F**	LOC100161473	GAACCTCGTTGGACTTGGAA	160
***vat1*-R**		ACCGAGTGAATAGGCTGACG	
***prt*-F**	LOC100163225	GTAGACTCTGCTCTGGTCCC	138
***prt*-R**		TTGACCGCCCAAAATAGGAC	
***Vmat*-F**	LOC100161228	TTCACGAAACGGTTGCAGTT	181
***Vmat*-R**		AATACCCCGTAACTCCGTCC	
***aaNAT*-F**	AANAT1	TCCGCGATGAACCATTGAAC	128
***aaNAT*-R**		ACCAGTTTGAGATACCGCCA	
***black*-F**	LOC100169332	CATAAGCACCTGTTGACCGG	92
***black*-R**		AACGTTGAGCACTGTTGAGG	
***ebony*-F**	LOC100167028	CCCAAACACAAAGACGACGT	108
***ebony*-R**		ATCGGTGTACTCGCTGTAGG	
***laccase*-F**	LOC100164049	GGAGACAACAAGCCAGCAAA	138
***laccase*-R**		GTGCAGATGGAACGGATGAC	
***yellow*-F**	Y-y	GTACGCGTCTGATGAGCTTG	222
***yellow*-R**		CTGGCTAGTGGGTGGAAGAA	

Table S3 - Primer used for *in situ* hybridization

Primer name	Gene ID	Primer sequence	Probe length
***pale*-F**	LOC100167369	GCAGACCAAACATTCCGTTT	1374
***pale*-R**		TCAGTGCGGTGTTTAGATGC	
***ddc*-F**	LOC100168964	TGGTCGCTGACATACTGAGC	973
***ddc*-R**		GGATCCCTTAAGTCGGAAGC	