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Dormancy awakened: aminergic control of diapause in Drosophila

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A mio nonno Natale, a Nathan e ai miei genitori.

… sopra la porta d'ingresso era appeso
un enorme cartello: ASTRONOMI. Lei ci passò sotto.
"Ok" pensò ironicamente "ora sono un'astronoma".
da "*il 4% dell'universo*", Richard Panek, Codice edizioni.

Contents

Abstract

Coping with adverse environmental conditions is one of the most crucial challenges for all living beings. The coupling between external cues and hormonal signaling is key to allow survivorship of individuals and insects in particular have been intensively studied to better understand this connection. Although the hormonal cascade that promotes insect development and reproduction is well known (insulin signaling - juvenile hormone – 20-Ecdysone), how this neuroendocrine axis is modulated by environmental stimuli remains still largely elusive. To deepen the molecular features of IIS-JH-20E axis regulation, we focused our attention on one of the best examples of physiological strategies triggered by environmental stimuli, diapause. Diapause is an inducible developmental arrest, which characterizes the life cycle of several species, from *Caenorhabditis elegans* to mammals. We have investigated in detail how adverse environmental signals are transduced molecularly to impair the IIS-JH-20E axis in the model organism *Drosophila melanogaster*. Two fundamental aspects need to be clarified: the regulation of neural insulin signaling (IIS) and the role of dopamine in insect dormancy. IIS down-regulation is necessary to enter diapause in several species, and in the fruit fly we know at least some of the molecules that regulate negatively brain insulin-like peptides (DILPs) expression/release (i.e. serotonin and GABA). First we demonstrated the key role of serotonin in modulating IIS under diapause-promoting conditions. The over-stimulation of serotonergic neurons leads to an increase in diapause levels, while the knock-down of the 5HT-1A serotonin receptor in the insulin-producing cells (IPCs) dramatically decreases diapause levels. Moreover, it is well known that GABAergic signaling inhibits neural IIS in Drosophila according to the feeding state (sensed by fat bodies, FB). Furthermore, a role for FB metabolism as a strong diapause modulator has been proposed in the cotton bollworm, *Helicoverpa armigera*. For this reason, we down-regulated GBR (metabotropic GABA receptor) on the IPCs, observing only slight phenotypic changes, whereas, the over-expression of *Upd2* (a molecule secreted from the FB which inhibits the IIS-suppressing GABA release, therefore promoting DILPs release) in the FB provided no effects on diapause levels.

Even dopamine seems to play an evolutionary conserved regulatory role in insect dormancy. We confirmed its relevance as a diapause-promoting factor, and extended its regulatory role also to reproductive/adult dormancy. Subsequently, we impaired Protein Kinase A (PKA) activity (increased also by dopamine via Dopamine Receptor 1, DopR1) within the *corpus allatum* (CA, the neurohemal gland that synthesizes JH) and the FB, and we observed a marked drop in diapause levels. A convergent result was obtained down-regulating *DopR1* in these tissues. This could represent the first direct evidence about a mechanism by which dopamine can modulate insect dormancy.

In conclusion, we propose a double regulation of the IIS-JH-20E axis induced by low temperatures, with serotonin that regulates IPCs physiology, whereas dopamine acts on downstream key metabolic organs such as the CA and the FB to regulate JH release and impair vitellogenesis, respectively. Our results shed new light on the regulation of key

neuroendocrine pathways for growth and development, and suggest how organisms couple environmental conditions with inner hormonal physiology.

Abstract italiano

Sopravvivere a drastici cambiamenti climatici rappresenta una delle sfide principali per gli esseri viventi. Gli insetti non solo forniscono esempi straordinari di adattamenti morfologici e fisiologici a climi sfavorevoli, ma sono anche molto studiati per comprendere quali sono i meccanismi molecolari responsabili di questi adattamenti. Negli insetti, i principali processi ormonali che promuovono lo sviluppo e la riproduzione sono ben noti e comprendono tre attori principali, il segnale insulinico (IIS), l'ormone giovanile (JH) e l'idrossi-ecdisone (20E). Nonostante questo importante asse neuroendocrino (IIS-JH-20E) sia ben studiato, poco si conosce riguardo i meccanismi molecolari che trasducono le informazioni ambientali ai componenti fondamentali del sistema endocrino, modulandone l'attività regolatoria. Per questo abbiamo rivolto la nostra attenzione ad uno degli esempi più interessanti di strategie fisiologiche evocate dagli stimoli esterni, la diapausa, un arresto dello sviluppo inducibile che rappresenta un evento estremamente diffuso nel regno animale. Al fine di comprendere appieno questo fenomeno, due sono gli aspetti principali che rimangono ancora da chiarire, la regolazione delle cellule neurosecretrici (IPCs) responsabili del rilascio degli "insulin-like peptides" (DILPs) ed il ruolo della dopamina nella regolazione della diapausa negli insetti. La riduzione dei livelli di IIS è un requisito necessario in molte specie per entrare in dormienza, e in Drosophila si conoscono almeno alcune delle molecole responsabili dell'inibizione dell'espressione e/o del rilascio dei DILPs (ad esempio serotonina e GABA), ed è sui potenziali effetti sulla diapausa di queste vie di segnalazione che abbiamo focalizzato la nostra attenzione. La stimolazione dei neuroni serotonergici, indotta geneticamente, induce un aumento dei livelli di diapausa, mentre al contrario, la riduzione dell'espressione del recettore per la serotonina *5HT-1A* nelle IPCs provoca un abbassamento delle percentuali di diapausa. E' stato dimostrato che il GABA è in grado di inibire il rilascio dei DILPs a seconda dello stato nutrizionale dell'individuo, ed inoltre, è stato suggerito che i corpi grassi (strutture che svolgono un ruolo chiave nell'accoppiare lo stato nutrizionale con la regolazione ormonale dello sviluppo) siano in grado di controllare l'ingresso o il mantenimento dello stato di dormienza nel lepidottero *Helicoverpa armigera*. Al contrario, inibendo l'espressione di *GBR* (recettore metabotropico del GABA) nelle IPCs, si osservano solamente lievi variazioni fenotipiche, mentre l'incremento dell'espressione di *Upd2* (una molecola secreta dai corpi grassi che inibisce l'azione del GABA sulle IPCs promuovendo il rilascio dei DILPs) nei corpi grassi non ha prodotto alcun effetto sui livelli di diapausa. Negli insetti, la dopamina sembra conservare un ruolo cruciale nella regolazione della dormienza. Abbiamo confermato la sua importanza come fattore che promuove la diapausa, estendendone l'attività regolativa anche alla diapausa riproduttiva. Successivamente, abbiamo compromesso l'attività del PKA signaling (innescato anche dal dopamine Receptor 1, DopR1) nel *corpus allatum* (la ghiandola neuroemale che sintetizza JH) o nei corpi grassi, riscontrando un drastico abbassamento delle percentuali di diapausa. Questi risultati sono stati successivamente riprodotti anche riducendo l'espressione di *DopR1* negli stessi tessuti, fornendo le prime evidenze riguardo un meccanismo mediante il quale la dopamina può modulare la dormienza

negli insetti. In conclusione, proponiamo un modello in cui una doppia regolazione dell'asse neuroendocrino IIS-JH-20E è innescata dalle basse temperature, con la serotonina responsabile della modulazione di IIS, mentre la dopamina agisce su altri organi chiave come il *corpus allatum* ed i corpi grassi per regolare rispettivamente il rilascio di JH e ridurre la vitellogenesi. I nostri risultati forniscono un contributo alla comprensione degli aspetti regolativi dei meccanismi neuroendocrini fondamentali per la crescita, lo sviluppo e la riproduzione, e suggeriscono alcune modalità con le quali gli insetti accoppiano la percezione delle condizioni ambientali con la loro fisiologia ormonale.

1. Introduction

1.1 Diapause

Diapause is an actively-induced developmental arrest (dormancy) that characterizes the life-cycle of several organisms from *C. elegans* to mammals. This phenomenon can be facultative or obligate and the dormant stages are species-specific; it occurs to prevent a developmental transition according to environmental cues perceived during earlier developmental stages, which signal to individuals whether an adverse seasonal condition is coming (Saunders *et al*., 2002). In several species, diapause is elicited to prevent desiccation (for instance, the aestivation of *Antheraea yamamai*) (Suzuki *et al*., 1990), starvation (*C. elegans*) (Wadsworth and Riddle, 1989) or to face hypoxia (killifish) (Myers, 1952; Wourms, 1972), but especially in insects, this phenomenon is mainly an overwintering strategy. In the latter case, diapause relies on the perception of reliable cues such as short photoperiods and low temperatures, typical features of autumn/early winter. Diapause onset, diapause termination, as well as the competence of the specific stage that will enter diapause and will overwinter, require genetically encoded mechanisms (Tauber *et al*., 1986), which can be different among species, although, as we will see, they comprise common hormonal signaling pathways. Diapause is not merely a block in development, but rather a complex physiological strategy considered a distinct life-history stage, which also increases survival potential as well as hibernation potential (promoting the synthesis of cryoprotectants, see Kostal *et al*., 2011; Li *et al*., 2014; Guz *et al*., 2014), regulates energy storage consumption, improves stress resistance, and reduces metabolic rate (see Hahn and Denlinger, 2007; 2011). Metabolic suppression during diapause varies among insects according to the intrinsic characteristics of the dormant stage: a little suppression of metabolic rate affects migrant diapausing individuals of the monarch butterfly (Chaplin and Wells, 1982), while in immobile pupal diapauses, even a 90% shut-down can be reached (Denlinger *et al*., 1972). In some extreme cases (like in the Colorado potato beetle, *Leptinotarsa decemlineata*), reduction in metabolic costs expects even the consumption of energetically costly tissues like flight muscles and the digestive tract (de Kort, 1990). Especially in those diapauses in which individuals do not feed (because of the developmental stage, if eggs or pupae undergo diapause, or, although diapause occurs during stages capable of feeding, if nutrient intake is reduced or even blocked), store a proper amount of nutritional reserves prior to diapause is key for survival. For instance, in the pink bollworm, *Pectinophora gossypiella*, diapause-destined individuals store 50% lipid reserves more if compared with non-diapausing ones as larvae (Adkisson *et al*., 1963), whereas in the mosquito, *Culex pipiens*, about twice lipids and carbohydrates stores are present in diapausing females with respect to their non-diapausing counterpart (Mitchell and Briegel, 1989). The most widely used molecules to improve cryopretective potential in insects are represented by sorbitol and glycerol, but some species synthesize also other polyols like ethylene glycol, erythritol, mannitol, ribitol, and threitol, or sugars such as trehalose and glucose (Denlinger

and Lee, 2010). In *Bombyx mori*, resistance to cold (also at -32°C) relies on the synthesis of glycerol and sorbitol (Horie *et al*., 2000; Lee and Denlinger, 1991), whereas trehalose plays an analogous role in Drosophila (MacRae, 2010). Sorbitol and glycerol are employed as cryoprotective agents also in the goldenrod gall fly, *Eurosta solidaginis*, and in the flesh fly, *Sarcophaga crassipalpis* (Storey and Storey, 1986; 1990; Denlinger and Lee, 2010). Interestingly, in both *Hyalophora cecropia* and *Philosamia cynthia*, a temperature-dependent activation under cold conditions of the glycogen phosphorylase (the enzyme that allows glycogen stores degradation, in this case to provide precursors for glycerol synthesis) occurs (Ziegler *et al*., 1979; Hayakawa and Chino, 1982). The most noteworthy examples about freezing tolerance and cryoprotectants role regards the drosophilid, *Chymomyza costata*, which enters a facultative diapause as mature third instar larva under short days (Riihimaa and Kimura, 1989). *C. costata* larvae exhibit an outstanding freeze tolerance that allows them to supercool between -15°C and -25°C but also to survive after immersion for 1 h in liquid nitrogen (-196°C) (Moon *et al*., 1997). Falling in diapause has been demonstrated to be fundamental to ensure high levels of survival to liquid nitrogen (an ability enhanced by cold acclimation), and a metabolites screening identified proline as a potential key cryoprotectant. During diapause transition and/or cold acclimation, the concentration of this amino acid raises from 20 to 147 mM, and increasing its levels by feeding larvae with a proline-enriched diet improves their freeze tolerance (Kostal *et al*., 2011). It has been suggested that high levels of proline, coupled with freeze dehydration, determine a water glass-like transition, giving rise to a physical state which helps to avoid cryoinjury. In conclusion, it seems to be present a direct correlation between proline content (either natural or artificial) and survival to adult stage for diapausing larvae of *C. costata*. Enhanced cold tolerance can be achieved also through modifications of biophysical properties of lipids in several tissues induced by diapause developmental program. In *Ostrinia nubialis*, fat bodies fatty acids in diapausing and non-diapausing individuals (larvae) show different features, and in general, in the first a higher level of unsaturation (a crucial characteristic to maintain membrane properties and to allow an effective lipid metabolism) occurs (Vukašinović *et al*., 2013). A similar change occurs also in *C. costata* to enhance freeze-tolerance: changes in phospholipids composition has been found in fat bodies and muscles cells of larvae in response to chilling (5°C) and diapause entry, as well as a concomitant decrease in the proportion of saturated fatty acids (Kostal *et al*., 2003). Thus, holometabolous insects provide the vast majority of diapause examples and, for this reason, we will focus our attention especially on them. In this taxon, all developmental stages can be exploited to overwinter, egg/embryo, larva, pupa or adult/imago, and below some examples are reported.

1.1.1 EMBRYONIC DIAPAUSE

Embryonic diapauses can be divided into two subgroups, depending on whether individuals undergo diapause as early embryos or as pharate first instar larvae (“hatch-ready” larvae) (Schiesari and O’Connor, 2013). In spite of this distinction, all of these dormancies rely on

ecdysone signaling modulation, even if the direction of the effects varies according to the developmental stage in which diapause occurs (Saunders *et al*., 2002; Denlinger *et al*., 2012). In the latter phenomenon, in the studied species, ecdysone promotes the developmental arrest, as described for *Lymantria dispar*, *Thymelicus lineola* and *A. yamamai* (Denlinger and Lee, 1997; McNeil and Fields, 1985; Suzuki *et al*., 1990). Dormant individuals of the gypsy moth, *L. dispar* terminate diapause solely after exposition to the cold, a stimulus necessary to reactivate growth (Schiesari and O'Connor, 2013). High levels of ecdysone induce and maintain diapause state in this species: ecdysone treatment prevents chilled/activated embryos to terminate diapause also if they were exposed to optimal conditions; on the other hand, the injection of an ecdysone antagonist impedes individuals to fall in dormancy (Lee and Denlinger, 1996; Denlinger and Lee, 1997). In *A. yamamai*, prolonged ecdysone pulses trigger diapause entry, while the shut-down of its signaling, as well as head-thorax excision from diapausing embryos, leads to precocious reactivation of development (Suzuki *et al*., 1990). On the contrary, whereas in these species diapause induction and maintenance is based on a general increase of ecdysone titers, in other embryonic dormancies, where an earlier arrest occurs (mid-embryonic development), diapause induction is caused by the impedance of the ecdysone pulse at this stage (Schiesari and O'Connor, 2013). The most documented example about this phenomenon concerns the silk moth, *Bombyx mori*: in nature, females that developed as embryos and larvae under long days and warm temperatures eclose in autumn and will lay melanized diapausing eggs (Schiesari and O'Connor, 2013). Also in this type of developmental arrest, a genetically defined period in the cold (2-3 months at 5°C) is needed for the termination of dormancy (Horie *et al*., 2000); this process depends on the restoration of ecdysone signaling, which relies solely on the action of the enzyme ecdysteroid-phosphate-phosphatase (EPPase) in transforming the reserves of inactive ecdysone-phosphate into its active form (Fujiwara *et al*., 2006). The activation of the EPPase is promoted by ERK signaling, a pathway responsible also for the activation of another key enzyme for Bombyx diapause termination, sorbitol dehydrogenase-2 (Iwata *et al*., 2005). This enzyme converts sorbitol (a cryoprotectant factor that is accumulated in diapause-destined eggs) into glycogen, which will provide the energy pool for developmental progression. Sorbitol does not act only as a cryoprotectant, but it appears also to cover a regulatory role, given that diapause induction can be elicited by the application of this molecule, whereas its absence from dormant embryos causes their precocious developmental reactivation (Horie *et al*., 2000). In 1951, Fukuda noted that the removal of the suboesophageal ganglion (SOG) from pupae, exposed during their development to diapause-promoting conditions, inhibited future females to lay dormant eggs, while the transplant of SOG from diapause-conditioned pupae into non-diapause-conditioned pupae induced the latters to lay diapausing eggs (Hasegawa, 1951). This suggests the possible existence of a SOG-released factor which action stimulates diapause induction. In subsequent decades, this key actor has been characterized and identified as diapause hormone (DH), a peptide that, produced by future females pupae, destines embryos to diapause (Nakagaki *et al*., 1991; Sato *et al*., 1994; Shiomi *et al*., 2007). Once released in the haemolymph, DH binds to the DH receptor located on developing eggs, increasing cryoprotectant leves (sorbitol and glycerol) (Homma *et al*., 2006; Horie *et al*., 2000), and

promoting thus diapause entry. The understanding of the molecular mechanism linking the perception of environmental (thermal) stimuli and neuroendocrine actors modulating diapause has been recently fueled thanks to the discover that in *B. mori*, the thermosensitive transient receptor potential TRPA1 affects diapause induction. In the silk moth, diapause can be elicited in laboratory conditions also exposing eggs to 25°C in DD (but no diapause has been observed at 15°C in DD) (Watanabe, 1924), underlying the strong thermosensitive feature of this response. The authors recorded the activation of BmTRPA1 under temperatures above ~21°C and surprisingly, the injection of dsRNA of *BmTRPA1* within embryos incubated at 25°C induces them to lay non-diapausing eggs once adult females. Moreover,

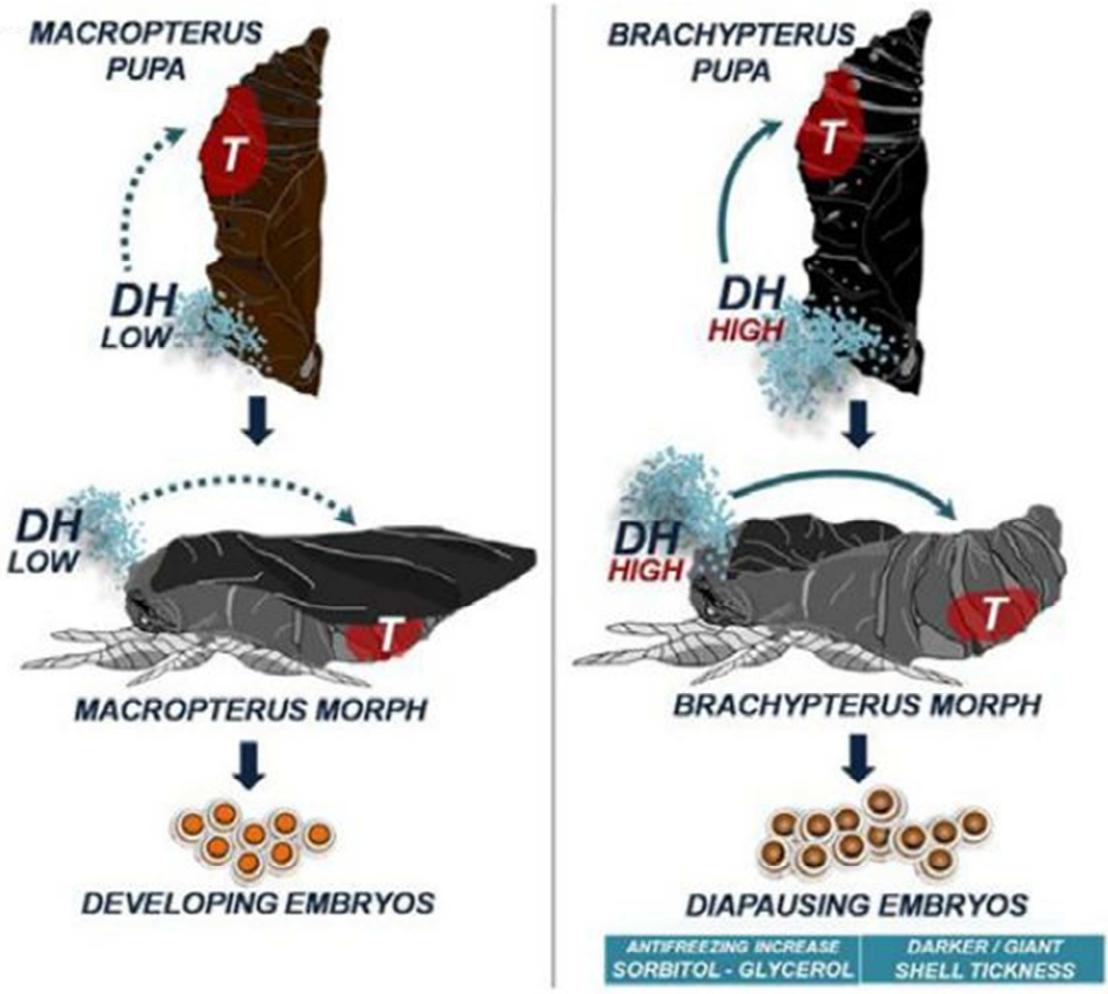

Figure 1. Normal and diapause-destined development in *O. thyellina* with the associated seasonal and stage-specific morphs. DH=Diapause hormone, T=target organs (from Schiesari and O'Connor, 2013).

immunohistochemical evidences demonstrated that the effect of the down-regulation via RNAi of *BmTRPA1* affects DH release during pupal-adult development (Sato *et al*., 2014). Interestingly, DH seems also involved in regulating Bombyx wing diphenism as well as energetic demands; indeed, DH injection in non-diapausing pupae leads to autumnal (with darker wings) or intermediate morphs (Yamanaka *et al*., 2000), whereas its up-regulation prolongs feeding period in diapause-programmed larvae (Xu *et al*., 1995). Another example

of DH modulation of dormancy regards the tussock moth, *Orgyia thiellina*. This species is characterized by a seasonal polyphenism, with a winged *Macropteres* (summer morph) that eclosed from pale pupae when larvae are reared under long days; on the contrary, under short days, from dark pupae a short wings autumnal morph called *Brachypteres* will hatch. While the first morph lays developing embryos, the second one deposits darker and heavier eggs in a dormant state (Kimura and Masaki, 1977) (Fig. 1). Also in this species, diapause is induced transgenerationally as a maternal effect, and also in this case by DH: the injection of DH in *Macropteres* pupae gives rise to females which will lay diapausing eggs, whereas *Brachypteres* pupae injected with a DH antagonist will lay developing embryos after eclosion (Uehara *et al*., 2011). The examples reported, although with the recognized differences, underline common pathways and actors involved in embryonic diapauses from several insect species. Recently, new pathways which regulate two key hormones for insect development and diapause have been identified in *B. mori*. Short Neuropeptide F (sNPF), a neuropeptide that promotes Insulin-like peptides (DILPs) expression in Drosophila (Lee *et al*., 2008), has been identified as a negative regulator of JH. sNPF synthesized by the *corpus cardium* seems to be involved in a fine-tuned stage-specific antagonistic action on JH production, and its expression is increased counter-intuitively by allatotropin (Kaneko and Hiruma, 2014). On the contrary, ecdysone synthesis has been demonstrated to be promoted by Pigment Dispersing Factor (PDF), a neuropeptide which represents an output of the circadian clock (Park *et al*., 2000). The authors suggested that PDF could affect ecdysone signaling through a translational and/or post-translational regulation, activating both Protein Kinase A (PKA) and phosphatidylinositol 3-kinase/Target of Rapamycin (PI3K/TOR) signaling pathways (Iga *et al*., 2014).

1.1.2 LARVAL DIAPAUSE

Some insects can overcome an unfavourable season eliciting a diapause phenomenon as larvae, an event that will block development preventing pupation and metamorphosis, and maintaining individuals in larval stages. Given that high levels of JH characterize insect larval life, and only a drop in its titers allows ecdysone pulse to initiate metamorphosis, it is plausible to expect an involvement of a prolonged JH action or a failure of ecdysone signaling in triggering larval diapause, and in fact, both mechanisms are employed. An example of larval diapause is represented by the pyralid, *Diatraea grandiosella*. Diapause entry in this moth is accompanied by a modification in the body colour pattern after the last larval instar, when the normal “spotted” morph (with direct/normal development) molts into an immaculate (diapausing) one (Schiesari and O’Connor, 2013). Although the latter morph maintains its size during subsequent possible extra “stationary” molts, its physiology dramatically changes, suppressing feeding, lowering respiration and improving dehydration, lipid storage and cold resistance (Chippendale, 1977; 1984). As in many other species which show larval diapause, also in *D. grandiosella* the block of development occurs via JH action, which, like during normal/direct development, prevents ecdysone signaling from triggering metamorphosis,

promoting larval-larval molts; instead, normal developmental progression in immaculate larvae is allowed just after the drop in JH levels (Fig. 2). Indeed, ecdysone injection into immaculate/diapausing larvae stimulates solely stationary molts (immaculate-immaculate) and not metamorphosis, because of JH activity, whereas JH application on "spotted"/non-diapausing individuals elicits the comparison of the immaculate/dormant morph. (Chippendale, 1977; 1984; Yin and Chippendale, 1973; 1974; 1979). A similar phenomenon

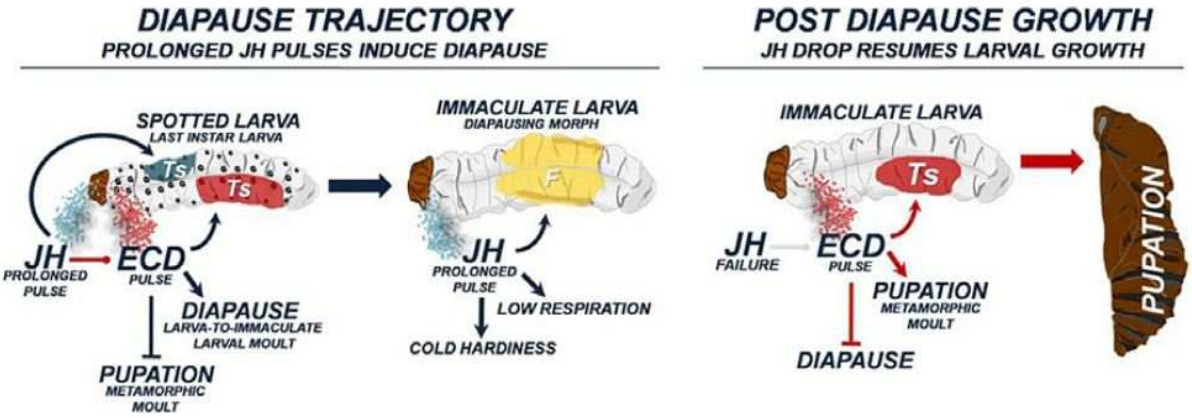

Figure 2. Endocrine mechanisms underlying diapause induction and diapause termination in *D. grandiosella*. JH=juvenile hormone, ECD=ecdysone, Ts=target organs, F=fat accumulation (from Schiesari and O'Connor, 2013).

of stationary molts, without an increase in size, occurs in *Sesamia nonagriodes*, with up to 12 events. Also in this noctuid, high levels of JH during larval diapause impede metamorphosis, but instead of promoting this developmental transition, the application of an ecdysone analog stimulates diapausing larvae to accelerate stationary molts (Eizaguirre *et al*., 1998; Eizaguirre *et al*., 2005). In larval dormancies which do not include extra molts, the loss of ecdysone signaling rather than high levels of JH seems to be responsible for diapause maintenance. In *Ostrinia nubialis*, JH injection in larvae did not elicit a developmental arrest, while injecting ecdysone in diapausing larvae, they undergo pupation (Denlinger *et al*., 2012; Schiesari and O'Connor, 2013). It is interesting to note that in larval diapause of both *Laspeyresia polmonella* and *O. nubialis*, the impairment of ecdysone signaling seems to be due to a suppression in prothoracicotropic hormone (PTTH) signaling (Saunders *et al*., 2002; Denlinger *et al*., 2012; Schiesari and O'Connor, 2013).

It is noteworthy the case of the parasitic wasp, *Nasonia vitripennis*, which shows a maternally-induced larval diapause. The adult female is the sensitive stage and if short photoperiods are perceived, 4th instar larvae of its progeny will enter diapause (Saunders, 1965). Clinal differences in photoperiodic perception have been demonstrated to be established in Europe. Northern populations exhibit an anticipate switch-point (the time in which females begin to give birth to diapause-destined progeny), higher levels of diapause within populations and longer critical photoperiods (the day-length at which diapause response is elicited in the 50% of a population, Kurota and Shimada, 2003; Wang *et al*., 2012; Paolucci *et al*., 2013).

1.1.3 PUPAL DIAPAUSE

Although there are some evidences in other insects, such as the flesh fly *Sarcophaga bullata*, which pupae enter diapause if these individuals perceive, as embryos or 1st instar larvae, short photoperiods (<13,5h of light per day) (Denlinger, 1972), the major part of the information related to pupal diapause comes from Lepidopterans. In pupal diapauses, the hallmark is represented by the prevention of metamorphosis completion through the suppression of ecdysone action, which peak marks the beginning of this process (Riddiford *et al*., 2000; Thummel, 2001) (see Fig. 3). In the hornworm moth, *Manduca sexta*, the perception of short days during larval period induces a developmental switch that will lead to dormant pupae. Although, in spite of short photoperiods, ecdysone levels remain unchanged during larval development, they markedly decrease in diapause-destined pupae, and this phenomenon prevents the continuation of metamorphosis (Bowen *et al*., 1984; 1985; Smith *et al*., 1986; Schiesari and O'Connor, 2013). A direct evidence of the prominent role of low ecdysone levels in triggering pupal diapause has been provided in *M. sexta* and *Pieris brassicae*: the injection of an ecdysone agonist in diapausing individuals of the first species stimulates the termination of dormancy (Sielezniew and Cymborowski, 1997), whereas the excision of the prothoracic gland (PG, which represents the main source of ecdysone during larval and pupal development) provokes the comparison of a permanent diapause in the second (Calvez, 1976; Pullin and Bale, 1989). In insects, ecdysone synthesis is stimulated by PTTH-producing neurons (Smith and Rybczynski, 2012), which likely connect the PG with higher integrational brain areas; thus, it is plausible to expect a block of PTTH signaling as one of the crucial points in in pupal diapause induction (Saunders *et al*., 2002; Denlinger *et al*., 2012; Smith and Rybczynski, 2012). Also in this case, evidences have been extensively provided by Lepidopterans: in both *M. sexta* and *Helicoverpa armigera*, *ptth* expression drops in dormant pupae (Xu and Denlinger, 2004; Wei *et al*., 2005), while the injection of PTTH breaks their diapause states (Shionoya *et al*., 2003; Wei *et al*., 2005) as well as in *Antheraea pernyi* (Sauman and Reppert, 1996). PTTH failure could be not due solely to an impairment in its synthesis or to the refractoriness of downstream targets. In *Mamestra brassicae*, although PTTH haemolypmphatic levels of diapausing pupae were lower than those characterizing non-diapausing ones, PTTH amount of brains after pupation was higher in the first, suggesting that the block of the release of PTTH rather than changes in its expression is responsible for diapause onset. Interestingly, PTTH deficiency (lower haemolymphatic titers and expression) characterizes already diapause-destined larvae, suggesting that the programmed fate is already established before pupariation (Mizoguchi *et al*., 2013). The identity of candidate inhibitors of PTTH-release have been elusive for a long time. Recently, in the Chinese silk moth, *A. pernyi*, the presence of PTTH with the 5HTRB serotonin receptor has been detected in two pairs of neurosecretory cells in the brain. Moreover, the injection of dsRNA-*5HTRB* in diapausing pupae elicited a precocious termination of dormancy. Thus, serotonin could shut-down PTTH production/secretion through 5HTRB, but whether this mechanism can regulate diapause induction/maintenance in other insects remains to be discovered (Wang *et al*., 2013). Many species of insects in which pupal diapause has been

investigated show a termination mechanism that is independent from the PTTH-ecdysone one previously mentioned. Indeed, de-brained diapausing pupae can resume metamorphosis in *M. sexta*, *P. brassicae*, *Antheraea polyphemus* and *Helicoverpa zea* (Judy, 1972; Wilson and Larsen, 1974; Maslennikova, 1970; McDaniel and Berry, 1967; Meola and Adikisson, 1977; Denlinger *et al*., 2012). The possibility that the PG can reactivate development autonomously,

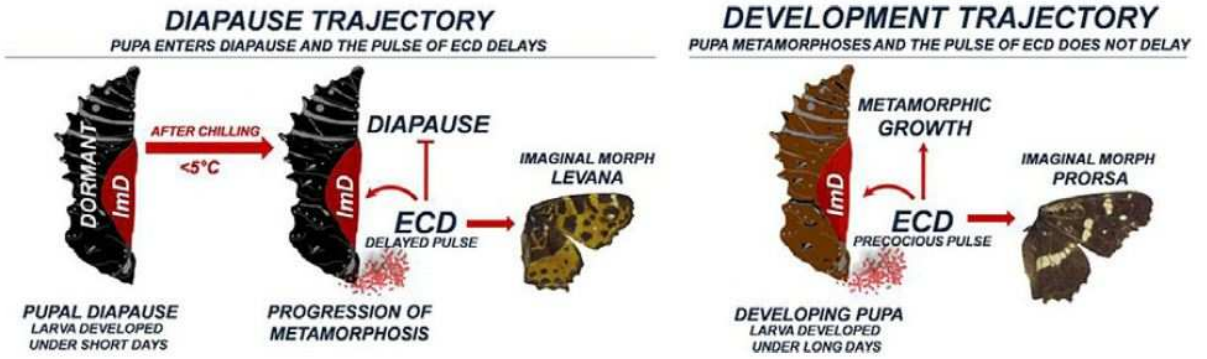

Figure 3. Diapause termination in the lepidopteran, *Araschnia levana*, and seasonal wing colour patterns of adults emerging from diapausing or non-diapausing pupae, respectively. ImD=Imaginal wing discs, ECD=ecdysone (from Schiesari and O'Connor, 2013).

responding directly to environmental stimuli, has been suggested (Saunders *et al*., 2002; Denlinger *et al*., 2012). Ozeki (1954) showed that the PG of *Papilio xuthus* is directly cold-responsive, whereas in other butterflies like *M. sexta*, *M. brassicae* and *P. brassicae*, the PG of diapausing pupae results refractory to the brain-derived PTTH signaling, and autonomous in terminating dormancy (Bowen *et al*., 1984; 1985; Agui, 1975; Calvez, 1976), although the molecular insights remain to be clarified. As we saw in embryonic diapause, also during pupal dormancy DH seems to have an important role (although with opposite direction), acting in synergism with PTTH to mediate diapause termination. In *M. sexta* and *H. armigera*, *DH* expression is down-regulated in diapausing individuals (Xu and Denlinger, 2004; Zhang and Denlinger, 2012) and interestingly, an increase in *DH* expression, as well as the perception of favourable temperatures (25°C), has been described to induce diapause termination in activated (chilled) pupae of *H. zea* (Zhang and Denlinger, 2012). Moreover, in several species of the Haliothis/Helicoverpa complex, DH injection promotes diapause termination in non-chilled diapauisng individuals exposed to 25°C but not 20°C (Xu and Denlinger, 2003; Zhang *et al*., 2008; Zhang *et al*., 2011). Taken together, these evidences underline the complexity of diapause termination, suggesting the need of a synergistic action of different cues (hormonal and environmental) to ensure the proper timing of development resumption.

1.1.4 ADULT/REPRODUCTIVE DIAPAUSE

Reproductive diapause can be commonly considered as a JH deficiency condition (Denlinger, 2002; Denlinger *et al*., 2012). The monarch butterfly (*Danaus plexippus*) is known especially for its outstanding autumnal migration of North America populations. What is probably less known is that these migrants exhibit a reproductive diapause, showing refractory mating behaviour and suppressed gonadic development to maintain the needed energy stores for the

exhausting journey (Oberhauser and Solensky, 2004), as well as increased fat reserves and lifespan, and reduced levels of JH (JH deficiency) (Herman and Tatar, 2001). Another example is provided by the Linden bug, *Pyrrhocoris apterous*, which shows an adult dormancy triggered by short photoperiods (Hodek, 1971). In Hodkova (1976), a mechanism for photoperiodic control of *corpus allatum* (CA) physiology through neurons of the *pars intercerebralis* has been depicted. Evidences suggest that under short days these neurons, with a double effect, could lock their stimulatory effect on JH production in the CA (blocking the release of specific neuropeptides), and at the same time exert an inhibitory action on CA. The best documented cases of reproductive dormancy regards the mosquito, *Culex pipiens*, and the fruit fly, *Drosophila melanogaster*. In *C. pipiens*, females enter an overwintering diapause according to short photoperiods and cold temperatures eventually perceived during the 4th larval instar/first pupal stage (Eldridge, 1966; Sanburg and Larsen, 1973; Spielman and Wong, 1973). During this transition, haemophagus females switch into a carbohydrates-rich diet (based on nectar, rotting fruits, etc…), repressing host-seeking behaviour, increase fat storage (fat body hypertrophy to provide the energy source for winter survival) and stop ovarian development (Eldridge, 1966; Sanburg and Larsen, 1973; Spielman and Wong, 1973; Mitchell, 1983; Mitchell and Briegel, 1989; Bowen, 1992). After spending winter in protected sites, diapausing mosquitoes will terminate diapause in spring, and they will start again to seek a blood meal. Robich and Denlinger (2005) found gene expression data which confirm this behavioural switch: in diapausing females, the expression of genes encoding *trypsin* and *chymotrypsin-like* enzymes (needed to digest a blood meal) dropped, whereas *fatty acid synthase* was up-regulated. This species has been extensively used to investigate the endocrine regulatory mechanisms of adult dormancy: application of JH on diapausing females breaks this developmental arrest (Spielman, 1974), while the removal of the CA from individuals reared under long photoperiods (and thus not programmed for diapause) induces diapause entry (Meola and Petralia, 1980; Readio *et al*., 1999). Recently, Kang *et al*. (2014) demonstrated that, in non-diapausing mosquitoes, allatotropin (an hormone responsible for the stimulation of JH production, see Unni *et al*., 1991; Li *et al*., 2005; Kang *et al*., 2014) transcript was more abundant than in diapausing individuals; furthermore, the down-regulation via RNAi of the *allatotropin*-encoding gene in non-diapausing flies halts ovarian development, confirming the key role of allatotropin shut-down in determining the JH deficiency that characterizes Culex diapause.

In the model organism *D. melanogaster*, which exhibits a shallow photoperiodic diapause (Saunders, 1989) mainly triggered by low temperatures, a decrease of JH levels, as well as a drop in ecdysone titers, is key to trigger diapause (like in Culex), even characterized by fat body hypertrophy (Gilbert *et al*., 1998; Saunders *et al*., 1989; Saunders *et al*., 1990; Richard *et al*., 1998; Saunders *et al*., 2002). Studies on Culex and Drosophila in the last decade revealed the nature of the brain signaling which likely links environmental perception with JH synthesis/release. Insulin signaling (IIS), a key neuroendocrine pathway involved in reproduction, development, growth and aging has been demonstrated to be responsible for reproductive/adult dormancy regulation. The down-regulation of *Insulin receptor* (*InR*) in non-diapausing females of *C. pipiens* leads to a shut-down of ovarian maturation, a block

rescuable via topical application of the JH analog methoprene *on dsInR* injected females. Moreover, the injection of *dsFOXO* reduces lipid content in diapausing individuals and shortens their lifespan (Sim and Denlinger, 2008). Culex *insulin-like peptides* (*ilp*) *1* (homolog of *dilp2* in *D. melanogaster*) and 5 mRNA were less abundant in diapausing mosquitoes compared to non-diapausing ones, and the down-regulation of *ilp1* (but not *ilp5*) in the latters resulted in a diapause-like block of ovarian growth, that also in this case can be restored through JH III application (Sim and Denlinger, 2009). Furthermore, in fat bodies (the organ responsible for vitellogenins production), FOXO content was higher in diapausing females (~7 times higher), and topical applications of JH III in diapausing individuals dramatically lower *FOXO* levels reducing fat storage (Sim and Denlinger, 2013). Williams *et al* (2006) identified the gene *Dp110* (encoding PI3K, a component of the InR downstream signaling cascade), as a candidate locus which modulates diapause incidence in Drosophila; lines with a deficiency in *PI3K* showed a general increase of dormant females, whereas the up-regulation of its expression within the central nervous system was sufficient to stimulate ovarian growth and the subsequent exit from diapause. Recently, other evidences about the importance of the IIS shut-down in altering physiology and hormonal homeostasis in Drosophila dormancy accumulated. Food intake and body weight decrease under diapause-inducing conditions, while glucose, trehalose (used as energy source and cryoprotectant) and triacyl-glycerols (TAG) levels increase, as well as amino acids (also potentially used as cryoprotectants) (Kubrak *et al*., 2014), all modifications depending on IIS level variations (Wu *et al*., 2005; Hong *et al*., 2012; Zhang *et al*., 2009; Grönke *et al*., 2010). As a confirmation, diapause significantly alters *dilps* and *akh* (a peptide with a glucagon-like function) gene expression, and elicits an up-regulation of transcripts encoding important metabolic regulators. Consistently with an improved stress resistance and survival, expression data revealed that also antimicrobial peptides (like *drosomycin* and *cecropin A1*) increased abundance (Kubrak *et al*., 2014). In our lab, in a previous study, we identified insulin producing cells (IPCs) as a key neuroendocrine component in diapause induction/termination. A higher proportion of dormant females was found in flies in which IPCs were genetically ablated (over-expressing two pro-apoptotic genes, *head involution defective*, *hid* and *reaper*, *rpr*) and when *dilp2* or *dilp5* were down-regulated in this neurons. On the contrary, a genetically-induced increased release of DILPs as well as *dilp2* and *dilp5* over-expression within IPCs led to a precocious exit from diapause (Schiesari *et al*., submitted). Counter-intuitively, diapausing females showed an up-regulation of *dilp2* and *dilp5* transcripts within the head; this mirrors brain *dilps* over-expression characterizing germline-less flies (Flatt *et al*., 2008) and flies with a systemic IIS impedance due to *ImpL2* (an insulin-binding protein that sequesters haemolymphatic DILP2 and 5) over-expression (Song *et al*., 2010). This result confirms a potential negative feedback loop from ovaries to the IPCs to modulate DILPs release. Interestingly, common cosmopolitan chromosomal inversions with a latitudinal clinal distribution, which characterizes also diapause frequencies in many natural populations, show associations with sets of genes belonging to IIS (Tab. 1) (De Jong and Bochdanovits, 2003). These findings suggest that at higher latitudes, Drosophila populations could have higher frequencies of genotypes/aplotypes associated with low levels of IIS, whereas at lower

Table 1. Common cosmopolitan chormosomal inversions associated with IIS and TOR pathway in Drosophila (from de Jong and Bochdanovits, 2003).

		Frequency correlation with latitude		
Inversion	Chromosome range	North America	Australia	Insulin-signalling pathway genes
In(2L)t	22D3-E1; 34A8-9	– 0.92	– 0.84	*chico, Pten, Tor*
In(2L)NS	23E2-3; 35F1-2			*chico, Pten, Tor*
In(2R)NS	52A2-B1; 56F9-13	– 0.90	– 0.67	
In(3L)P	63C; 72E1-2	– 0.74	– 0.74	*S6k, Ilp1, . . . , Ilp5, Pi3K68D*
In(3L)M	66D; 71D			*Ilp1, . . . , Ilp5, Pi3K68D*
In(3R)P	89C2-3; 96A1-19	– 0.80	– 0.72	*Pi3K92E, InR, Tsc1*
In(3R)K	86F1-87A1; 96F11-97A1			*Akt1, Pi3K92E, InR, Tsc1*

latitudes genomes which correspond to higher IIS levels are favoured. Moreover, genome-wide-next generation sequencing of DNA from flies sampled along the Eastern North American Coast (flies which showed clinal variations in life-history traits like diapause frequencies, see Schmidt *et al*., 2005a; 2005b) revealed intense sequence variation in genes involved in insulin/TOR signaling, as well as ecdysone, torso (PTTH receptor), EGFR, TGFβ/BMP, JAK/STAT, immunity and circadian rhythm pathways (Fabian *et al*., 2012). Taken together these results underline the deep connection between IIS and diapause fate in insects. In our work previously mentioned, we also proposed a model to explain diapause inducibility evolution: mutations in *dilps* promoters/cis-regulatory regions, or the rise of specific aplotypes (characterized by specific patterns of chromosomal inversions, for instance) could set lower baseline IIS levels, which, after an environmental perturbation (like the fall of temperatures), will drop under the hormonal thresholds necessary to allow normal development, promoting diapause induction through a genetic accomodation mechanism. In *C. pipiens*, the first evidences have been provided about a possible role in adult dormancy modulation of another hormone which effects on pupal diapause are well known, PTTH. *ptth* transcript cycles daily during the 4th (and final) larval instar, and the differences in terms of longer developmental time and increased size of individuals exposed to short days have been explained with a further *ptth* cycle. Counter-intuitively, in both pupae and adults, *ptth* levels slightly increase in individuals reared under short days. The interesting result raised from this study is the dramatic increase in *ptth* transcript in both diapausing and non-diapausing females (up to 7 fold change in the latters) after a blood meal (Zhang and Denlinger, 2011). It is noteworthy that, in Drosophila, PTTH is a key regulator of developmental time (McBrayer *et al*., 2007), likely connecting sensory and/or higher integrational brain structures with ecdysone production in the PG (on which PTTH-producing neurons impinge), and promotes larval light avoidance before wandering (Yamanaka *et al*., 2013), another key developmental process. In adults, the PG disappears, and some axons of PTTH-producing neurons seem to impinge on the ellipsoid body (McBrayer *et al*., 2007), a structure that, to our knowledge, does not represent neither a neuroendocrine site nor an ecdysone producing tissue, but which is involved in regulating walking and flight behaviour (Ilius *et al*., 1994; Martin *et al*., 2001). Recently, Hentze *et al*. (2013) demonstrated that genes encoding key enzymes for ecdysone (but not 20-ecdysone) synthesis are expressed in male accessory glands of *Tribolium castaneum*; in Drosophila males, the expression of the PTTH receptor, torso, was also

detected in the same organs, where it regulates ecdysteroids levels. Nevertheless, there are no evidences about potential connections between PTTH signaling and tissue responsible for ecdysteroids production in adult females, the ovary (Riddiford, 1993; Chavez *et al*., 2000; Niwa *et al*., 2004; Warren *et al*., 2002; 2004). Finally, in *L. decemlineata*, low levels of short neuropeptide F (sNPF, a Drosophila NPY peptide ortholog) characterize brains from diapausing individuals (Huybrechts *et al*., 2004). Interestingly, in *D. melanogaster* larvae, sNPF regulates positively IIS, acting on IPCs to stimulate the expression of *dilp1* and *dilp2* through ERK activation (Lee *et al*., 2008).

1.1.5 DOPAMINE AND DIAPAUSE

The deepening of the relationship between insect diapause and dopamine deserves a discussion aside. As in all the metazoans, also in insects, dopamine acts as neurotransmitter, neuromodulator or endocrine factor. In *D. melanogaster*, dopamine is involved in many physiological processes: locomotor activity (Draper *et al*., 2007), low quality food rejection (Bjordal *et al*., 2014), wake-sleep modulation (van Swinderen and Andretic, 2011; Liu *et al*., 2012), odors-induced aversive reinforcement (Schwaerzel *et al*., 2003; Riemensperger *et al*., 2005; Aso *et al*., 2010; Galili *et al*., 2014), courtship learning (Keleman *et al*., 2012), temperature-preference behaviour regulation (Bang *et al*., 2011), appetitive odor memory formation and rewarding memory reinforcement (Liu *et al*., 2012), appetitive motivation (Burke *et al*., 2012), pigment synthesis (Sugumaran, 1988; Prota, 1992; Wright, 1987), stress response (Neckameyer and Weinstein, 2005; Argue and Neckameyer, 2013a; Argue and Neckameyer, 2013b) and several developmental processes (Neckameyer, 1996). In Drosophila, dopamine is synthesized in a two-step process; first tyrosine is converted into L-3,4-dihydroxy-phenylalanin (L-DOPA) by the enzyme tyrosine-hydroxylase (encoded by the gene *pale*), and subsequently L-DOPA is in turn modified into dopamine by dopa-decarboxylase (DDC) (Liu *et al*., 2009; Riemensperger *et al*., 2011; Livingstone and Tempel, 1983). This biogenic amine acts through two types of receptors, D1-like and D2-like which are positive and negative regulators, respectively, of the PKA signaling pathway that mediates dopamine effects within cells (Brody and Cravchik, 2000; Gotzes *et al*., 1994; Feng *et al*., 1996; Han *et al*., 1996; Hearn *et al*., 2002). In the fruit fly genome, there are 4 dopamine receptors: *Dop1R1* (also called *dumb* or *DA1*) and *Dop1R2* which belong to the first class, and *Dop2R* (also known as *D2R*) of the second one. Dop1R1 and Dop2R are mainly (but not exclusively) expressed in the fly brain whereas Dop1R2 localization is restricted to a brain region with functions in learning, memory formation and temperature preference setting, the mushroom bodies (McGuire *et al*., 2001; Akalal *et al*., 2006; Berry *et al*., 2012; Bang *et al*., 2011). The fourth receptor, dopamine/ecdysteroid receptor (DopEcR), deserves to be treated separately. Like the others, also this receptor is a G-protein-coupled receptor (GPCR), and like Dop1R1 and Dop1R2 is coupled with a $G_{\alpha s}$ that activates the adenylate-cyclase activity increasing intracellular cAMP levels. Through this receptor, dopamine triggers the activation of both PKA and PI3K signaling pathways, whereas ecdysone and 20-ecdysone can inhibit

dopamine effects and elicit the activation of the mitogen-activated protein kinase pathway (MAPK) (Srivastava *et al*., 2005). After the release in the synaptic cleft, dopamine re-uptake is allowed by the dopamine transporter (DAT), encoded by the gene *fumin* (Porzgen *et al*., 2001).

Dopamine seems also to have an evolutionary conserved role in regulating insect diapause (see Fig. 4). In the cabbage armyworm, *M. brassicae* (a species with a photoperiodically determined pupal diapause), higher levels of dopamine in central nervous system, integument and haemolymph characterize the whole pre-pupal/pupal development in individuals exposed to diapause-promoting conditions (LD 10:14, 18°C) compared to those reared at long photoperiod and higher temperatures (LD 16:8, 22°C). Interestingly, dopamine levels peaked

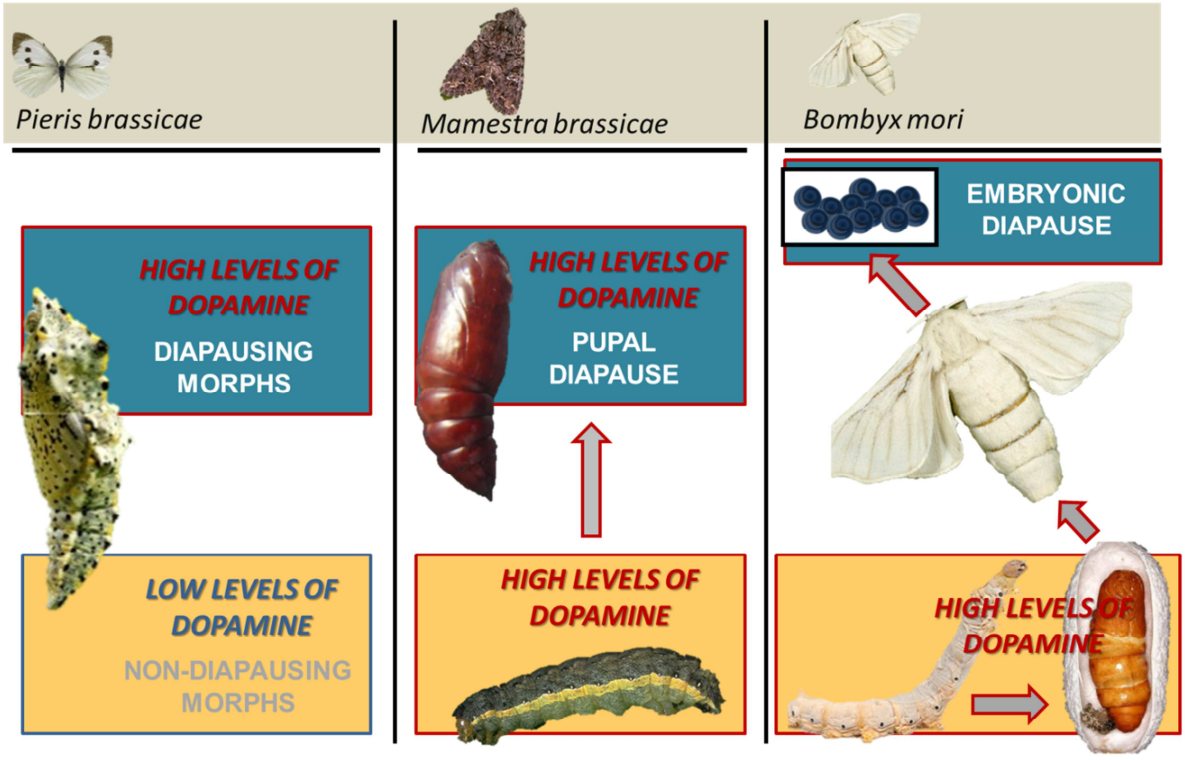

Figure 4. Schematic representation of the role of dopamine in regulating diapause in several holometabolous insects (see bibliography for pictures references).

during the transition between pre-pupa/pupa in both conditions, but always with higher levels in diapausing individuals. Both DDC activity and mRNA abundance followed the same pattern in the integument, confirming the enhanced dopamine synthesis in diapausing pupae. Moreover, the increase in dopamine titers, by feeding last instar larvae with L-DOPA, promoted a diapause onset in more than 50% of pupae, despite they were reared under long photoperiods (Noguchi and Hayakawa, 1997). Taken together, these results led to the hypothesis that dopamine could gate the release of growth-promoting factors from the brain, such as PTTH, and thus inhibiting development and eliciting dormancy. In *P. brassicae* (another species with a photoperiodically-determined pupal diapause), both dopamine and serotonin are involved in diapause modulation. In the last period of the photosensitive phase

(that lasts from the beginning of the 3rd larval instar to the middle of the 5th) dopamine content in the haemolymph was two-fold higher in dormant individuals, and serotonin detectable only in these individuals. In diapausing pupae, dopamine was 28 times higher than in non-diapausing ones, whereas serotonin was 13 times higher in the firsts compared with the latters (Isabel *et al*., 2001). Dopamine role as a pro-diapause factor is shown also in the embryonic diapause of the silk worm, *B. mori*. Larvae and pupae which were exposed during embryonic stages to diapause-promoting conditions (long photoperiod, LD 14:10 and high temperature, 25°C) showed higher dopamine titers in both haemolymph and brain-suboesophageal ganglion complex (Br-SOG) compared to non-diapause-programmed individuals (DD, 18°C). Moreover, female adults exposed to optimal conditions, and in which dopamine concentration was increased by feeding L-DOPA during last-instars larvae, laid between 31% and 59% of diapausing eggs, while those whose dopamine content was increased by L-DOPA/dopamine injection in 2 days old pupae laid 71% and 67% of diapausing eggs, respectively (in both feeding and injection experiments, the controls laid 0% of diapausing eggs). In this study also the activity and expression of DDC (the last step enzyme in dopamine biosynthesis) have been investigated in diapause and non-diapause types, and the results were consistent with the previous ones, with both activity and mRNA levels higher in the Br-SOGs of pupae exposed to diapause-promoting conditions during development. Interestingly, the incubation with L-DOPA or dopamine of isolated Br-SOGs from non-diapause type pupae increased the expression of *diapause hormone* (*DH*) (Noguchi and Hayakawa, 2001). In the Chinese oak silk moth, *A. pernyi*, a saturniid moth that overwinters as pupa, long days are required to promote the escape from diapause in both univoltine (reproduction once per year) and bivoltine (reproduction twice per year) strains, but in the latters, diapause intensity is reduced, and the addition of a period of chilling is also needed to resume development. Interestingly, the photoperiodic-dependent and the chilling-dependent diapause terminations seem to rely on two independent mechanisms (Tohno *et al*., 2000). Although under long days (LD 16:8, 25°C) dopamine content, as well as norepinephrine and epinephrine-like ones, was higher than that under diapause-maintaining conditions (LD 12:12, 25°C), after a period of chilling (DD, 5°C) it decreased progressively, suggesting that a drop in dopamine levels could be required to trigger the temperature-dependent diapause termination (Matsumoto and Takeda, 2002). Evidences regarding dopamine as a diapause-promoting factor have been found also in the photoperiodically-induced larval dormancy (3rd instar larvae) of the drosophilid, *C. costata*, where non-diapausing mutants (incapable to enter diapause and programmed to normal development) exhibit lower levels of dopamine in the integument during the 2nd and the 3rd larval instar. On the contrary, wild-type strains maintained relatively high levels of dopamine titers during these developmental events (Kostal *et al*., 1998). Moreover, a similar decrease in dopamine levels has been observed also in wild-type individuals exposed to long photoperiods (LD 16:8, 18°C) compared to those reared under short days (LD 10:14, 18°C), and in larvae kept under diapause-inducing conditions, where dopamine and serotonin levels gradually increased with time in the central nervous system (Kostal *et al*., 1999). In spite of these findings, the role of dopamine and serotonin in the regulation of diapause in this species seems to be not straightforward along development (for instance, individuals with a dopamine

depletion in the central nervous system showed delayed development and reduced growth), suggesting perhaps a stage-specific or tissue-specific aminergic developmental regulation.
The relationship between dopamine and diapause seems to cross the border between insects and other taxa. In *C. elegans* for instance, the impairment of dopamine signaling (but even its increase) via the ablation of dopamine-producing neurons stimulates the movement of quiescent individuals called *dauer* larvae (a diapause-like state) (Gaglia and Kenyon, 2009). In mammals, there are some evidences regarding a possible role of dopamine in blastocyst diapause. The injection of cabergoline or bromocriptine, two dopamine synthetic agonists, has been demonstrated to be sufficient to induce the reactivation of diapausing blastocysts (inhibiting prolactin secretion) in the tammar wallaby, *Macropus eugenii* (Tyndale-Biscoe and Hinds, 1984; Fletcher *el al*, 1990; Renfree, 1994; Hearn *et al*., 1998). The effects of bromocriptine and another dopamine agonist, domperidone, on prolactin secretion and diapause have been reported also for the Bennett's wallaby (*Macropus rufogriseus rufogriseus*), and they are consistent with the previously exposed ones (Curlewis *et al*., 1986). In the mink, *Mustela vison*, a dopamine antagonist, pimozide, has been demonstrated to stimulate the precocious termination of diapause, elevating prolactin levels (Marks *et al*., 2006). Hence, we can argue that in this species, dopamine should promote diapause rather than suppress this phenomenon like in marsupials. It seems clear from these examples of embryonic diapause in mammals that, both in marsupials and eutherians, dopamine retains its role of prolactin secretion-inhibiting factor; on the contrary, prolactin changes the direction of its effect in promoting or inhibiting the reactivation of the dormant blastocyst in eutherians and marsupials, respectively. In conclusion, although a sufficient amount of information to generalize the role of dopamine in the regulation of mammalian embryonic diapause is lacking, it is interesting to note that the function of dopamine as a prolactin inhibiting factor seems conserved across mammals.

1.1.6 DIAPAUSE AS A WIDESPREAD PHENOMENON IN ANIMAL KINGDOM: THREE EXAMPLES FROM NON-INSECTS METAZOANS SPECIES

Probably the most documented example of diapause regards the round worm, *C. elegans*. During its life-cycle, this nematode normally passes through 4 larval stages (L1-L4), but when L1 individuals are exposed to adverse environmental conditions like starvation, high temperatures and high levels of *dauer* pheromone (a lipophilic factor released constitutively by individuals, which conveys the information about conspecifics density, Jeong *et al*., 2005; Butcher *et al*., 2007), they enter an alternative developmental pathway and arrest their development as a L3 diapausing stage called *dauer* (Cassada and Russell, 1975; Golden and Riddle, 1984). Like its insect counterpart, this dormant stage is associated to typical morphological and physiological changes, like metabolic suppression, suspension of feeding and lifespan extension (also till several months) (Riddle and Albert, 1997). Compared to insect diapauses, the molecular mechanisms and the diverse signaling pathways involved in *C. elegans dauer* formation are better known, and rely mainly on Transforming Growth

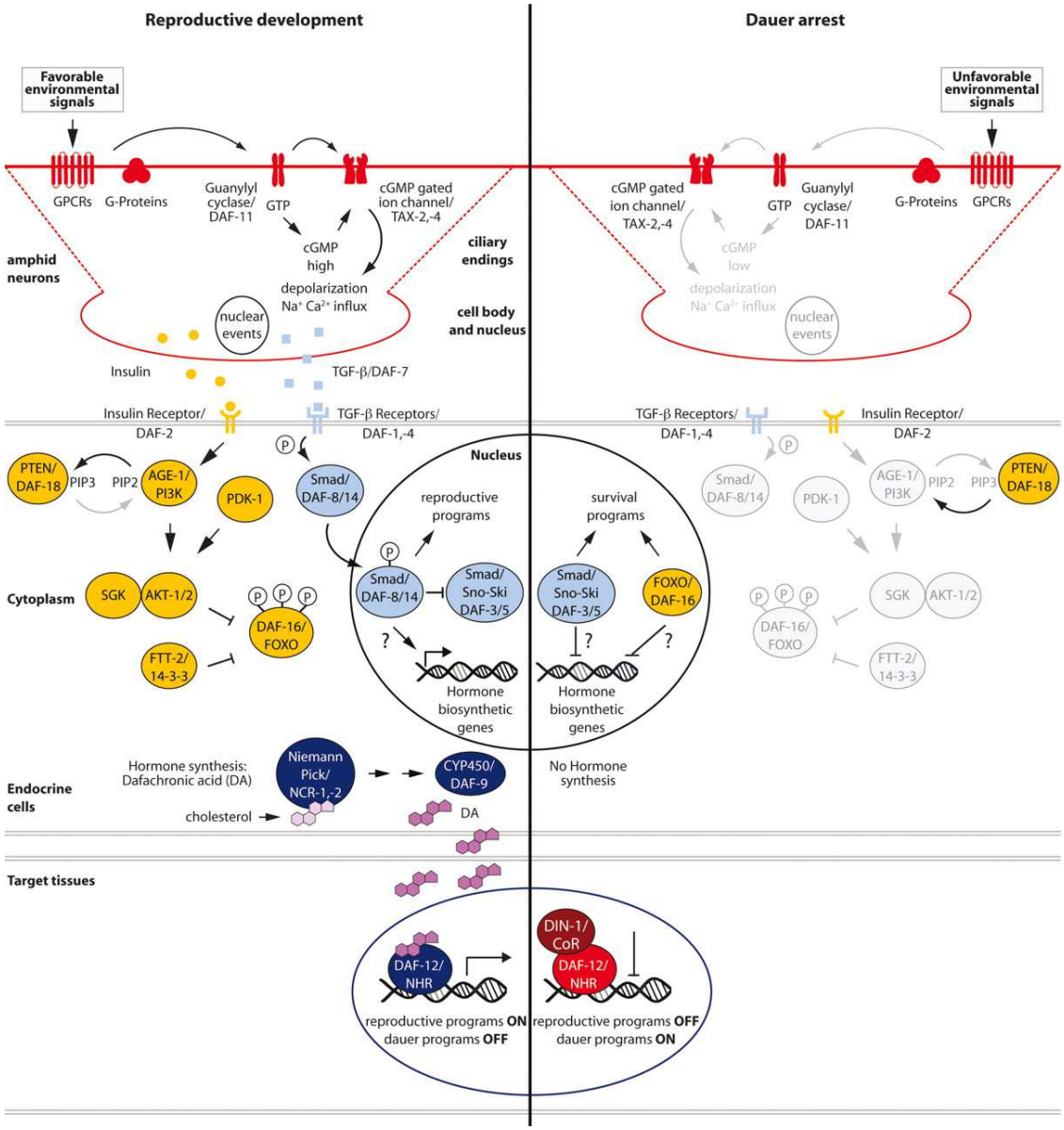

Figure 5. A model for the regulatory signaling network involved in normal development/*dauer* formation switch (from Fielenbach and Antebi, 2008).

Factor β (TGF-β) and insulin signaling (IIS) pathways. *Dauer* pheromone, for instance, activates ASJ and ASI sensory neurons (Riddle and Albert, 1997); in the first set, it stimulates *dauer* formation through the guanylyl cyclase, DAF-11 (Schackwitz *et al*., 1996; Birnby *et al*, 2000), whereas acting on the second one, *dauer* pheromone blocks DAF-7 (TGF-β) release (Bargmann and Horvitz, 1991; Schackwitz *et al*., 1996). When activated, ASI chemosensory neurons, which express *daf-7* and *daf-28* (an insulin-like peptide), release these growth factors (Ailion *et al*., 1999; Pierce *et al*., 2001; Li *et al*., 2003), inhibiting *dauer* formation (Schackwitz *et al*., 1996; Ren *et al*., 1996; Li *et al*., 2003; Kulalert and Kim, 2013; Reiner *et al*., 2008). Instead, AFD sensory neurons perceive and convey temperature stimuli, and the impairment of their downstream signaling in *daf-7* mutants decouples temperature stimuli from diapause induction or termination (Hobert *et al*., 1997; McKemy, 2007). Low levels of nutrients can also promote *dauer* formation (Golden and Riddle, 1984), but the molecular

mechanism which underlies this response remains not completely understood. What is known, regards TOR signaling involvement in diapause-linked metabolic switch like higher fat storage. Moreover, mutations in genes encoding *TOR* (*let-363*) and its binding partners *Raptor* (*daf-15*) result in *dauer* formation and lifespan extension (Jia *et al*., 2004). On the contrary, the loss of LKB1/AMPK (adenosine monophosphate-activated protein kinase, a stress-responsive TOR inhibitor, activated in Drosophila by energy break-down, nutrient restriction, or DNA damage, see Hay and Sonenberg, 2004; Towler and Hardie, 2007) signaling in fat tissues allows larvae to enter diapause, but in this case, stored energy consumption during *dauer* stage is not slowed, leading to precocious death (Narbonne and Roy, 2009). Like in Drosophila, IIS plays a key role in regulating *dauer* formation: for instance, mutations in the *age1* and *daf-2* genes (which encode, respectively, the mammalian homologs of *PI3K* and *InR*) cause diapause formation and promote longevity (Morris *et al*., 1996; Kimura *et al*., 1997). On the contrary, *dauer* formation fails in *daf-16* (a *C. elegans* FOXO transcription factor) mutants (Kenyon, 2005; Oh *et al*., 2005). Both TGF-β signaling and IIS (which can exert its effect independently or activated by upstream TGF-β, see Narasimhan *et al*., 2011; Shaw *et al*., 2007) converge on DAF-12 (a nuclear hormone receptor whose mutants show a *dauer*-defective phenotype, which retains high homology with vertebrate vitamin D and LXR receptors, Antebi *et al*., 2000; Snow and Larsen, 2000) to suppress its *dauer*-promoting function (Kimura *et al*., 1997; Ren *et al*., 1996; Schackwitz *et al*., 1996). In detail, TGF-β and IIS are thought to act directly or indirectly on DAF-9, a cytochrome P450 responsible for the synthesis of DAF-12 ligands called "dafachronic acids" (Gerisch *et al*., 2001; Jia *et al*., 2002). Liganded DAF-12 promotes reproduction and inhibits *dauer* program, whereas on the contrary, unliganded DAF-12 in target tissues induces *dauer* formation and, associated with its co-repressor DIN-1/CoR (homolog of mammalian SHARP co-repressor) halts reproduction (Ludewig *et al*., 2004) (Fig. 5) (see Fielenbach and Antebi, 2008). *daf-9* is expressed in sensory neurons, XXX neuroendocrine cells, hypodermis and somatic gonadal cells, revealing this tissues as potential endocrine sites for dafachronic acids production (Gerisch *et al*., 2001), whereas *daf-12*, which expression characterizes several tissues during the whole life, is expressed in nervous system and in the somatic part of the adult gonad, suggesting its key role in regulating aging and reproduction (Antebi *et al*., 2000). Motola *et al*. (2006) identified two steroids, Δ4 and Δ7 dafachronic acids, as metabolites of DAF-9 and ligands for DAF-12. Interestingly, also DAF-36, a Rieske-like oxygenase which Drosophila homolog is involved in ecdysone synthesis (Yoshiyama *et al*., 2006), represents an enzyme of the dafachronic acids biosynthesis pathway, and in turn a key actor in *dauer*/normal developmental switch (Rottiers *et al*., 2006).

Also within vertebrates several examples of diapause developmental arrest exist. The annual killifish, *Austrofundulus limanaeus*, lives in waterholes that during the dry season expire. In this period, individuals can survive entering diapause as embryo, a phenomenon triggered by hypoxia; once the rainy season comes, embryos can re-start development in refilled puddles (Berois *et al*., 2012; Podrabsky and Hand, 1999; Podrabsky *et al*., 2007). Killifishes can block development at three different embryonic stages (Berois *et al*., 2012; Podrabsky *et al*., 2010; Schiesari and O'Connor, 2013), and like has been described for several insect dormancies,

also in this case diapause is associated with changes in developmental time (Podrabsky *et al*., 2010). Diapausing embryos are characterized by dehydration and hypoxia stress resistance and they are able to survive for months without oxygen (Podrabsky *et al*., 2010; Podrabsky and Hand, 1999). Intriguingly, like in the major part of insects, also in these species, diapausing individuals show lower levels of steroid hormones (in this case 17-β-estradiol), and they terminate diapause if exposed to 17-β-estradiol (Pri-Tal *et al*., 2011). Moreover, in both *A. limanaeus* and *Nothobranchius guentheri*, diapause can be maternally-induced; in the latter species, molecules similar to pheromones and secreted by adults seem responsible for eliciting diapause (Inglima *et al*., 1981). Given that embryonic diapause can occur also in other species of Cyprinodontiformes (Inglima *et al*., 1981; Levels *et al*., 1986; Murphy and Collier, 1997), whereas in some non-diapausing species a delay in hatching occurs, it has been suggested that killifish diapause could derive from the eliciting of a pre-exisisting developmental plasiticity (Berois *et al*., 2012; Schiesari and O'Connor, 2013).

Diapause is a phenomenon which characterizes also the life-cycle of several species of mammals. In these animals, an embryonic diapause occurs, which consists in a reversible suspension of embryonic development within the uterus with a postponed blastocyst implantation (Renfree and Shaw, 2000). Diapause within mammals is maternally induced, and it can be stimulated by lactation (lactational diapause) or by seasonal environmental cues like photoperiod (seasonal diapause) (Flint *et al*., 1981). This phenomenon shows a wide distribution across mammals, in both placentals and marsupials (see Tab. 2), and can be facultative, like in many rodents, or obligate (which characterizes many species of mustelids), while marsupials show both types (Fenelon *et al*., 2014). The facultative variant of diapause embryonic arrest is induced by lactation, and the implantation delay increases with the increasing of the progeny in the suckling litter (Pritchett-Corning *et al*., 2013). Although the cellular mechanism that causes embryonic developmental arrest in mice remains elusive, Liu *et al*. (2012) reported a significant up-regulation of several miRNAs in arrested blastocysts compared with the non-dormant ones. Moreover, the increased abundance of one of these miRNAs, *Lethal-7a* (whose target genes are involved in cell proliferation, Gurtan *et al*., 2013), counteracts blastocyst implantation. On the contrary, carnivores, especially mustelids, provide many examples of obligate diapause. In minks and skunks, an increase in day-length is accompanied by an enhanced release from the pituitary gland of prolactin (Murphy, 1983), which exogenous administration reactivates embryonic development and in turn halts diapause (Papke *et al*., 1980; Murphy *et al*., 1981). Both prolactin and luteotropic hormone (LH, another pituitary hormone) seem to concur in progesterone synthesis from the *corpus luteum* (Murphy and Rajkumar, 1985; Douglas *et al*., 1998), which increase is necessary for blastocyst implantation (Stoufflet *et al*., 1989; Murphy, 2012). It is noteworthy that, also in rats, LH promotes diapause termination: LH treatment of hypophysectomized females stimulates implantation (Macdonald *et al*., 1967), whereas its immunological inactivation impedes this phenomenon (Maneckjee and Moudgal, 1975). Also in marsupials diapause represents a widespread and extensively studied phenomenon especially in kangaroos like the tammar wallaby, *Macropus eugenii*, which is able to enter both facultative and obligate diapause (Renfree and Shaw, 2000). Like eutherian facultative diapause, also in marsupials

TABLE 1

LIST OF CURRENTLY KNOWN SPECIES WITH PRE-IMPLANTATION EMBRYONIC DIAPAUSE

Order	Family	Common name	Species
OBLIGATE			
EUTHERIA			
Artiodactyla	Cervidae	Roe deer	*Capreolus capreolus*
Carnivora	Mephitidae	Hooded skunk	*Mephitis macroura*
		Striped skunk	*Mephitis mephitis*
		Western spotted skunk	*Spilogale gracilis*
	Mustelidae	Hog-badger	*Arctonyx collaris*
		American hog-nosed skunk	*Conepatus mesoleucus*
		Sea otter	*Enhydra lutris*
		Wolverine	*Gulo gulo*
		North American otter	*Lontra canadensis*
		Neotropical river otter	*Lontra longicaudis*
		American marten	*Martes americana*
		Yellow-throated marten	*Martes flavigula*
		Beech marten	*Martes foina*
		Nilgiri marten	*Martes gwatkinsii*
		European pine marten	*Martes martes*
		Japanese marten	*Martes melampus*
		Fisher	*Martes pennanti*
		Sable	*Martes zibellina*
		European badger	*Meles meles*
		Honey badger	*Mellivora capensis*
		Short tailed weasel (stoat)	*Mustela erminea*
		Long tailed weasel	*Mustela frenata*
		European mink	*Mustela lutreola*
		American mink	*Neovison vison*
		American badger	*Taxidea taxus*
		European marbled polecat	*Vormela peregusna*
	Odobenidae	Walrus	*Odobenus rosmarus*
	Otariidae	New Zealand fur seal	*Arctocephalus forsteri*
		Antarctic fur seal	*Arctocephalus gazella*
		South African fur seal	*Arctocephalus pusillus*
		Subantarctic fur seal	*Arctocephalus tropicalis*
		Northern fur seal	*Callorhinus ursinus*
		Stellar sea lion	*Eumetopias jubatus*
		Australian sea lion	*Neophoca cinerea*
		Southern sea lion	*Otaria byronia*
		California sea lion	*Zalophus californianus*
	Phocidae	Hooded seal	*Cystophora cristata*
		Bearded seal	*Erignathus barbatus*
		Grey seal	*Halichoerus grypus*
		Weddell seal	*Leptonychotes weddellii*
		Crabeater seal	*Lobodon carcinophagus*
		Northern elephant seal	*Mirounga angustirostis*
		Southern elephant seal	*Mirounga leonina*
		Ross seal	*Ommatophoca rossi*
		Harp seal	*Pagophilus groenlandicus*
		Ribbon seal	*Phoca fasciata*
		Ringed seal	*Phoca hispida*
		Spotted seal	*Phoca largha*
		Baikal seal	*Phoca sibirica*
		Harbour seal	*Phoca vitulina*
	Ursidae	Giant panda	*Ailuropoda melanoleuca*
		Spectacled bear	*Tremarctos ornatus*
		Black bear	*Ursus americanus*
		Brown bear	*Ursus arctos*
		Sun bear	*Ursus malayanus*
		Polar bear	*Ursus maritimus*
		Asiatic black bear	*Ursus thibetanus*
		Sloth bear	*Ursus ursinus*
Chiroptera	Miniopteridae	Little bent-winged bat	*Miniopterus australis*
		Common bent-winged bat	*Miniopterus schreibersii*
	Pteropidae	Straw-colored fruit bat	*Eidolon helvum*
Cingulata	Dasypodidae	Southern long-nosed armadillo	*Dasypus hybridus*
		Nine-banded armadillo	*Dasypus novemcinctus*
Eulipotyphla	Talpidae	Siberian mole	*Talpa altaica*
Pilosa	Myrmecophagidae	Giant anteater	*Myrmecophaga tridactyla*

Order	Family	Common name	Species
FACULTATIVE			
Eulipotyphla	Soricidae	Eurasian water shrew	*Neomys fodiens*
		Common shrew	*Sorex araneus*
		Pygmy shrew	*Sorex minutus*
Rodentia	Cricetidae	Red tree vole	*Arborimus longicaudus*
		Small vesper mouse	*Calomys laucha*
		Northern collared lemming	*Dicrostonyx groenlandicus*
		Field vole	*Microtus agrestis*
		Bank vole	*Myodes glareolus*
		Northern grasshopper mouse	*Onychomys leucogaster*
		Cotton mouse	*Peromyscus gossypinus*
		White-footed mouse	*Peromyscus leucopus*
		Deer mouse	*Peromyscus maniculatus*
		Pinyon mouse	*Peromyscus truei*
		Campbell's hamster	*Phodopus campbelli*
		Dzhungarian hamster	*Phodopus sungorus*
		Chiriqui brown mouse	*Scotinomys xerampelinus*
	Muridae	Lesser short-tailed gerbil	*Gerbillus simoni*
		Common water rat	*Hydromys chrysogaster*
		Natal mastomys	*Mastomys natalensis*
		Sundevall's jird	*Meriones crassus*
		Shaw's jird	*Meriones shawi*
		Mongolian gerbil	*Meriones unguiculatus*
		House mouse	*Mus musculus*
		Spinifex hopping mouse	*Notomys alexis*
		Fawn hopping mouse	*Notomys cervinus*
		Sandy island mouse	*Pseudomys hermannsburgenesis*
		New holland mouse	*Pseudomys novaehollandiae*
		Bush rat	*Rattus fuscipes*
		Brown rat	*Rattus norvegicus*
		Indian gerbil	*Tatera indica*
MARSUPIALIA			
Dasyuromorphia	Dasyuridae	Brown antechinus	*Antechinus stuartii*
Diprotodontia	Acrobatidae	Feathertail glider	*Acrobates pygmaeus*
		Feathertail possum	*Distoeuchurus pennatus*
	Burramyidae	Western pygmy possum	*Cercartetus concinnus*
		Little pygmy possum	*Cercartetus lepidus*
		Eastern pygmy possum	*Cercartetus nanus*
	Macropodidae	Banded hare wallaby	*Lagorchestes fasciatus*
		Western hare wallaby	*Lagorchestes hirsutus*
		Agile wallaby	*Macropus agilis*
		Black-striped wallaby	*Macropus dorsalis*
		Tammar wallaby*	*Macropus eugenii*
		Eastern grey kangaroo	*Macropus giganteus*
		Western brush wallaby	*Macropus irma*
		Parma wallaby	*Macropus parma*
		Pretty-faced wallaby	*Macropus parryi*
		Common wallaroo	*Macropus robustus*
		Red-necked wallaby*	*Macropus rufogriseus*
		Red kangaroo	*Macropus rufus*
		Bridled nailtail wallaby	*Onychogalea fraenata*
		Allied rock wallaby	*Petrogale assimilis*
		Narbarlek	*Petrogale concinna*
		Black-footed rock wallaby	*Petrogale lateralis*
		Brush-tailed rock wallaby	*Petrogale pencillata*
		Prosperine rock wallaby	*Petrogale persephone*
		Purple-necked rock wallaby	*Petrogale purpureicollis*
		Yellow-footed rock wallaby	*Petrogale xanthopus*
		Quokka	*Setonix brachyurus*
		Red-bellied pademelon	*Thylogale billardierii*
		Red-necked pademelon	*Thylogale thetis*
		Swamp wallaby	*Wallabia bicolor*
	Potoroidae	Rufous bettong	*Aepyprymnus rufescens*
		Tasmanian bettong	*Bettongia gaimardi*
		Burrowing bettong	*Bettongia lesueur*
		Brush-tailed bettong	*Bettongia penicillata*
		Northern bettong	*Bettongia tropica*
		Gilbert's potoroo	*Potorous gilbertii*
		Long-nosed potoroo	*Potorous tridactylus*
	Tarsipedidae	Honey possum	*Tarsipes rostratus*

Table 2. Currently known mammal species which exhibit embryonic diapause (from Fenelon *et al*., 2014, reproduced with permission from *The International Journal of Developmental Biology* (2014), *58*,163-174.

this phenomenon is regulated by neural inputs to the hypothalamus triggered by the feeding of the newborns, while melatonin release from the pineal gland modulates the photoperiodically-induced obligate diapause, which can reinforce lactational diapause eventually delaying much more blastocyst reactivation (Renfree *et al*., 1981; Renfree, 1979; Tyndale-Biscoe *et al*., 1986; Renfree and Shaw, 2000). Although prolactin does not affect embryonic diapause in rodents, in both mustelids and marsupials its secretion plays a key role, but while in the first group this pituitary hormone induces diapause termination and in turn blastocyst reactivation, in the latters it promotes developmental arrest in both facultative and obligate variants (Hinds, 1989; Hinds and Tynkale-Biscoe, 2013). Moreover, in these species prolactin blocks the progesterone titer increase necessary to allow implantation (Hearn, 1974; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989), and the suppression of its action favours blastocyst reactivation.

In both placentals and marsupials, steroid hormones like estradiol and progesterone often play a key role in diapause termination. In rodents, diapause can be arrested by an increased release of estradiol from the ovary (necessary to allow implantation) (McCormack and Greenwald, 1974) or via its administration (Cha *et al*., 2013), whereas progesterone concentration increases in *Mustela vison* prior to implantation, when the blastocyst resumes growth (Stoufflet *et al*., 1989). In the tammar wallaby, both estradiol and progesterone can stimulate blastocyst reactivation, but this process can be further carried on solely by progesterone (Renfree and Tyndale-Biscoe, 1973; Fletcher *et al*., 1988). In mammals, like in insects, it is likely that diapause evolved independently many times, given the presence/absence of diapause in species belonging to the same genus and the great variety of species characterized by this phenomenon (Sandell, 1990). Despite this hypothesis, it has been recently demonstrated that ovine embryos unable to enter diapause can arrest reversibly their development if inserted into mouse uteri of females which were exposed to diapause-promoting stimuli, suggesting contrarily that embryonic diapause, or at least its potential, was not secondarily acquired, but it could have been present in the mammalian common ancestor (Ptak *et al*., 2012).

1.2 Drosophila IIS-JH-20E axis: an overview

1.2.1 INSULIN/IGF SIGNALING

Insulin/IGF signaling (IIS) is a widely evolutionary conserved signaling pathway which regulates growth and development in a multitude of organisms, from *C. elegans* to chordates (Broughton *et al*., 2005; Grönke *et al*., 2010) In Drosophila, 8 insulin-like peptides are known (DILP 1-8) which, although acting through a unique Insulin Receptor (InR) (Ruan *et al*., 1995; Fernandez *et al*., 1995; Yenush *et al*., 1996; Chen *et al*., 1996; Brogiolo *et al*., 2001; Shingleton *et al*., 2005), show temporal and spatial specific expression patterns. Brain insulin-Producing Cells (IPCs) consist in two neuronal clusters which retain functional and developmental analogies with vertebrate hypothalamus and pituitary axis, showing also homology with pancreatic β cells (de Velasco *et al*., 2007; Clements *et al*., 2008; Okamoto *et al*., 2012; Wang *et al*., 2007). This subset of neuroendocrine cells is responsible for the production and release of DILP2 (from 1st instar), DILP5 (from the 2nd) and DILP3 (from the 3rd) (in larvae also DILP1) (Brogiolo *et al*., 2001; Ikeya *et al*., 2002; Rulifson *et al*., 2002; Grönke *et al*., 2010) (Fig. 6). In adults, *dilp2* is expressed in the IPCs (but during development also in salivary glands and imaginal discs), *dilp5* is expressed also in Malpighian tubules (organs analogous to human kidney) with a supposed role in oxidative stress response (Söderberg *et al*., 2011) and in the follicular cells of the ovary (Brogiolo *et al*., 2001), whereas *dilp3* also in glial cells, with *dilp6*, where they promote the reactivation of quiescent neuroblasts (Sousa-Nunes *et al*., 2011). DILP2 is likely the most potent growth promoting DILP: *dilp2* mutant flies exhibit longer developmental time, decreased body weight and increased threalose levels (Grönke *et al*., 2010), whereas *dilp2* over-expression in the IPCs

accelerates developmental rate (Walkiewicz and Stern, 2009). Furthermore, It has been extensively demonstrated that the knock-out of *dilp2* extends lifespan (Hwangbo *et al*., 2004; Wang *et al*., 2005; Bauer *et al*., 2007; Lee *et al*., 2008; Grönke *et al*., 2010). On the contrary, *dilp3* and *dilp5* mutants do not show effects on developmental time and lifespan, but like *dilp2* knock-out flies, they are characterized by a decreased lifetime fecundity (Grönke *et al*., 2010). IPCs-derived DILPs are also responsible for coupling nutrient uptake with systemic growth. Upd2, a humoral factor secreted by fat cells in response to sugars and fats nutritional uptake, promotes the release of DILP2 and 5; moreover, *dilp3* and *dilp5* are down-regulated in larvae under starvation conditions (Ikeya *et al*., 2002; Colombani *et al*., 2003), whereas *dilp5* also in adult flies exposed to dietary restriction (Min *et al*., 2008). Expression data, shown in Grönke *et al*., 2010, reveal that a compensatory mechanism is established within the IPCs between DILP2, DILP3 and DILP5 (Broughton *et al*., 2008; Grönke *et al*., 2010). Moreover, in Drosophila, DILPs share a high degree of functional redundancy and, like other organisms, also the fruit fly genome contains several *ilps*-encoding genes; these two mechanism, as well as the compensatory scenario previously mentioned, could provide an evolutionary advantage (Zhang *et al*., 2009; Gronke *et al*., 2010), creating a robust growth-regulatory system, less fragile in case of potential environmental perturbations or mutations that could affect *dilps* gene expression. For IPCs-derived DILP3 has been proposed a role as autocrine regulator of *dilp2* and *dilp5* transcription, according to *dilp2* and *dilp5* down-regulation in *dilp3* single null mutant (Grönke *et al*., 2010). From IPCs, DILPs are released into the haemolymph through the *corpus cardium* (part of a neurohaemal complex) and the aorta (Rulifson *et al*., 2002) (see Fig. 6); after their secretion, DILPs can bind to IGF Binding Proteins (IGFBPs) which modulate their action. For instance, dALS and ImpL2 can bind both

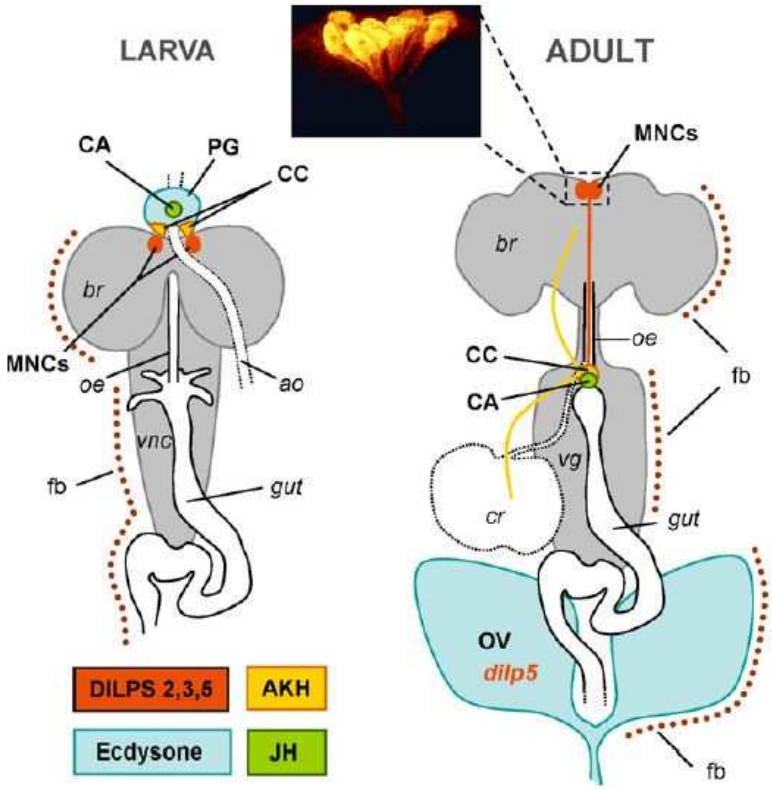

Figure 6. Simplified anatomical representation of the larval and adult IIS-JH-20E neuroendocrine axis regulating Drosophila growth, aging and reproduction, with key organs responsible for the release of these endocrine factors. br=brain, MNCs=Median Neurosecretory or Insuling Producing Cells (IPCs), CA=*Corpus allatum*, CC=*Corpus cardium*; PG=Prothoracic gland, oe=oesophagus, vnc=ventral nerve cord, vg=ventral ganglia, ao=aorta, cr=crop, fb=fat bodies, OV=ovaries, DILPS=Drosophila Insulin-like peptides, AKH=adipokinetic hormone, JH=juvenile hormone (from Toivonen and Partridge, 2009).

DILP2 and DILP5, counteracting their role as growth-promoting factors and metabolic regulators (Colombani *et al*., 2003; Arquier *et al*., 2008; Song *et al*., 2010). Recently, another DILPs binding partner has been identified by Okamoto *et al* (2013), called Secreted decoy of InR, which shows affinity for several DILPs. *dilp1* is co-expressed with *dilp2,3,5* in larval IPCs (Brogiolo *et al*., 2001; Rulifson *et al*., 2002), but no information is available about its expression in adults. Grönke *et al*. (2010) demonstrated a slight decrease in body weight of *dilp1*$^{-/-}$ flies, but this was not coupled with differences in developmental time or in other modifications in related traits. However, the decrease in body weight is consistent with the reduction of body length in adults characterizing *dilp1* interfered flies (Lee *et al*., 2008). A lack of information characterizes DILP4: this peptide is expressed in embryonic midgut and mesoderm (Brogiolo *et al*., 2001), revealing its presence also within the brain (Grönke *et al*., 2010), but no evidences have been provided to award a role to DILP4. Interestingly, another DILP relays growth-promoting signals when feeding is suppressed (for instance during metamorphosis, starvation or dietary restriction), DILP6, mainly expressed in fat cells. Its expression in both conditions depends on FOXO, whereas during pupal stages it is governed by ecdysone (Slaidina *et al*., 2009; Okamoto *et al*., 2009). In adults, DILP6 released from fat bodies also promotes longevity, impairing both DILP2 production and release as well as *dilp5* expression in the IPCs (Bai *et al*., 2012). *dilp7* expression is limited to some neurons (within suboesophageal ganglion, ventral nerve cord and thoracico-abdominal ganglion) which impinge on the reproductive tract; this localization as well as the sterility caused by silencing these neurons leads to the conclusion that DILP7 acts as a relaxin (peptide involved in egg-laying behaviour) (Yang *et al*., 2008; Miguel-Aliaga *et al*., 2008); nevertheless, this inference is not consistent with fertility data of *dilp7* mutants (Grönke *et al*., 2010). The recently discovered *dilp8*, expressed within larval imaginal discs, delays metamorphosis when larval organs are not developing harmoniously or tissues underwent abnormal growth, suppressing ecdysone production and allowing to coordinate tissues growth progression with the proper developmental time (Colombani *et al*., 2012; Garelli *et al*., 2012). To mediate DILPs different actions, it has been suggested that InR could form complexes with still unknown co-receptors (Taniguchi *et al*., 2006; Belfiore *et al*., 2009; Teleman, 2010; Grönke *et al*., 2010). Insulin Receptor (InR) binding with DILP ligands triggers the phosphorylation of Tyr sites, allowing the recruitment of two scaffod Insulin–Receptor Substrates (IRSs), CHICO and SH2B. CHICO binding activates PI3K, which converts phosphatidylinositol 4,5-bisphosphate (PIP2) in PIP3, a molecule that activates Phosphoinositide-dependent kinase-1 (PDK1), which in turn phosphorylates AKT/PKB (Protein kinase B), triggering its activation. At the end of this pathway, AKT/PKB can phosphorylate Forkhead Box-O (FOXO) (a transcription factor which activates the transcription of genes involved in stress response, blocking growth and development), preventing its nuclear translocation (Hwangbo *et al*., 2004; Teleman, 2010; Tang *et al*., 2011; Wang *et al*., 2011).

Although direct axonal connections as well as clear developmental repercussions are still elusive, the relationship between IIS and JH reflects in low JH titers characterizing *InR* mutants (Tatar *et al*., 2001). Furthermore, it has been demonstrated that IPCs-derived DILPs modulate JH production in the adult, up-regulating the *3-hydroxy-3mehylglutaryl CoA*

reductase (*hmgcr*, one of the enzymes involved in JH biosynthesis) gene expression (Belgacem and Martin, 2007; Jones *et al*., 2010). Recently, Mirth *et al*. (2014) have shown that the ablation of the *corpus allatum* (the organ responsible for JH synthesis) affects growth rate and in turn final body size; this action was found to be mediated by both FOXO (thus IIS) and ecdysone. IIS effects on the timing of metamorphosis also suggest a functional connection between DILPs release from the IPCs and ecdysone. Genetic ablation of IPCs or a partial loss of function mutation of *InR* decrease growth rate, delaying metamorphosis; on the contrary, over-expression of *dilp2* within these cells increases larval growth rate and shortens developmental time (Shingleton *et al*., 2005; Rulifson *et al*., 2002). Moreover, the activation of PI3K in the prothoracic gland (the site of ecdysone production) leads to a precocious metamorphosis through an early ecdysone synthesis, whereas suppression of IIS (expressing *PTEN*, Phosphatidylinositol-3,4,5-triphosphate 3-phosphatase, a negative regulator of IIS, or a dominant negative form of PI3K) lengthens developmental time, attenuating ecdysone synthesis (Colombani *et al*., 2005; Caldwell *et al*., 2005; Mirth *et al*., 2005; Layalle *et al*., 2008). In 2009, Walkiewicz and Stern demonstrated that the impairment of PKA signaling pathway in the IPCs up-regulated systemic IIS, which resulted in an early ecdysone synthesis, and in turn accelerated larval growth rate and anticipated metamorphosis. Interestingly, a recent study showed that IIS in larval mushroom bodies (structures which govern learning, memory and temperature-preference setting) regulates food intake and growth (Zhao and Campos, 2012).

1.2.1.1 Insulin-Producing Cells regulation and physiology

As previously exposed, IPCs are a pivotal neuroendocrine center for the modulation of insect growth, development and reproduction. Given the plethora of physiological processes involving DILPs, it is easy to explain the great amount of neurotransmitters or hormones whose producing neurons impinge on the IPCs to regulate DILPs expression and/or release. This regulation is extremely complex and fine-tuned, according to the diverse environmental signaling that these neurons receive. In the Table below (Tab. 3) a schematic overview about the nature of the different neurotransmitters/neuromodulators which control brain-IIS is reported (reviewed in Nässel *et al*., 2013).

Table 3. Neuromodulators or endocrine factors affecting IPCs physiology.

Factor	*Receptor on the IPCs*	*Effect on IIS*	*Effects within the IPCs*	*Nature of the signaling*	*References*
sNPF	sNPFR1	+	↑ *dilp1*[1]*-2-5* expression	Nutrition-derived?*	Lee *et al*., 2008; Kapan *et al*., 2012
DILP3	InR	+	↑ *dilp2-5* expression	IPCs autocrine regulation	Broughton *et al*., 2008; Gronke *et al*., 2010
DILP6	InR	-	↓ *dilp2-5* expression; ↓ DILP2 release	Fat body-derived signal[§]	Bai *et al*., 2012
GABA	GBR	-	↓ *dilp2* expression[†]; ↓ DILP2-5 release	Low levels of nutrition-derived sugars/fats	Enell *et al*., 2010; Rajan and Perrimon., 2012
DTK	DTKR/NKD	-	↓ *dilp2-3* expression	Nutrition-derived?*	Birse *et al*., 2011
*Adiponectin-like**	Adiponectin Receptor	+	↑ *dilp3* expression; ↑ DILP2 release	Fat body-derived signal[§]	Kwak *et al*., 2013
Serotonin	5-HT1A	-	↓ *dilp2* expression[†]; ↓ *dilp2-5* expression	Nutrition- derived?*	Luo *et al*., 2012; Luo *et al*., 2014
Octopamine	OAMB	**?**	↑ *dilp3* expression	?	Luo *et al*., 2014
SLOB	SLO*	+	↑ *dilp3* expression	Ca^{+}-activated K^{+}channel binding partner	Sheldon *et al*., 2011

[1] only in larval stages;

* Not confirmed;

[§] The environmental stimulus which induces the release remains unknown;

[†] measured via immunocytochemistry.

As can be noted from the information reported in Tab. 3, environmental cues triggering the signaling of some of these factors remain still elusive. Further studies have to be performed to better understand the nature of various signaling pathways affecting IPCs physiology.

1.2.2 JUVENILE HORMONE AND ECDYSTEROIDS

JHs and ecdysteroids (like ecdysone and 20-ecdysone) are two classes of lipophilic hormones which retain pleiotropic roles in insect growth, development, reproduction, aging and behaviour (Toivonen and Partridge, 2009) and rely also on functional IIS, see above (Bownes, 1982; Carney and Bender, 2000; Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; Soller *et al*., 1999).

JHs are acyclic sesquiterpenoids mainly known for their role as developmental modulators, but they are also crucial in regulating life-histories and fitness trade-off (Flatt *et al*., 2005). In Drosophila, the *corpus allatum* synthesizes three JHs (JH III, JHB3 and methyl farnesoate), but the differences in their signaling are elusive (Flatt *et al*., 2005; Richard *et al*., 1989; Toivonen and Partridge, 2009) (thus, we will refer to JH to include all of them). Apart from *hmgcr* (see above), genes involved in JH synthesis are still unknown, as well as the exact JH transduction mechanism. To explain how JH can affect transcription, both a direct regulation within the nucleus and a cell-surface receptor-mediated interaction have been proposed (Wheeler and Nijhout, 2003). Methoprene-tolerant (Met) nuclear transcriptional regulator (Wilson an Fabian, 1986; Ashok *et al*., 1998) and Ultraspiracle (USP), a nuclear receptor which constitutes also an EcR binding partner (Jones and Sharp, 1997; Jones *et al*., 2006) have been suggested as putative JH receptors (perhaps working as a dimer, like the EcR-USP complex, see Yao *et al*., 1992; Yao *et al*., 1993). JH is involved in a multitude of phenomena, including imaginal disc growth, ovarian maturation, diapause, polyphenisms, memory, learning and courtship behaviour, but it is mainly known for its involvement in the regulation of insect development (see Flatt *et al*., 2005). Although in distinct insect groups JH shows different effects on developmental processes (Truman and Riddiford, 2002), in holometabolous insect development, like the Drosophila one, JH exhibits a role as a "*status quo*" factor. While 20-ecdysone peaks dictate the tempo of different developmental transitions, like larval molts, the maintenance of high levels of JH during larval life allows 20-ecdysone increase to lead towards a further larval stage instead of metamorphosis. In other words, drops in JH titers determine if the developmental transition will give rise to a new morph or not (Nijhout, 1994; Riddiford, 1993; Riddiford, 1994). Before metamorphosis, the *corpus allatum* blocks JH synthesis, whereas in other tissues JH degradation becomes more efficient to provide an effective JH clearance mechanism, allowing 20-ecdysone to promote metamorphosis. Reproduction and lifespan represent just other few extraordinary examples in which JH plays a key role. Applications of a JH analog during Drosophila larval development induces a dramatic reduction of adult female survivorship as well as an increased early life fecundity (Flatt and Kawecki, 2007). JH promotes vitellogenesis in concert with IIS, directly or indirectly, promoting ecdysone synthesis from ovarian follicular cells (Soller *et al*., 1999; Gilbert *et al*., 1998; Bownes, 1982; Drummond-Barbosa and Spradling, 2001), whereas in males, it induces the production of specific peptides in male accessory glands (Wilson *et al*., 2003) like in *Tribolium castaneum* (Parthasarathy *et al*., 2009).

Ecdysteroids, which in Drosophila comprise ecdysone (E) and 20-hydroxy-ecdysone (known also as 20-ecdysone, 20E), are steroid hormones which elicit the transcription of specific genes (Thummel, 2002; Riddiford *et al*., 2000). During larval life, E is produced in the prothoracic gland (PG), and its secretion into the haemolymph, controlled by diverse neuropeptides such as PTTH and DILPs (which dictate developmental timing and final body size, McBrayer *et al*., 2007, Walkiewicz and Stern, 2009, Caldwell *et al*., 2005; Colombani *et al*., 2005; Mirth *et al*., 2005), triggers key events for development. In adult females, ovaries represent the main source of E (Riddiford, 1993; Chavez *et al*., 2000; Niwa *et al*., 2004; Warren *et al*., 2002; 2004), while in males, the site of ecdysteoids production, although elusive for a long time, has been recently reported as male accessory glands in *T. castaneum* and Drosophila (Hentze *et al*., 2013). E released is further converted into its active form 20E in peripheral tissues by 20-monooxygenase (encoded by *shade*, Petryk *et al*., 2003), and the latter provides the ligand for a receptor complex which involves EcR and USP (Koelle *et al*., 1991; Yao *et al*., 1993; Baker *et al*., 2000). These receptors represent the vertebrate farnesoid X receptor (FXR) or liver X receptor (LXR), and retinoid X receptor (RXR) homologs, respectively (King-Jones and Thummel, 2005). In females, the importance of ecdysteroids in fueling yolk protein synthesis and uptake from the ovary is well known (Gilbert *et al*., 1998; Buszczak *et al*., 1999; Carney and Bender, 2000), but also in males it exhibits a key role in gonads maturation, spermatogenesis and reproductive behaviour (Wismar *et al*., 2000). Moreover, combined with JH, ecdysteroids controls also courtship behaviour (Ringo *et al*., 1992; Ganter *et al*., 2007) as well as longevity (Tatar *et al*., 2001; 2003). Although the shut-down of both IIS and ecdysteroids signaling decreases fertility and increases lifespan in Drosophila (Clancy *et al*., 2001; Tatar *et al*., 2001; Tu *et al*., 2002; Simon *et al*., 2003; Carney and Bender, 2000), the coordination of these pathways in modulating insect aging seems more complex, given that, for instance, *chico* mutants (which exhibit lifespan extension) showed neither impairment in E release nor reduced haemolymph ecdysteroids levels (Richard *et al*., 2005).

Considering that Drosophila reproductive diapause consists in a shut-down of ovarian development, it seems quite important to have a detailed perspective of the hormonal control of fruit fly oogenesis. The current endocrine model for Drosophila oogenesis is based on a complex interaction between IIS, JH and 20E signaling pathways. The release of DILPs from IPCs promotes JH synthesis (Belgacem and Martin, 2007; Jones *et al*., 2010), and JH release from the *corpus allatum* is responsible for both fuel early synthesis of vitellogenins from fat cells and stimulate ovarian follicular cells to produce E, which will carry on late vitellogenesis, promoting vitellogenins production in fat bodies. Finally, vitellogenins secreted into the haemolymph will be uptaken by the growing oocytes (Richard *et al*., 1998; Richard *et al*., 2001) (see Fig. 7). It has been also demonstrated that DILPs release into the haemolymph is required for ovarian maturation independently from JH and ecdysone (Tatar *et al*., 2001; LaFever and Drummond-Barbosa, 2005; Richard *et al*., 2005), according to *InR* expression in the ovary. Taken together, these evidences underline the deep connection and

synergism between IIS, JH and 20E signaling pathways, even thought suggesting the possible existence of molecular mechanisms by which they can exploit their functions independently.

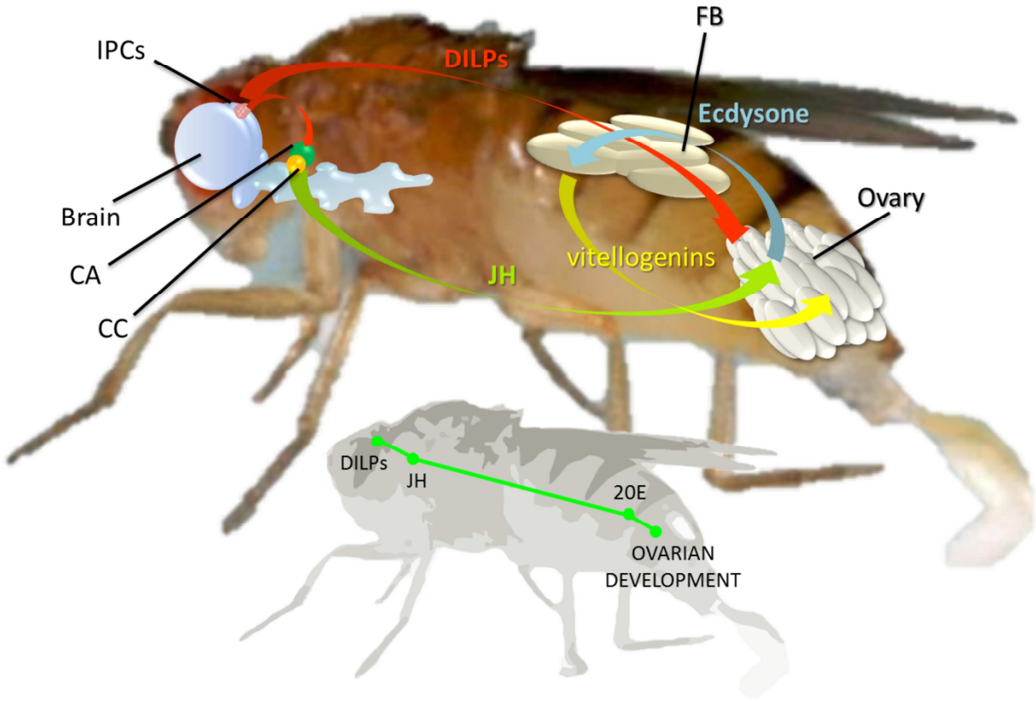

Figure 7. Current model for Drosophila endocrine regulation of vitellogenesis. IPCs=Insulin Producing Cells; CA=Corpus allatum; CC=Corpus cardium; FB=fat bodies; DILPs=Drosophila Insulin-like peptides; JH=juvenile hormone.

2. Results

2.1 Serotonin but not octopamine signaling promotes diapause in Drosophila

Taking into consideration all the information about insect reproductive diapause reviewed in the Introduction, it appears plausible to hypothesize that diapause-promoting environmental stimuli, are they cold, day-length, desiccation/humidity or starvation, should modulate (through still unknown neural networks) insulin-producing cells (IPCs) physiology, to induce or prevent the expression and/or the release of insulin-like peptides (ILPs, in Drosophila DILPs), thus regulating the neuroendocrine axis responsible for the diapause/non-diapause fate. For this reason, we first focused our attention on the different receptors located on the IPCs, searching potential candidate receptors which activation can impair brain insulin signaling (IIS). As previously reported, at the moment three receptors are known which signaling contribute to DILPs secretion shut-down: Drosophila Tachykinin-related peptide receptor (DTKR), 5HT1A serotonin receptor, and the metabotropic GABA receptor, GBR. More complex is the role of the Insulin receptor, InR in these neuroendocrine cells: it has been proposed to fuel IIS through its activation by DILP3 (Grönke *et al*., 2010), but also inhibit *dilp2*/*dilp5* expression and DILP2 release via DILP6 binding (Bai *et al*., 2012). We decided to include the OAMB octopamine receptor in the first screening, because, although its involvement in IIS regulation remains unclear (Erion *et al*., 2012), in the IPCs this receptor plays its function triggering the activation of the PKA signaling pathway (Balfanz *et al*., 2005; Nässel *et al*., 2013), which impairment within larval IPCs has been demonstrated to increase IIS (Walkiewicz and Stern, 2009). In many insects, evidences about the key role of aminergic signaling in diapause accumulated in the last years, therefore we focused our attention on potential aminergic effects on IPCs physiology. We started over-stimulating specific subsets of aminergic neurons expressing a bacterial sodium channel (*Na^+ChBac*) in order to stimulate an enhanced release of serotonin and octopamine respectively under diapause-promoting temperatures (12°C), but under long days (a conditions that should promote diapause termination), in order to counteract possible positive results, making them more robust. The over-stimulation of serotonergic neurons, employing a driver based on the *Trh* (the gene encoding the first step of serotonin byosynthesis) promoter (*Trh>Na^+ChBac*), induces a bigger proportion of flies to undergo diapause (87,5% ± 5,1) with respect to the controls (*Trh>+*, 61,5% ± 6,4 and *UAS-Na^+ChBac/+*, 42,4% ± 4,2 (Fig. 8a). On the contrary, an increased octopamine signaling (*Tdc2>Na^+ChBac*, driver built on the *Tdc* promoter, where *Tdc* encodes the enzyme responsible for the first step of octopamine biosynthesis), based on the same approach, leads to a decreased response to lower temperatures, causing a precocious exit from diapause state (26,0% ± 7,5) with respect to the controls (*Tdc2>+*, 44,7% ± 3,8; *UAS-Na^+ChBac/+*, 42,4% ± 4,2) (Fig. 8a). In order to confirm our previous evidences about serotonergic and octopaminergic signaling in diapause, we first impaired serotonin signaling to the IPCs down-regulating the expression of the *5HT1A* serotonin receptor via RNAi, expressing a *5HT1A* dsRNA, specifically in the IPCs. We used two independent *Gal4* lines to direct the dsRNA expression, the first *dilp2(p)-Gal4* which expression starts during the

second larval instar and the *dilp2-Gal4* that allow UAS-dependent expression from the mid-late third larval instar. The results of this genetic manipulation under diapause-inducing conditions (12°C and LD 8:16) are consistent using these two different drivers, with interfered flies that show a marked drop in dipause levels (*dilp2(p)>5HT1A-RNAi*, 15,9% ± 2,3 and *dilp2>5HT1A-RNAi*, 11,7% ± 4,8) compared with control flies (*dilp(p)>+*, 47,5% ± 3,7; *dilp2>+*, 37,5% ± 1,9 and *UAS-5HT1A-RNAi/+*, 49,9% ± 4,3) (Fig. 8b). With the same *Gal4*

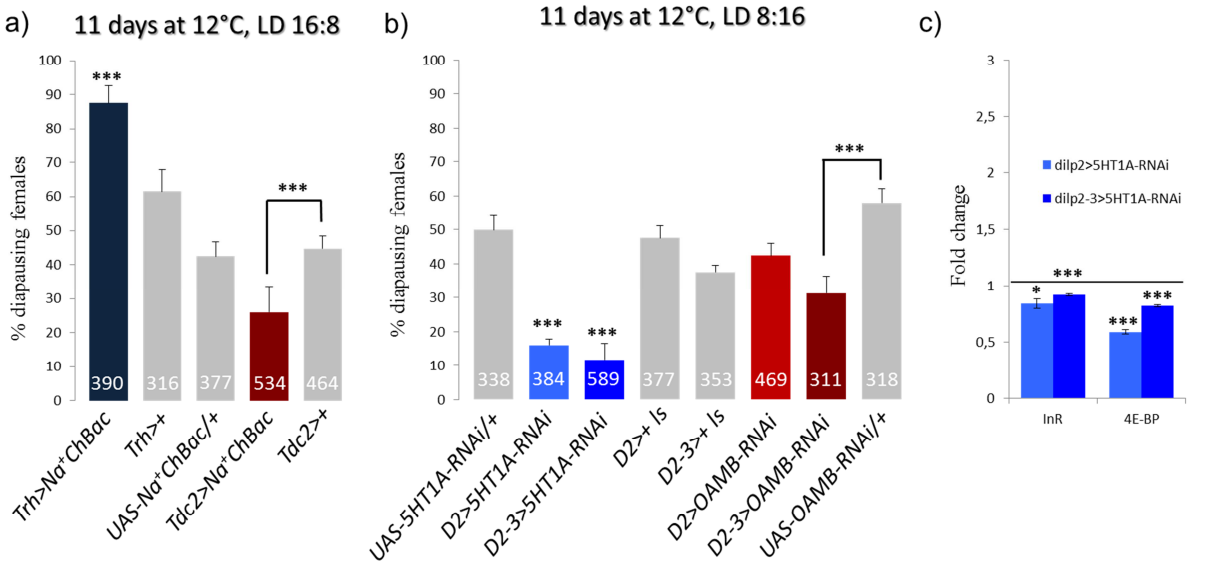

Figure 8. a) Diapause levels of flies characterized by an increased release of serotonin (blue) or octopamine (red); b) diapause proportion of flies with *5HT1A* (blue) or *OAMB* (red) down-regulation in the IPCs; c) gene expression of *InR* and *4E-BP* used as proxy for IIS suppression. Numbers within bars refer to sample size. Diapause data are shown as Mean ± SD, 5-7 rep. n≥60; expression data are shown as Mean ± SE, 3 rep. n=14. 2-Way-ANOVA-Interaction: **p*<0.05; ***p*<0.01; ****p*<0.001.

lines we knocked-down *OAMB*, the gene encoding the octopamine receptor located on the IPCs, to down-regulate the octopaminergic signaling directed toward the IPCs, observing negligible effects on diapause response (*dilp2(p)>OAMB-RNAi*, 42,3% ± 3,7; *dilp2>OAMB-RNAi*, 31,3% ± 4,9; controls were: *dilp(p)>+*, 47,5% ± 3,7; *dilp2>+*, 37,5% ± 1,9 and *UAS-OAMB-RNAi/+*, 57,9% ± 4,2) (Fig. 8b). Afterwards, to confirm the role of serotonin as a potential candidate in mediating the block of DILPs release transduced into a systemic IIS down-regulation (which characterizes diapause) we measured in bodies of *5HT1A* interfered flies, transctipt levels of *InR* and *4E-BP*, which expression can be used as a proxy for IIS activity (they are both under the control of FOXO) (Miron *et al.*, 2001; Puig *et al.*, 2003). Flies with an impaired serotonin signaling to the IPCs exhibit a statistically significant decrease of transcript levels of both *InR* and *4E-BP*, under diapause-promoting conditions (*dilp2(p)>5HT1A-RNAi*: *InR*, 0,84 ± 0,04; *4E-BP*, 0,59 ± 0,02; *dilp2>5HT1A-RNAi*: *InR*, 0,84 ± 0,04; *4E-BP*, 0,59 ± 0,02. *dilp2(p)>+* and *dilp2>+* flies were used as controls) (Fig. 8c). Also in this case, at the molecular level, performing the analysis with two different drivers did not affect the uniqueness of the results. Taken together, these evidences suggest that serotonin but not octopamine promotes diapause entry and contributes to IIS down-regulation characterizing the dormant state.

2.2 The modulation of the nutrient signaling connecting fat bodies and brain shows weak or no effects on diapause

In *Helicoverpa armigera*, the injection of a mixture of fat bodies catabolites (Tricarboxylic acids intermediates) in dormant pupae has been demonstrated to be sufficient to stimulate a precocious termination of diapause increasing *PTTH* expression (Xu *et al*., 2012). Moreover, Rajan and Perrimon (2012) identified in Drosophila a fat bodies-released molecule that promotes DILPs (DILP2 and DILP5) release from the brain as a cytokine-like protein called

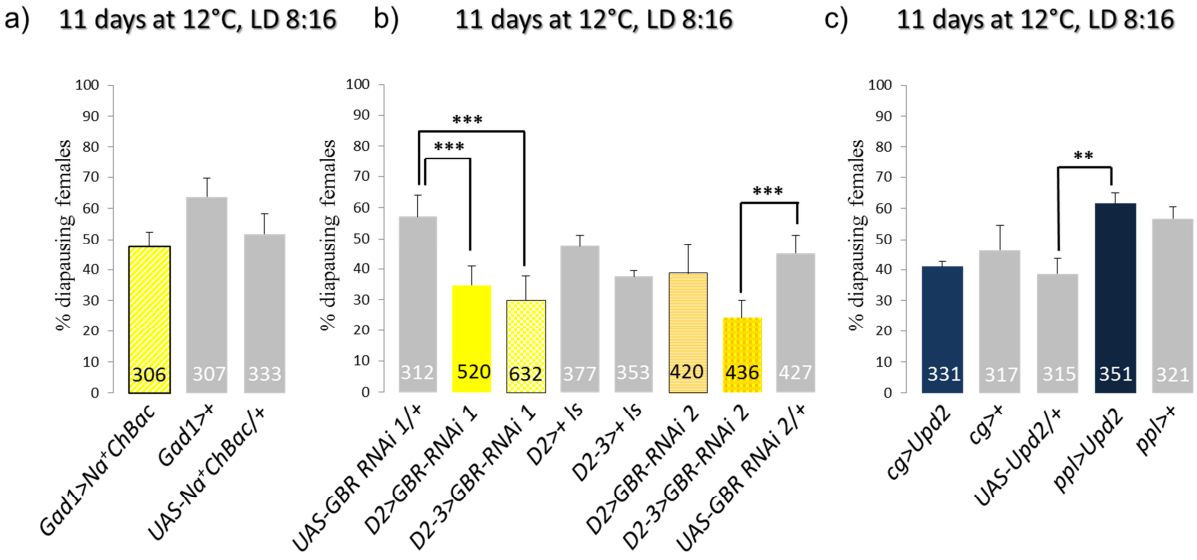

Figura 10. Diapause levels of flies with a) genetycally-induced over-stimulation of GABA release; b) down-regulation of GBR in the IPCs; c) over-expression of Upd2 within fat cells. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-8 rep. n≥ 60. 2-Way-ANOVA-Interaction: **p*<0.05; ***p*<0.01; ****p*<0.001.

Unpaired 2 (Upd2). This peptide plays a role analogous to that of the human Leptin (satiety hormone), and its expression is strongly down-regulated under starvation conditions, whereas it is up-regulated if high sugar or high fat diets (but not high amino acids diets) were administered. This suggest that Upd2 signals to the IPCs nutritional information about nutrition-derived sugars and fats. Taken together, these evidences suggest that insect fat cells could have a key role in shaping diapause acting as a metabolic sensor and not simply as a downstream effector of the IIS-JH-20E axis. Thus, we wondered if fat bodies metabolism could affect diapause response modulating brain neuroendocrine activity also in *D. melanogaster*. In Drosophila, Upd2 released from fat bodies promotes indirectly DILPs secretion, acting on GABAergic neurons juxtaposed to the IPCs: here, it binds to the receptor Domeless (Dome), triggering the activation of the JAK/STAT signaling pathway, whose activation suppresses GABA release form this subset of neurons. As we previously said, GABA, through its metabotropic receptor (GBR) on the IPCs impedes DILPs secretion, thus the release of Upd2 is translated into an enhanced IIS. In order to investigate the potential role of the Upd2-GABA nutrient signaling connecting fat bodies and brain in diapause modulation, we started over-stimulating specifically GABAergic neurons to increase GABA

release, expressing the bacterial sodium channel (*Na$^+$ChBac*). As shown in Fig. 9a, no effects on the proportion of dormant females have been observed (*Gad1>Na$^+$ChBac*, 46,0% ± 4,4; controls: *Gad1>+*, 61,9% ± 5,8 and *UAS-Na$^+$ChBac/+*, 50,2% ± 6,5) (Fig. 9a). Furthermore, we impaired GABA signaling to the IPCs to verify whether also this perturbation could be responsible for IIS down-regulation under diapause-promoting conditions. The down-regulation via RNAi of the *GABA receptor*, *GBR*, in the IPCs, leads to weak effects on diapause response (*dilp2(p)>GBR-RNAi* (1), 34,7% ± 6,3; *dilp2>GBR-RNAi* (1), 29,9% ± 7,8; *dilp2(p)>GBR-RNAi* (2), 38,6% ± 9,3; *dilp2>GBR-RNAi* (2), 24,1% ± 5,7), with significant differences only with respect to the UAS controls (*UAS-GBR-RNAi* (1)*/+*, 57,1% ± 6,9; *UAS-GBR-RNAi* (2)*/+*, 45,1% ± 6,0; *Gal4* controls were *dilp2(p)>+*, 47,5% ± 3,7; and *dilp2>+*, 37,5% ± 1,9) (Fig. 9b). Finally, we over-expressed *Upd2* in fat cells to induce the over-activation of the fat bodies-brain nutrient signaling in diapause-promoting conditions, employing two independent *Gal4* lines, *cg-Gal4* and *ppl-Gal4*, which expressions (anyhow, mainly driven in fat cells) overlap solely in fat bodies. Also in this case we found weak or no effects on diapause (*cg>Upd2*, 41,0% ± 1,6; *ppl>Upd2*, 61,6% ± 3,3; *cg>+*, 46,4% ± 8,3; *ppl>+*, 56,8% ± 3,7; *UAS-Upd2/+*, 38,7% ± 5,1) (Fig. 9c). Our results suggest that an enhanced nutrient sensitivity to sugars and fats provokes little or no effects on diapause and likely GABA action is not responsible for the major shut-down of DILPs release caused directly or indirectly by low temperatures.

2.3 Modulation of TOR signaling in the fat bodies affects diapause

The effects due to IIS pathway activation on organismal and cellular physiology are very often linked to the activation of another pathway, that shares some actors with IIS, but which can be activated also independently from IIS. Target of Rapamycin (TOR) signaling represents a nutrient sensor in Drosophila fat bodies: here, amino acids can enter in the cells through the amino acids channel SLIMFAST and trigger TOR activation disabling the two negative TOR regulator complexes, Tuberous Sclerosis Complex 1 (TSC1) and TSC2, which, as an heterodimer,

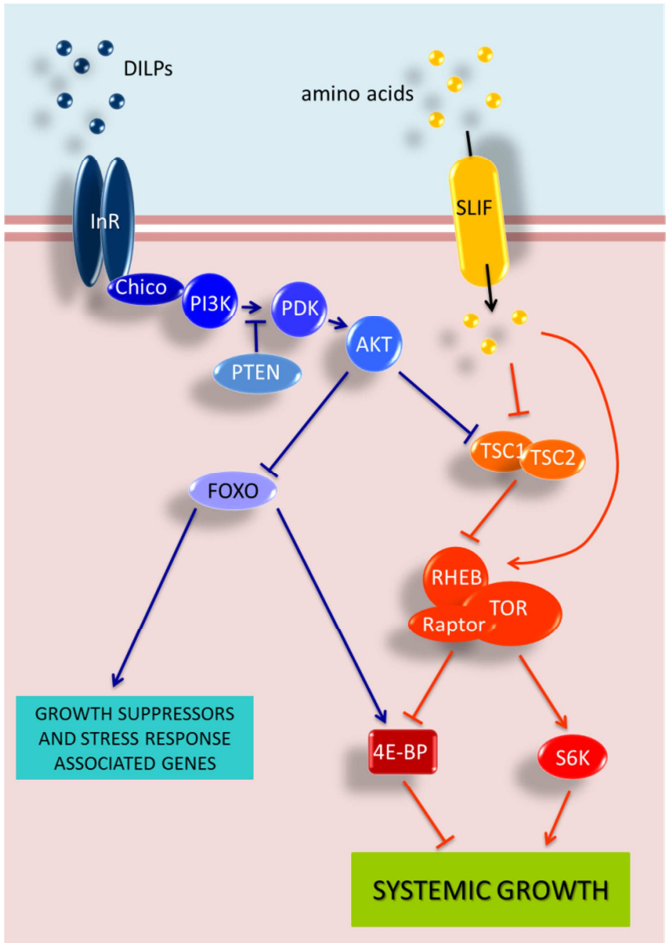

Figura 10. Simplified representation of Insulin and TOR signaling pathways in Drosophila fat bodies.

inhibit the GTPase Rheb, the factor required for TOR activation (Colombani *et al*., 2003). Together with Rheb and other two binding partners (Raptor and LST8), TOR can activate

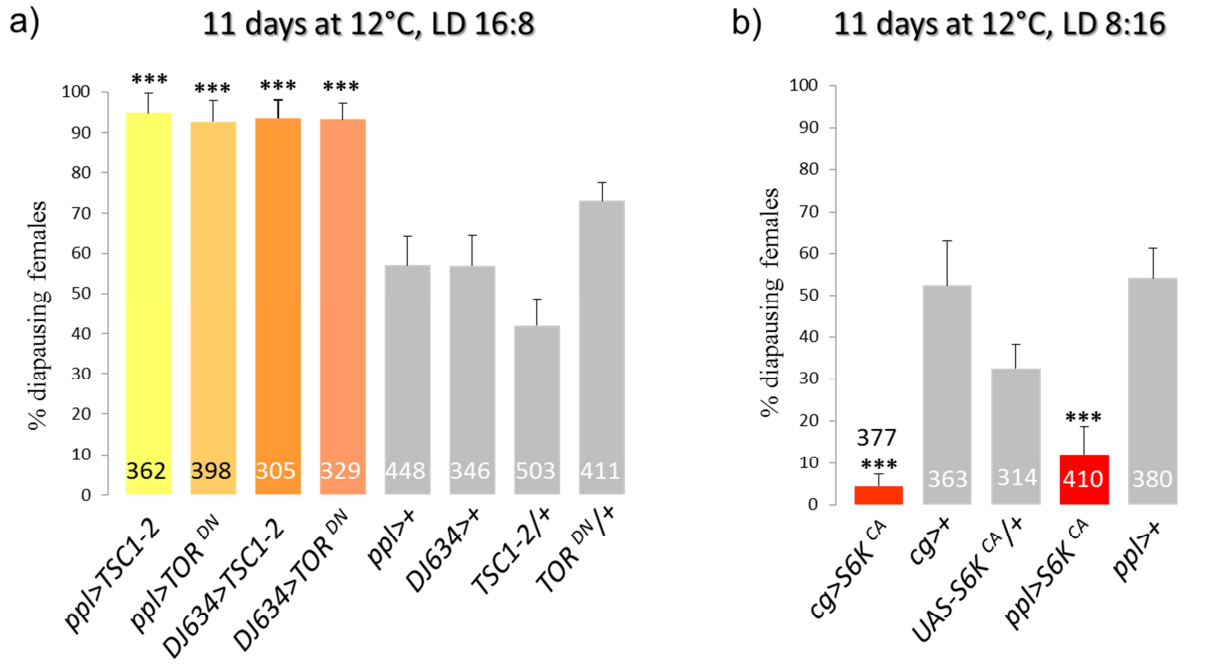

Figura 1. Diapause levels of flies characterized by a) suppressed TOR activity in fat cells; b) TOR activity up-regulation within fat bodies. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-8 rep. n≥60. 2-Way-ANOVA-Interaction: * p<0.05; ** p<0.01; *** p<0.001.

S6K to stimulate protein synthesis, TIF1A to induce the transcription of specific genes, and repress 4E-BP, coordinating growth with nutrition. On the contrary, when TOR pathway is suppressed, 4E-BP is unabled to repress translation, blocking protein synthesis and thus cellular and organismal growth (Colombani *et al*., 2001) (see Fig. 10). Moreover, an increased TOR activity within the fat cells has been demonstrated to drive the release of a humoral factor able to promote DILPs release from the brain. Nevertheless, at the moment no potential candidates have been suggested (Géminard *et al*., 2009). Although IIS and TOR pathway are deeply linked, functional studies in a diapause fashion on the latter are lacking. In order to investigate the potential role of TOR pathway in the reproductive diapause of Drosophila, we first down-regulated TOR activity specifically within the fat bodies, using two approaches, the over-expression of *TSC1* and *TSC2*, or the expression of a dominant negative form of TOR protein (TOR^{DN}, a mutated form of TOR that contributes to the formation of a disabled TOR complex). Also in this case, to strengthen our analysis, we employed two independent *Gal4* lines, which expression is mainly driven in fat bodies, *ppl-Gal4* and *DJ634-Gal4*. As showed in Fig. 11a, the inhibition of TOR activity, using both approaches and both drivers raises the percentage of dormant females beyond 90% (*ppl>TSC1-2*, 94,8% ± 5,0; *ppl>*TOR^{DN}, 92,6% ± 5,2; *DJ634>TSC1-2*, 93,4% ± 4,7; *DJ634>*TOR^{DN}, 93,2% ± 3,9) with respect to their controls (*ppl>+*, 57,0% ± 7,1; *DJ634>+*, 56,9% ± 7,4; *UAS-TSC1-2/+*, 42,0% ± 6,6; *UAS-*TOR^{DN}*/+*, 73,1% ± 4,5). Subsequently, to provide evidences confirming TOR key role in diapause regulation, we tested whether an induced over-activation of TOR within fat cells could lead to the opposite phenotype, in other words, lower levels of diapause. Thus, we

expressed a constitutively active form of *S6K* (*S6K*^{CA}) which acts downstream to TOR. We found a marked decrease in diapause incidence with both drivers (*cg*>*S6K*^{CA}, 4,5% ± 2,8; *ppl*>*S6K*^{CA}, 12,1% ± 6,5) while controls showed higher proportion of dormant females (*cg*>+, 52,4% ± 10,6; *ppl*>+, 54,1% ± 7,1; *UAS-S6K*^{CA}/+, 32,5% ± 5,7) (Fig. 11b). These results suggest a functional connection between the activity of the amino acids sensor TOR within the fat bodies and diapause.

2.4 Loss of dopamine signaling represses diapause

As reported in the chapter "Dopamine and diapause", high levels of dopamine in several tissues characterize diapausing morphs in many insects exhibiting embryonic, larval and pupal diapauses, but evidences are lacking about effects on reproductive dormancies. To investigate the role of dopamine in the reproductive diapause of Drosophila, we started testing in diapause-promoting conditions (12°C, LD 8:16) flies with mutated *ple* and *Ddc* genes, encoding for the two enzymes responsible for dopamine synthesis, TH and DDC. The *ple*^{4} *null-mutant* was tested as heterozygous (*ple*^{4}/+) given that in homozygosis it causes lethality. Exposed to cold temperatures, *ple*^{4}/+ flies showed extremely low levels of diapause (12,0% ± 4,9), as well as *Ddc* hypomorphic mutant (*Ddc hyp*, which causes a reduced functionality and not a gene knock-out) females (6,4% ± 3,5) (Fig. 12a); the latters showed strikingly high levels of vitellogenesis (also oocytes at stage 14, the last and ready-to-be-laid) even if exposed to a diapause-inducing temperature, resembling the ovarian development of flies maintained at optimal conditions (Fig. 12b). Interestingly, the results about *ple*^{4} and *Ddc hyp* flies can be partially phenocopied analyzing the diapause response of an hypomorphic mutant for *DopR1*

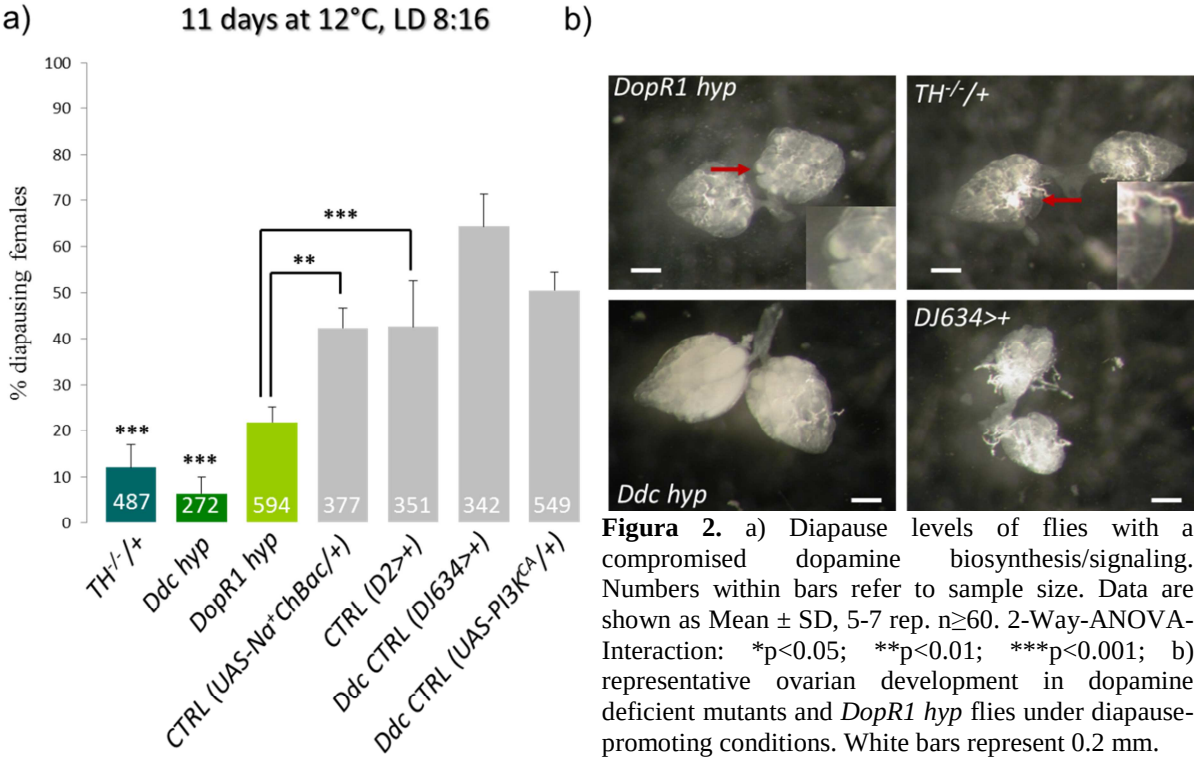

Figura 2. a) Diapause levels of flies with a compromised dopamine biosynthesis/signaling. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-7 rep. n≥60. 2-Way-ANOVA-Interaction: *p<0.05; **p<0.01; ***p<0.001; b) representative ovarian development in dopamine deficient mutants and *DopR1 hyp* flies under diapause-promoting conditions. White bars represent 0.2 mm.

(*DopR1 hyp* or *dumb*^{3}), one of the four dopamine receptors (21,8% ± 3,3) (Fig. 12a). To exclude potential effects due to the genetic background of these mutants, in this experiment we used multiple controls: both *white mutants* (w^{1118}) ($w^{1118}s$, 39,2% ± 2,2; $w^{1118}ls$, 57,6% ± 6,0), representing the genetic background of our mutants, and transgenic flies with a $w^{-/-}$ background but in which at least a partial rescue of the *w* gene is present (*UAS-Na*$^{+}$*ChBac/+*, 42,4% ± 4,2; *dilp2(p)>+*, 42,7% ± 9,8; *DJ634>+*, 62,8% ± 5,6; *UAS-PI3K*^{CA}*/+*, 50,5% ± 3,9), to prevent possible pleiotropic effects caused by mutations at the *w* locus. These results demonstrated that the loss of dopamine signaling promotes ovarian maturation, affecting, in turn, diapause response.

2.5 The increase in dopamine levels promotes diapause

We wondered if an increase in dopamine endogenous levels is sufficient to induce an increase in diapause response. Thus, we analyzed the ovarian maturation at low temperatures (12°C, but under LD 16:8 in order to promote photoperiodic ovarian development) of the mutant *ebony*, e^{1}, characterized by a double dopamine content (Hodgetts and Konopka, 1973; Ramadan *et al*., 1993). The proportion of dormant females in this strain settled around 90% (88,1% ± 3,1), while the controls spanned between 57,8% ± 2,9 (*P0206>+*) and 51,6% ± 3,7 ($w^{1118}ls$) (Fig. 13a). The ovaries of the e^{1} females appeared extremely atrophic, as shown in Fig. 13b. Subsequently, we used another genetic strategy to induce an increase in dopamine titers, exploiting the *UAS-Gal4* binary system. Using the same strategy employed for

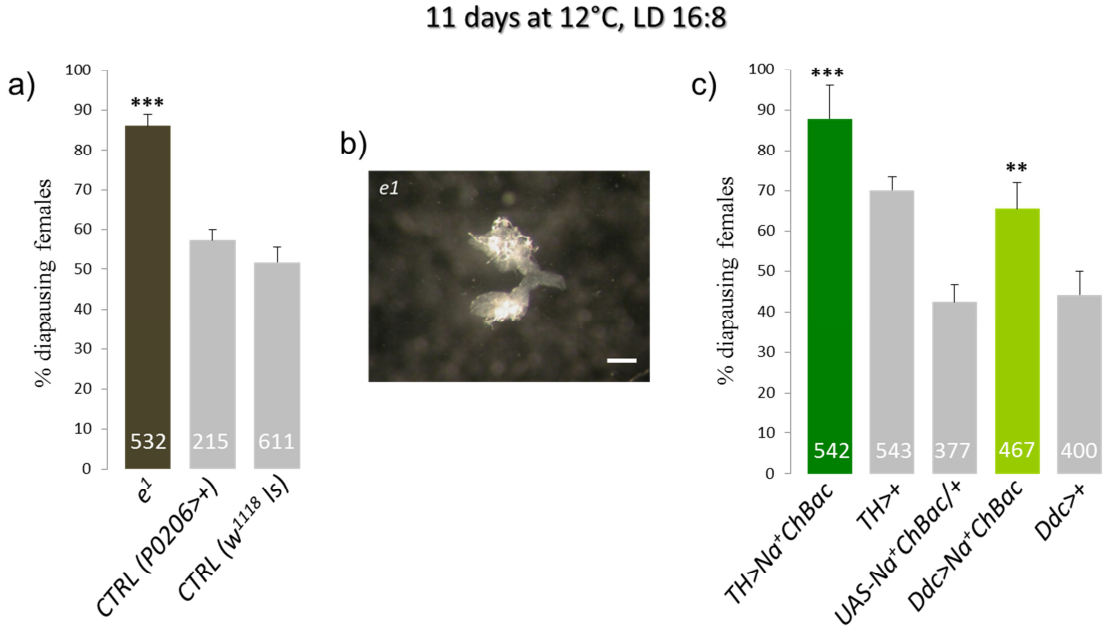

Figura 13. a) Diapause levels of ebony mutants; b) representative ovarian development of ebony gonads under diapause-promoting conditions. White bars represent 0.2 mm; c) diapause proportion of females showing a genetically-induced increased release of dopamine. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-7 rep. n≥60. 2-Way-ANOVA-Interaction: **p*<0.05; ***p*<0.01; ****p*<0.001.

serotonin and GABA, we over-stimulated specifically dopaminergic neurons expressing a bacterial sodium channel (*Na^+ChBac*) in these neurons, using a dopaminergic neurons specific driver, *TH/ple-Gal4* and a second one, *Ddc-Gal4*, specific for dopaminergic but also serotonergic neurons (the expression of these two drivers overlaps solely in dopaminergic neurons). The induced enhanced dopamine release leads to a statistically significant increase in diapause response in both cases (*TH>Na^+ChBac*, 87,8% ± 8,2 and *Ddc>Na^+ChBac*, 65,5% ± 6,4), whereas controls maintained lower percentages of dormant females (*TH>+*, 70,1% ± 3,2; *Ddc>+*, 44,1% ± 5,8; *UAS-Na^+ChBac/+*, 42,4% ± 4,2) (Fig. 13c). Thus, we demonstrated that a genetically-driven increase of dopamine levels is sufficient to elicit diapause.

2.6 Diapause-inducing conditions increase endogenous dopamine levels

At this point, we hypothesized that diapause-promoting conditions are responsible for a physiological (non artificially-induced) increase of endogenous dopamine titers. For this reason, we measured dopamine levels via High-Performance-Liquid-Chromatography (HPLC) in flies maintained for 11 days at 12°C or at 23°C (control flies). In detail, flies exposed to low temperatures were divided into two batches, exposed to different day-lengths (LD 8:16 and LD 12:12) to check for possible photoperiodic effects. Flies exposed under diapause-inducing temperatures (at both LD 8:16 and LD 12:12) showed more than a double dopamine content (2,07 ± 0,32 and 2,49 ± 0,28 µg dopamine/g flies, respectively) with respect to flies kept in optimal thermal conditions (0,90 ± 0,07 µg dopamine/g flies) (Fig. 14). Finally, we demonstrated that low temperatures are sufficient to determine the endogenous dopamine increase responsible for diapause induction/maintenance.

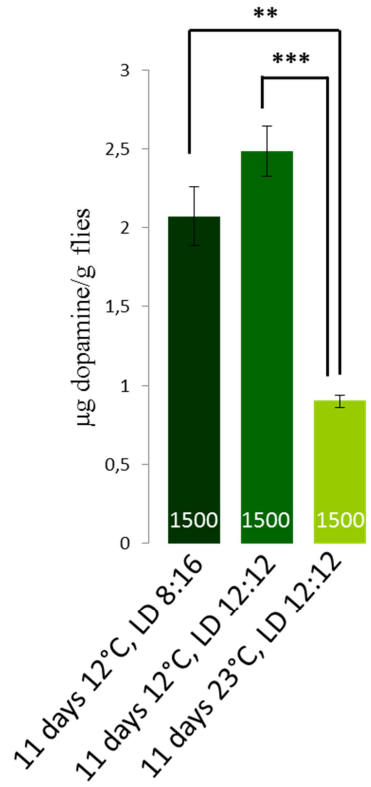

Figura 14. Dopamine whole body content of flies exposed for 11 days under diapause-inducing conditions (12°C, LD8:16 or LD12:12) or 23°C (LD12:12). Numbers within bars refer to sample size. Data are shown as Mean ± SE, 3 rep. n=1500. Student t-test: *p<0.05; **p<0.01; ***p<0.001.

2.7 Impairment of PKA signaling in the *corpus allatum* decreases diapause response and is phenocopied by *DopR1* down-regulation

As we previously demonstrated in *D. melanogaster*, dopamine exerts a potent role in the modulation of diapause, as well as in other kinds of dormancies. Moreover, increased dopamine levels have been found also in the haemolymph in moths, suggesting an

endocrine inhibitory action likely on the IIS-JH-20E axis rather than one as a neurotransmitter within interneuron networks. In order to understand at which level of this axis dopamine acts to molulate this complex life-history trait in Drosophila, we have reviewed the expression and immunohistochemical data reported in the literature, and we found no confirmed evidences about the presence of dopamine receptors in the IPCs. Interestingly, two dopamine receptors, DopR1 and D2R seem to be located on other organs with key functions for hormonal system integrity: *corpus allatum* and fat bodies (Gruntenko *et al*., 2012a) (see Fig. 15a). Although in opposite directions, these two receptors, when activated, perform their functions modulating the same intracellular signaling cascade, the Protein Kinase A (PKA) pathway. Indeed, DopR1 is coupled with a G-protein with a stimulatory α subunit (α_s) (Gotzes *et al*., 1994) (see Fig. 15b), which activates the adenylate cyclase enzyme (AC). Once activated, this enzyme converts ATP in cAMP, that will bind to the regulatory subunit of PKA (PKAR). This subunit works as a dimer, inhibiting another dimer formed by catalytic subunits (below PKA). When PKAR is not bound with cAMP, it represses PKA, blocking this signaling; on the contrary, when cAMP binds PKAR it provokes the detachment between catalytic and regulatory subunits, enabling PKA to phosphorylate the cAMP-Responsive Element Binding protein (CREB). Phosphorylation activates this transcription factor that now can enter the nucleus to bind CRE DNA sequences to promote the expression of specific genes. D2R, instead, has been suggested to be coupled with a G-protein with an inhibitory α subunit (α_i) (Ren *et al*., 2002), which blocks AC activity, suppressing the signaling pathway. Given the previously reported results about *DopR1 hyp* females and the ambivalence of dopamine signaling in these tissues, we decided to start impairing PKA signaling within one of these organs, the *corpus allatum*. Surprisingly, the over-expression of *PKAR* in the *corpus allatum* caused an almost complete absence of diapause (*Aug21>PKAR33*, 0,3% ± 0,6; controls were *Aug21>+*, 69,5% ± 5,2 and *UAS-PKAR33/+*, 34,4% ± 4,6) (Fig. 16a and b). To demonstrate a functional connection between the endogenous increase of dopamine levels caused by low temperatures and the effects on ovarian development due to PKA modulation in the *corpus allatum*, we down-regulated *DopR1* (between the two dopamine receptors the only which could activate PKA) in this tissue. We expressed a dsRNA of *DopR1* specifically in the *corpus allatum* finding a pronounced decrease of diapause incidence (*Aug21>DopR1-RNAi*, 22,6% ± 2,3) if compared

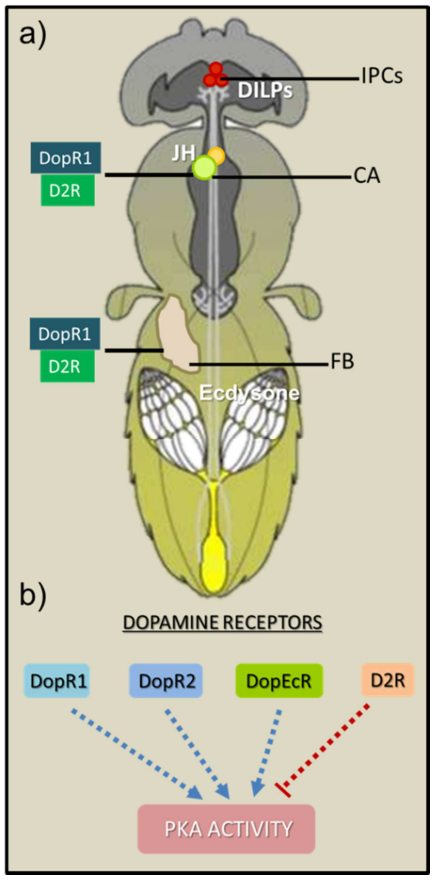

Figura 15. a) Distribution of dopamine receptors along the IIS-JH-20E neuroendocrine axis of Drosophila. IPCs=Insulin Producing Cells; CA=*Corpus allatum*; FB=fat bodies; DILPs=Drosophila Insulin-like peptides; JH=juvenile hormone. b) Effect of the four dopamine recpetors of *D. melanogaster* on PKA signaling pathway (modified from Clyne and Miesenböck, 2009).

with controls (*Aug21*>+, 62,8% ± 3,3; *UAS-DopR1-RNAi*/+, 50,0% ± 5,2) (Fig. 16c). Taken together, these evidences suggest that dopamine modulates diapause response acting through

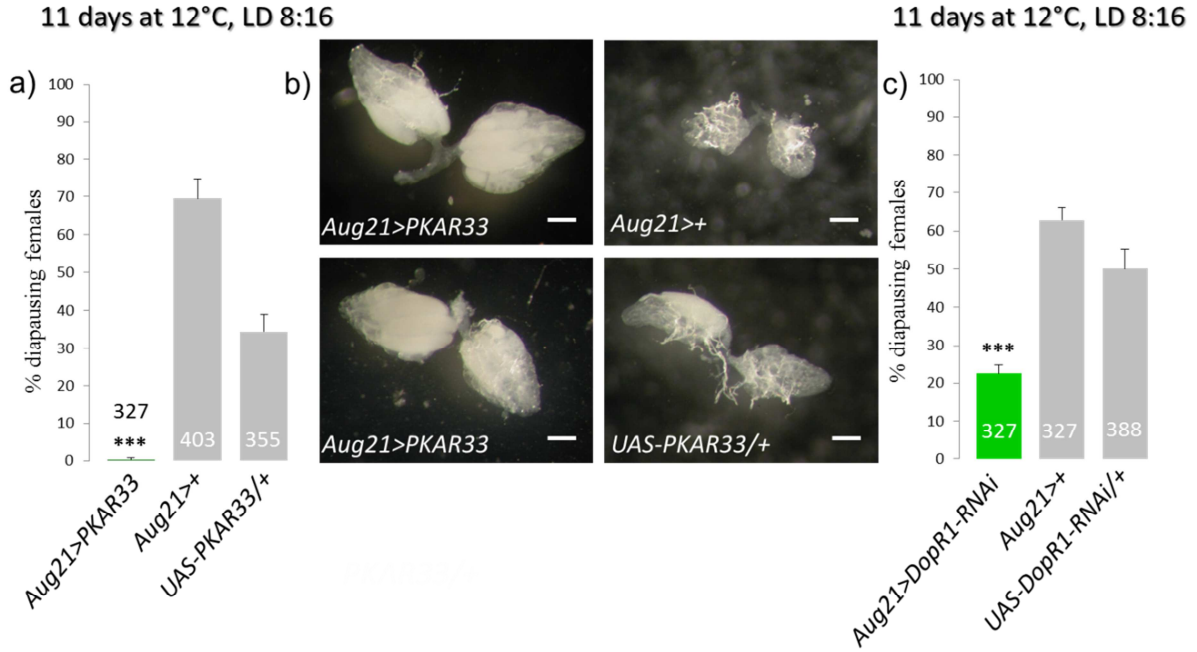

Figura 3. a) Diapause levels of flies with reduced activity of the PKA pathway within the corpus allatum; b) representative ovarian development of Aug21>PKAR33 gonads under diapause-promoting conditions compared to controls. White bars represent 0.2 mm; c) diapause proportion of DopR1-interfered females. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-8 rep. n≥60. 2-Way-ANOVA-Interaction: **p*<0.05; ***p*<0.01; ****p*<0.001.

DopR1 in the *corpus allatum*.

2.8 Shut-down of PKA signaling in the fat cells decreases diapause incidence and is phenocopied by *DopR1* down-regulation

Focusing our attention on the other organ involved in IIS-JH-20E axis regulation where dopamine receptors are expressed, fat bodies, we employed an analogous approach, but using two different drivers which expression, although almost fat body-specific, converges solely in fat bodies (see above). We first shut-down PKA pathway over-expressing *PKAR*, obtaining a lower percentage of dormant females in *cg>PKAR33* flies (10,1% ± 6,2) with respect to the appropriate controls (*cg*>+, 46,4% ± 8,3; *UAS-PKAR33/+*, 39,8% ± 4,1), whereas *ppl>PKAR33* manipulation showed a significant decrease in diapause incidence (24,8% ± 6,7) compared to *ppl*>+ (56,8% ± 3,7) but not to *UAS-PKAR33/+* (39,8% ± 4,1) (Fig. 17a). A clearer response was observed impairing PKA pathway expressing a dominant negative form of CREB (CREB^{DN}). In this case, both *cg>CREB*^{DN} and *ppl>CREB*^{DN} responses were significantly down-regulated (13,4% ± 2,6 and 21,2% ± 1,3, respectively), whereas controls ranged between 45-60 % (*cg*>+, 46,4% ± 8,3; *ppl*>+, 56,8% ± 3,7; *UAS-CREB*^{DN}/+, 60,2% ± 6,4) (Fig. 17b). Also in this case, DopR1 is the only dopamine receptor whose directional

action can fit with both diapause-driven dopamine increase and PKA-mediated effects on ovarian growth. Thus, we down-regulated specifically in the fat bodies *DopR1* expression (using two drivers also in this case). Both *cg>DopR1-RNAi* and *ppl>DopR1-RNAi* flies showed very low levels of diapause (3,5% ± 3,1 and 28,6% ± 2,4, respectively), phenocopying low proportion of dormant females exhibited by flies with impaired PKA signaling within this tissue (controls were *cg>+*, 46,4% ± 8,3; *ppl>+*, 56,8% ± 3,7 and *UAS-DopR1-RNAi/+*, 50,0% ± 5,2) (Fig. 17c). As previously proposed for the *corpus allatum*, also in this case our results suggest that dopamine modulates diapause phenotype via DopR1/PKA signaling also acting on fat cells.

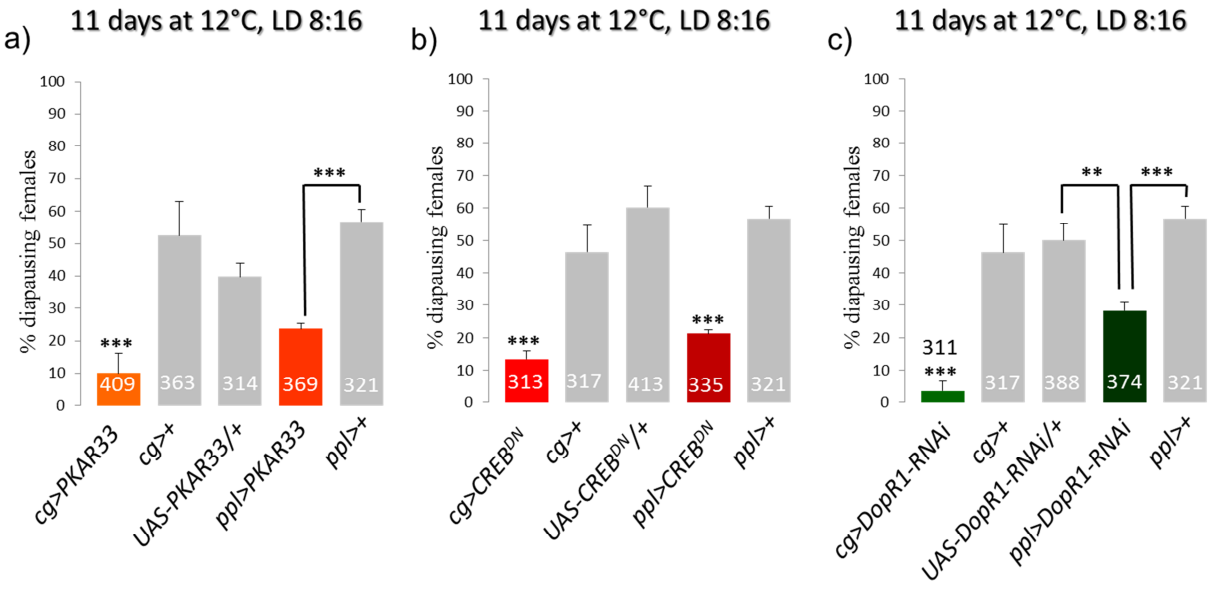

Figure 4. Diapause levels of flies with a) and b) an impairment in PKA activity in fat cells; c) DopR1 down-regulation within fat bodies. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 5-8 rep. n≥60. 2-Way-ANOVA-Interaction: **p*<0.05; ***p*<0.01; ****p*<0.001.

2.9 PKA signaling within *corpus allatum* and fat bodies alters gene expression of JH-related and vitellogenins genes, respectively

The *corpus allatum* is known solely to be the JH producing gland, thus, it is reasonable to envisage changes in JH release to explain the effects on diapause of the dopamine-PKA signaling shut-down. Given that measuring directly JH titers is extremely complex (the vast majority of papers on JH employ indirect assays like qPCR on JH-target genes or estimation of JH degradation, see Mirth *et al*., 2014 and Rauchenbach *et al*., 2011), we measured mRNA levels of three genes which expression has been demonstrated to be strongly affected by *corpus allatum* genetic ablation (and thus JH deficiency) (Yamamoto *et al*., 2013). As shown in Fig. 18a, expression levels of *Odorant binding protein 99b* (*Obp99b*), *Jonah 25Bii* (*Jon25Bii*) and *Krüppel homolog 1* (*Kr-h1*), measured in fly body (thorax + abdomen) of flies with impaired dopamine-PKA signaling within the *corpus allatum* (*Aug21>PKAR33*;

Aug21>CREB^{DN}; *Aug21>DopR1-RNAi*; *Aug21>+* as control) maintained at 12°C for 11 days showed deep alterations (Fig. 18a). These data suggest at least a partial involvement of JH in mediating the effects on diapause of dopamine-PKA signaling manipulation.

Fat bodies are key organs to couple nutrition with systemic growth, but also for metabolic regulation and yolk production. Thus, to investigate the role of PKA signaling in fat cells, we hypothesized its potential involvement in regulating yolk proteins production and/or release. We measured transcripts levels of the three vitellogenin genes encoded in the Drosophila genome (*yp1*, *yp2*, *yp3*) in flies with a shut-down of PKA signaling within fat cells. We employed the most widely used *ppl-Gal4* line to drive the over-expression of *PKAR* or *CREB*^{DN}, and we exposed flies under cold temperature (12°C) for 11 days. We found a marked and significant up-regulation of almost all the three *yp* genes (Fig. 18b), a result which demonstrates that PKA signaling could block vitellogenesis in fat cells and thus promote diapause.

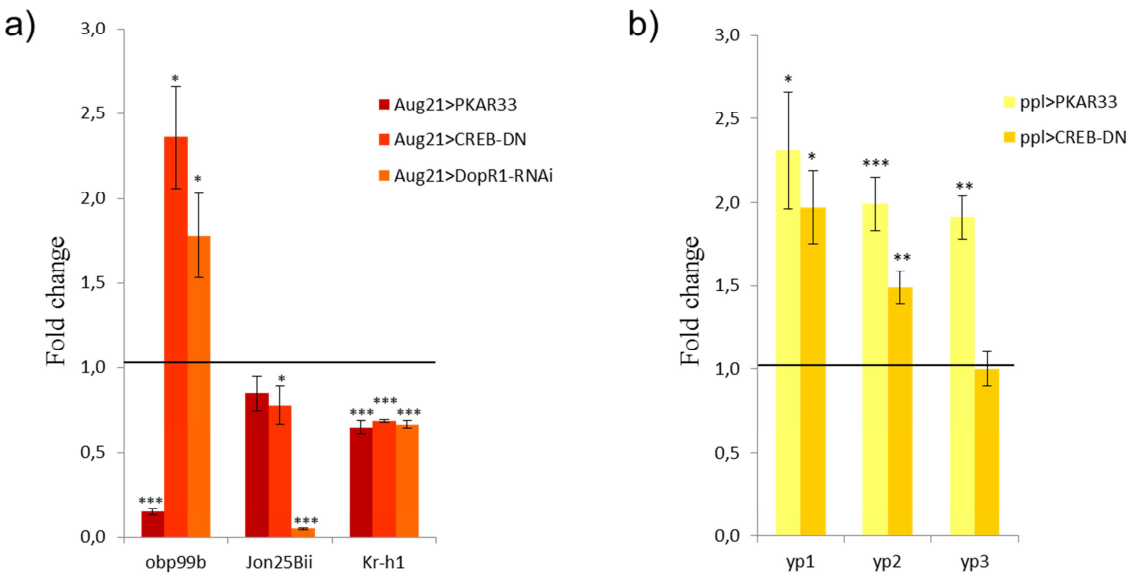

Figure 5. a) Expression levels of JH-regulated genes in females with impaired PKA activity or DopR1 down-regulation in the corpus allatum (Aug21>+ flies were used as controls); b) Expression levels of Drosophila vitellogenins genes in females with PKA impairment in the fat bodies (*ppl>+* flies were used as controls). Data are shown as Mean ± SE, 3 rep. n=14. Student t-test: **p*<0.05; ***p*<0.01; ****p*<0.001.

3. Discussion

3.1 Aminergic control of brain IIS and diapause in Drosophila

Like the mosquito *C. pipiens*, the model organism *D. melanogaster* exhibits a reproductive diapause caused by a systemic Insulin signaling (IIS) shut-down. Insulin Producing Cells (IPCs) physiology is regulated by several factors (neuropeptides, biogenic amines, other humoral factors) which exert a positive or a negative effect on Drosophila Insulin-like peptides (DILPs) secretion from these neuroendocrine cells. Although the understanding of many of these mechanisms is well established, and in spite of the well acknowledged role of IIS in modulating many life-history traits, nothing is known about the identity of the IPCs regulators whose effect triggers the activation of adaptive responses in nature such as diapause. For this reason we focused our attention on the identification of the negative regulator(s) of brain IIS which action on IPCs determines an impaired systemic IIS, a condition necessary for entering diapause. We focused mainly on aminergic signaling which represent an outstanding example of signaling co-option, given the involvement of many biogenic amines in the regulation of dormancy across metazoans. In Drosophila, serotonin, GABA and octopamine signaling converge on IPCs affecting their physiology, and a role in IIS regulation has been demonstrated for all of them. In detail, the knock-down of *5HT-1A* (serotonin receptor) increases DILP2 production (Luo *et al*., 2012) and *dilp2* and *dilp5* expression (Luo *et al*., 2014), whereas *GBR* (metabotropic GABA receptor) down-regulation results in an enhanced production and release of DILPs (Enell *et al*., 2010; Rajan and Perrimon, 2012). Recently, *dilp3* up-regulation has been found in flies expressing a *OAMB* dsRNA (an octopamine receptor) within the IPCs (Luo *et al*., 2014). Thus, we tested flies characterized by a down-regulation of each of these receptors in the IPCs in diapause-promoting conditions (12°C, LD 8:16), with the aim to identify which of these genetic manipulations have the potential to increase brain IIS even under unfavourable conditions for growth and development, and therefore to affect diapause response. The down-regulation of *5HT-1A* in the IPCs led to a remarkable drop in the percentage of dormant females using two different drivers, while *GBR* and *OAMB RNAi* flies exhibited weak or no effects on diapause incidence. To confirm these results, we also induced genetically an increased stimulation of the different aminergic neurons in order to promote a constitutive release of specific biogenic amines. The over-stimulation of serotonergic neurons promotes consistently high levels of diapause whereas, GABAergic neurons excitation showed no significant effects; on the contrary, an increased release of octopamine led to significantly lower levels of diapause percentages. Phenotypic evidences suggest that serotonin could mediate diapause-inducing stimuli to the IPCs, causing the suppression of IIS. In fact, in *5HT-1A* interfered flies, both *4E-BP* and *InR* expression (used as read out for IIS shut-down) were down-regulated, confirming also molecularly the enhanced IIS activity in flies with an impairment in serotonin signaling, and in turn, the pivotal role of this molecule in diapause signaling cascade. As

demonstrated by Schiesari *et al*. (submitted), *dilp2* and *dilp5* expression within the fly brain is up-regulated in females exposed to diapause-promoting conditions, a scenario which seems to clash with the role of serotonin/5HT-1A as negative regulators of *dilp2* and *dilp5* expression. Luo *et al*. (2012) did not focused their attention on axonal terminations (in which DILP2 immunofluorescence could have provided more reliable data to infer a role in serotonin also in gating DILPs release), nevertheless, all the phenotypic effects observed in both their and our experiments on *5HT-1A-RNAi* flies are consistent even with an impaired DILPs secretion, a conclusion supported by our expression data about *4E-BP* and *InR* in fly bodies, which suggest a systemic IIS up-regulation in *5HT-1A* interfered flies. Unfortunately, there is no information about the nature of serotonin signaling to the IPCs. Although Luo *et al*. (2012) showed an impaired survival under both starvation and heat-stress (39°C) conditions of *5HT-1A* interfered flies, these results do not necessarily prove a serotonin involvement in the conveyance of thermal or nutritional information to the IPCs. In fact, DILPs share pleiotropic functions, and the modulation of their production/release could affect indirectly phenotypes in which IIS results somehow involved. However, Luo *et al*., (2012), on the basis of studies accomplished on the larger blowfly and in Drosophila larvae (Nässel, 1988; Kaplan *et al*., 2008; Agrawal *et al*., 2009), suggested that serotonin signaling to the IPCs could be part of the suboesophageal ganglion-brain neuronal network, which mediates chemosensory inputs and regulates feeding as well as neuroendocrine functions. Nevertheless, further studies are required to understand what are the environmental information triggering serotonin signaling towards the IPCs. We should also consider the possibility that the activity of serotonergic neurons on the IPCs could be affected by an elusive and still unknown fat body-derived humoral factor, able to stimulate DILPs release from the brain in relation to amino acids sensing (Géminard *et al*., 2009) preventing serotonin release, in a circuit similar to the sugars/fats-Upd2-GABA one. Another intriguing and possible explanation infers that serotonin signaling could mediate thermal information rather than being involved in nutrient signaling networks. Although the regulatory pattern of IPCs physiology have been significantly dissected, no evidences have been provided so far about the existence of factors mediating cues not related to nutrition. Given the strong effects on growth, development and reproduction exerted by temperature and the prominent role of IPCs as neuroendocrine regulators of these biological processes, it is plausible to hypothesize the existence of a temperature-dependent neural signaling which communicates favourable or unfavourable thermal conditions to the IPCs. The analysis of a potential temperature-dependent regulation of *dilps* transcripts is difficult due to the existence of feedback regulatory mechanisms from other organs such as ovaries. 12°C represents a low temperature which anyway allows a basal metabolism, although reduced. Thus, temperature-dependent effects on *dilps* expression can be masked by the ovarian feedback likely acting on the IPCs, and demonstrated by brain *dilps* up-regulation under diapause-promoting conditions (Schiesari *et al*., submitted) or in germline-less flies (Flatt *et al*., 2008). Moreover, our results about *OAMB* down-regulation within the IPCs are consistent with the existing literature. Octopamine has been argued to counteract the expression of *dilp3* (Luo *et al*., 2014), a DILP which, although likely involved in a positive autocrine feedback to stimulate *dilp2* and *dilp5* expression (Grönke *et al*., 2010),

if knocked-out, shows no increase in diapause response contrarily to *dilp2* and *dilp5 null mutants* (Schiesari *et al*., submitted). Apparently, this result does not fit with the autocrine role proposed by Gronke *et al*. for DILP3, but could be easily explained by the likely temperature-dependent gating of DILPs release under low temperature. If we take for granted why DILP3 effect is dispensable under diapause conditions, it appears clear why *OAMB-RNAi* flies showed no changes in the proportion of dormant females with respect to the controls. More complex it seems to find an explanation for the low diapause levels characterizing flies with increased octopamine secretion. It has been demonstrated that flies with enhanced activity of octopaminergic neurons are characterized by an up-regulation of trehalose and glucose haemolymphatic levels, as well as higher amounts of total triglycerides, a metabolic effect partially decoupled from IIS (Erion *et al*., 2012). Thus, these evidences suggest that octopamine, affecting sugar and lipid metabolism (which changes concur in insect diapause) also independently from IIS, can potentially influence diapause phenotype in Drosophila. Alternatively, given that octopamine seems to increase ecdysone production and conversion into the active form 20-hydroxy-ecdysone (Rauschenbach *et al*. 2007; Rauschenbach *et al*., 2008), the significantly lower diapause levels observed in *Tdc>Na*$^{+}$*ChBac* flies could be also explained via an increased ecdysone titer. In 2012, Xu *et al*. provided interesting data about precocious diapause termination in *H. armigera* due to the direct or indirect action of tricarboxylic acids (TCAs) intermediates on brain neuroendocrine activity (PTTH release). TCAs are key components to fuel the Krebs cycle, which represents the central core of both sugars, fats and amino acids metabolism. In spite of its effect on DILPs expression and release, a shut-down in GABA signaling does not seem to affect strongly diapause behaviour, as well as a genetically-induced increase in GABA release. Moreover, also the over-expression of *Upd2* (the cytokine-like protein released by fat bodies according to nutrition-derived fats and sugars able to prompt DILP2 and DILP5 release) within the fat bodies using two independent drivers, showed no or weak effects on the percentage of diapausing females. Data about diapause response obtained employing manipulations of GABA-Upd2 signaling are not sufficient to exclude the role of fat bodies as regulators of insect brain neuroendocrine activity depicted by Xu *et al*. (2012). In fact, the activation of this signaling is triggered only by sugars and fats obtained from feeding, but not by amino acids sensing (Rajan and Perrimon, 2012), which represents the most prominent nutrition-derived cue able to affect growth, development and lifespan (see Colombani *et al*., 2003; Min and Tatar, 2006; Grandison *et al*., 2009; Bjedov *et al*., 2010; Katewa *et al*., 2012). As already mentioned, the amino acids-based mechanism is mediated by TOR, a key modulator of cellular growth, which, in fat cells, plays a nutrient-sensing role. For this reason, we down-regulated TOR activity within fat bodies via *TSC1-TSC2* over-expression or expressing a dominant negative form of TOR (TOR^{DN}), using two different drivers, showing a strong diapause phenotype. Although we can not exclude completely a developmental effect, at least for the *ppl-Gal4* driver, given that this *Gal4* line employed to induce *UAS*-mediated expression in fat cells drive *Gal4* expression also during larval stages (see Colombani *et al*., 2003), no obvious phenotypic differences between these females and controls have been observed. Nevertheless, TOR-S6K activity promotes vitellogenesis in the hard tick, *Haemaphysalis longicornis*

(Umemyia-Shirafuji *et al*., 2012), in the yellow fever mosquito, *Aedes aegypti* (Hansen *et al*., 2005; Roy and Raikhel, 2011) and in the German cockroach, *Blattella germanica* (Maestro *et al*., 2009), but, although surprisingly, there is no information about this mechanism in the model organism *D. melanogaster*. All these findings suggest that our results on TOR role as diapause regulator could be explained as due to a decrease or increase in vitellogenins production and in turn to an extension or an early termination of the diapause state; the other possibility relies on an indirect effect of TOR activity within fat bodies on vitellogenesis, exerted through its control of DILPs secretion. In conclusion, the fat body-derived humoral factor released upon TOR activation must be identified in order to investigate the role of TOR in diapause, and to better understand fat bodies contribution in insect dormancy as downstream effector or main control structure.

3.2 Dopamine signaling is key for diapause onset/maintenance in Drosophila

As previously discussed, dopamine signaling shows a widespread regulatory role in dormancy across insects. Although this role has been established for a long time, no evidences have been provided in the model organism *D. melanogaster*, despite the huge amount of genetic toolkits available to study this mechanism. In a great variety of species, dopamine exhibits a powerful role in promoting diapause onset and/or maintenance, nevertheless nothing is known about the target organs of diapause-induced dopamine release, as well as the molecular mechanisms through which dopamine exerts its effect. To investigate the role of dopamine in the regulation of Drosophila diapause, we started analyzing diapause behaviour of flies with impaired dopamine production or signaling. In diapause-promoting conditions (12°C, LD 8:16), both $TH^{-/-}$ and *Ddc hyp* mutants (mutants for the two enzymes involved in dopamine synthesis) exhibited very low levels of diapause with respect to the controls, suggesting that the disruption of dopamine biosynthesis results in the failure of diapause entry or maintenance, favouring a precocious termination. Interestingly, low levels of dormant females characterized also the hypomorphic mutant for *DopR1*, one of the four dopamine receptors encoded in the Drosophila genome. Subsequently, we demonstrated that a genetically-induced up-regulation of dopamine titers, employing the mutant *ebony* or exploiting the *UAS-Gal4* binary system to stimulate an increased secretion from dopaminergic neurons, led to high percentages of diapausing females, confirming the role of dopamine as a positive regulator of insect diapause. Furthermore, we measured dopamine levels in flies exposed to diapause-promoting conditions, observing higher dopamine levels with respect to control flies kept in optimal conditions for growth and reproduction (23°C, LD 12:12). Taken together, these results show robust evidences about the importance of dopamine production and release in determining diapause/normal developmental fate in Drosophila. Dopamine absence or anyhow a disruption in its synthesis can disable females to enter diapause properly, in spite of the perception of diapause-inducing environmental cues, whereas an enhanced dopamine release seems to halt or slow-down ovarian maturation. Moreover, the remarkable vitellogenic levels exhibited by *Ddc hyp* females, with many oocytes at stage 14 (ready-to-be-laid),

provide a strong evidence about the pivotal role of both dopamine and serotonin as diapause-inducing factors, given that an impairment in the synthesis of these amines gives rise to phenotypes similar to those characterizing females exposed to favourable conditions for oogenesis. Considering dopamine strong effects on developmental arrest across insects, it is plausible to envisage that this biogenic amine can affect in some way the IIS-JH-20E neuroendocrine pathway, and supporting evidences for such a role, although with some inconsistencies, have been provided by Gruntenko and colleagues, in several papers. Intriguingly, a juvenile hormone (JH) deficiency characterizes the adult *ebony* mutant females (see Gruntenko *et al*., 2012a), which has a double dopamine content compared to wild-type flies (Hodgetts and Konopka, 1993), providing another evidence about a potential involvement of dopamine in regulating key steps of the neuroendocrine axis governing fly development, growth and reproduction, as confirmed by our data on diapause in these mutants. Thus, we wondered at what level dopamine could interact with the IIS-JH-20E regulatory network. To our knowledge, examining the existing literature and thanks to some personal communications kindly shared by Prof. Frederik Wolf and Prof. Dick Nässel (see also Kim *et al*., 2007), no dopamine receptors seem to be present on the IPCs, whereas two of them, *DopR1* and *D2R*, are expressed in *corpus allatum* and fat bodies (Gruntenko *et al*., 2012a). The *corpus allatum* is the neurohaemal gland responsible for JH synthesis and release in insects, and its removal or genetic ablation cause severe developmental or reproduction-defective phenotypes (Meola and Petralla, 1980; Readio *et al*., 1999; Riddiford *et al*., 2010; Gruntenko *et al*., 2010), while fat bodies represent key structures for metabolic regulation, energy storage and vitellogenesis. Although we can not exclude the involvement of other dopamine receptors (like DopEcR, which expression perhaps has not been analyzed in detail in these tissues yet), our data suggest that dopamine effects on Drosophila dormancy are mediated, at least partially, by DopR1, given the low proportion of dormant individuals shown by the hypomorphic mutant for this receptor. For this reason, we tested diapause response in flies in which we down-regulated specifically *DopR1* in each of these tissues, driving the expression of a dsRNA *DopR1* within *corpus allatum* or fat cells. As previously exposed, both *corpus allatum* and fat bodies *DopR1* interfered flies showed lower proportion of dormant females compared to the controls. Moreover, genetic manipulations of PKA signaling (which acts downstream to DopR1) within these structures, confirmed our previous results. In fact, PKA signaling impairment within the *corpus allatum*, obtained over-expressing the *PKA regulatory subunit*, *PKAR*, resulted in extremely well developed ovaries even under diapause-promoting conditions. Focusing on fat bodies, experiments of PKA signaling impairment (both over-expressing *PKAR* or expressing a dominant negative form of CREB, $CREB^{DN}$, a PKA downstream effector) resulted in a consistent reduction in diapause proportion with respect to the controls. Dopamine role as a diapause-promoting actor is widespread across insects, nevertheless, no regulatory networks have been proposed to place diapause-induced dopamine increase in a biological context. Our experiments provide the first evidence about a potential regulatory mechanism which involves dopamine action in insect diapause. The diapause governing mechanisms that are now emerging appear much more complex than those previously expected, based on the almost hierarchical relationship

between the main actors involved in insect development and reproduction, IIS, JH and ecdysteroids. The potential adaptive value of having at least a double control, at two different levels, of the neuroendocrine cascade governing Drosophila reproduction seems undoubted if we consider the fundamental role of diapause not simply to block or delay reproduction, but even for survival of individuals themselves. Indeed, as we have already reported, diapause program enhances survival potential, slowing-down metabolic rate and energy storage consumption, as well as increasing thermotolerance via cryoprotectants synthesis. Thus, this redundant control mechanism could ensure hormonal lock-down in conditions where, if not gated, the effect of just one of these actors could irreversibly compromise individual survival. Moreover, although closely related, DILPs, JH and ecdysteroids retain also independent functions, as reported in the Introduction, and hence, this double mechanism can be exploited to sunder at least partially IIS and lipophilic hormones signaling even under sub-optimal or optimal conditions. In this view, the depicted regulatory network could allow a harmonious ovarian growth, coupling egg chamber growth and vitellogenins up-take with the proper hormonal balance. Thus, the double regulatory aminergic mechanism described by us could also provide a fine tuned hormonal homeostasis as well as ensure a suitable hormonal secretion gating which fit the environmental conditions experienced.

DopR1 and *D2R* are differentially expressed in *corpus allatum* and fat bodies depending on fly age: in young females (1 day old), DopR1 and D2R are more abundant in the *corpus allatum* and fat bodies, respectively, whereas in mature females (6 days old), these expression pattern is overturned, with *D2R* more expressed in the *corpus allatum* and *DopR1* in fat cells (Gruntenko *et al*., 2012a). Both the receptors act on the PKA signaling pathway but in different directions. Indeed, DopR1 is coupled with a G-protein carrying a G_{α} subunit, which activates adenylate cyclase and then PKA, while D2R represses PKA activity. In spite of the expression pattern observed by Gruntenko *et al*. (2012a), the dopamine effects that we have observed on Drosophila diapause could be explained solely through its interaction with DopR1, which leads to the activation of PKA signaling, a process that, in case of impairment, induces diapause loss in the majority of females exposed to low temperatures. However, it is difficult to assess the aging delay which characterizes females exposed to low temperatures like 12°C. In the future, it could be useful to analyze the dopamine effects on *corpora allata* of diapausing females, analyzing dopamine receptors abundance on this gland at the time of the ovarian dissection (after 11 days at 12°C) using immunocytochemistry, and then compare the results with the pattern shown by Gruntenko *et al*. (2012a). A biological explanation for the concomitant presence (although with differences in abundance) of two dopamine receptors (with opposite effects on PKA signaling) on the same tissues remains puzzling. Perhaps, this scenario could be part of a fine-tuned hormonal or metabolic regulation in which a constitutively low basal expression of the two receptors provides a sort of buffering system in the case of important perturbations in dopamine contents which could in turn cause dramatic effects on hormonal homeostasis. In addition, these two receptors could trigger also the activation of other, still unknown, intracellular signaling pathways specific for each dopamine receptor.

Dopamine role as an endocrine modulator of both JH and ecdysone production has been reported in several insect species, like *Blattella germanica*, where dopamine promotes JH synthesis during the early days of adult life but shows already opposite effects at the end of the first week (Pastor *et al*., 1991). A similar pattern has been observed, in larvae of *M. sexta*, with dopamine stimulating JH production during the last larval instar but counteracting JH synthesis once the individual reaches the pre-pupal stage (Granger *et al*., 1996). Also in Drosophila JH and ecdysteroids titers vary according to dopamine signaling, and even in this organism, dopamine seems to induce JH and ecdysone synthesis in young females but not in the sexually mature ones, where this amine plays the opposite role (Gruntenko *et al*., 2000; Rauschenbach *et al*., 2007). This kind of scenario, with an ambiguous and sometimes opposite role of dopamine signaling depending on the tissue or on the developmental stage, is not surprising even in Drosophila. For instance, in Drosophila dopamine has been demonstrated to promote developmental progression and reproduction (Neckameyer, 1996), although in adults its synthesis is induced after heat stress exposure (Gruntenko *et al*., 2004). To complicate this scenario, dopamine seems to be involved in a complex feedback interplay with JH and 20-hydroxy-ecdysone (20E) to ensure proper hormonal balance. The genetic ablation of the *corpus allatum* causes a drop in tyrosine hydroxylase (TH) activity (consistent with the opposite result provided by JH application, see Rauschenbach *et al*., 2011a), but at the same time an unexpected higher dopamine content (Gruntenko *et al*., 2012b). In young females of *D. melanogaster* and *D. virilis*, TH activity is increased by 20E or JH administration (Gruntenko *et al*., 2009), whereas in mature *D. virilis* females, these two hormones reduce and enhance TH activation, respectively (Rauschenbach *et al*., 2011a). Furthermore, in *D virilis*, even dopamine content appears mutable in time according to 20E regulation: indeed, 20E administration results in higher dopamine levels in young females (3 days old) but lower in 7 days old females (Gruntenko *et al*., 2005b) (For other details about this potential mechanism of hormonal balance see also Gruntenko *et al*., 2005a; Gruntenko and Rauschenbach, 2008; Gruntenko *et al*., 2009). In *D. melanogaster*, the activation of DopR1 seems to reduce JH degradation (Rauschenbach *et al*., 2011b). The authors stated that dopamine stimulates JH production, a conclusion based on the measurement of JH degradation (and other indirect assays such as tyrosine decarboxylase activity, and JH stress-reactivity). However, this conclusion clashes with the results about JH degradation in stress conditions obtained by the same group, given that this parameter counter-intuitively drops in heat stressed flies (Gruntenko *et al*., 2004), potentially favouring JH rise in adverse environmental conditions. These authors showed an opposite pattern of JH degradation in flies after a pharmacological inhibition or activation of D2R. In 2 days old females, JH degradation was higher, while, in 6 days old females, this parameter was lower with respect to the controls (Karpova *et al*., 2010). Moreover, in females expressing a *D2R*-dsRNA in the *corpus allatum*, JH synthesis, estimated by the same indirect assays was prompted in both young and mature females (Gruntenko *et al*., 2012c), a result which does not mirror the previous one. Taken together, all these studies reveal at least three paradoxes: 1) both DopR1 and D2R activation in young females leads to higher JH levels, in spite of the opposite effects on PKA pathway; 2) pharmacological inactivation of D2R and its down-regulation via RNAi

show opposite patterns of JH degradation; 3) JH degradation levels are surprisingly higher in control flies with respect to flies exposed to heat stress, another puzzling result if we consider the role of JH in adult Drosophila. In conclusion, these data reveal how some indirect parameters adopted to estimate JH titers are not sufficiently reliable to infer putative effects on its metabolism and claim the urgence of further studies to clarify some aspects about dopamine effects on the *corpus allatum* physiology.

Corpus allatum is mainly known as the structure responsible for JH production and release, thus we hypothesized that dopamine-PKA action on this neurohaemal gland could result in changes in JH levels. As the direct measurement of JH levels shows several technical difficulties, we analyzed mRNA levels of genes (*Obp99b*, *Jon25Bii* and *Kr-h1*) whose expression is regulated by JH (see Yamamoto *et al*., 2013) in flies with an impaired DopR1-PKA signaling within the *corpus allatum*. Our results suggest an implication of this signaling pathway in JH secretion. In detail, two out of three of the selected genes, *Jon25Bii* and *Kr-h1*, showed a consistent expression pattern between flies bearing a *DopR1* down-regulation or a PKA shut-down (achieved employing *PKAR* over-expression or the expression of a dominant negative form of CREB), although *Jon25Bii* down-regulation in *DopR1* interfered flies was more severe. Yamamoto *et al*. (2013) did not specify the tissues they used for RNA extraction, likely the whole body (given that in the same study also *dilp2*, *dilp3* and *dilp5* transcripts are measured), whereas we used solely bodies (and not heads) for our analysis, to avoid potential feedback mechanisms (like those established for brain *dilps*, see Flatt *et al*., 2008; Yamamoto *et al*., 2013; Schiesari *et al*., submitted). This difference could potentially affect the expression of the selected genes, making at least in part unrelaible a comparison between the results obtained by Yamamoto *et al*. (2013) and ours. Indeed, both *Obp99b* and *Kr-h1* are even expressed in the head (the latter even across insects) (Liu *et al*., 2009; Abdou *et al*., 2011; Grozinger and Robinson, 2007; Minakuchi *et al*., 2009; Kayukawa *et al*., 2012; Yamamoto *et al*., 2013; Flybase modENCODE). Moreover, Yamamoto *et al*. (2013) induced the genetic ablation of the *corpus allatum* from the adult stage, while the expression of our transgenes driven by *Aug21-Gal4* (the same driver used by those authors) starts during larval stages, and perhaps even this difference could reflect in altered transcripts levels.

Finally, it is worthnoting that their expression data concern a scenario different from ours, with a complete or partial ablation of the *corpus allatum*, and not the impairment of a signaling pathway within the same tissue, although we propose PKA as an upstream modulator of JH synthesis/release. *Obp99b* expression shows a non univocal pattern comparing *DopR1-RNAi* females and flies characterized by an impaired PKA signaling within the *corpus allatum*. In flies over-expressing *PKAR*, *Obp99b* levels result extremely low, consistently with the expression in flies bearing a genetic ablation of the *corpus allatum* (see, Yamamoto *et al*., 2013), whereas on the contrary, in both $CREB^{DN}$ and *DopR1-RNAi* flies, this gene is surprisingly up-regulated. One possibility relies on the different effect of the transgenes employed. Unfortunately, there are no data available about the PKA role within the *corpus allatum*, which could be useful to disentangle this complex scenario. Thus, surprisingly, we obtained an unexpected expression pattern of JH-dependent genes which, in some cases, is consistent with a JH gain rather than a JH deficiency induced by PKA

activation in the *corpus allatum*, something that both the only known role of *corpus allatum* and our phenotypic results strongly suggest. Taken together, our expression data potentially reveal a complex regulation of JH metabolism mediated by dopamine and PKA signaling rather than a simple JH lock-down.

Fat bodies are key structures for diapause regulation. Indeed, these key organs couple feeding with tissue growth, regulate metabolism and energy storage consumption, represent lipid and glycogen store tissues as well as the site of vitellogenins synthesis and secretion. To investigate dopamine signaling role on fat bodies, we started focusing on the latter processes, measuring transcript levels of the three yolk protein genes (*yp1*, *yp2*, *yp3*) in flies with an impaired PKA signaling within fat cells. Females expressing in fat cells *PKAR* or $CREB^{DN}$ showed a significant up-regulation of almost all yolk protein genes (only *yp3* in $ppl>CREB^{DN}$ was not significantly increased). These evidences, together with our phenotypic data, suggest that dopamine, acting through DopR1-PKA signaling in the fat bodies could promote vitellogenins production and consequently diapause impairment. It is possible that changes in vitellogenins expression are not the unique modifications induced by PKA shut-down within fat cells, considering the multiple roles of these tissues, some of which have been listed above. In the future, it would be interesting to analyze further this possibility, for instance measuring expression levels of genes involved in those biological processes (i.e., genes encoding the enzymes involved in JH degradation, produced specifically in the fat bodies, see Gruntenko *et al*., 2012a). Finally, to explore further potential metabolic changes induced by an impaired dopamine signaling within fat cells, it could be worth to estimate free sugar levels in the haemolymph, glycogen and lipid stores, and metabolic rate (for instance evaluating oxygen consumption).

The upstream neural network as well as the specific environmental signaling which triggers dopamine release in diapause-promoting conditions, remain elusive. A recent finding shows that some dopaminergic neurons are activated in response to an incomplete diet to promote food rejection (Bjordal *et al*., 2014). In this study, the authors found that larvae in the middle of their 3rd instar with a genetically-induced activation of dopaminergic neurons fed less than controls. Although this finding could suggest that $TH>Na^{+}ChBac$ flies exhibited higher levels of diapause because of a reduced food intake during larval stages (but we observed no evident morphological differences with respect to controls), another study has demonstrated that starvation stimulates an increased dopamine secretion towards sugar-sensing Gustatory Receptor Neurons (GRNs) to enhance proboscis extension reflex (PER), a behaviour related to food intake (Inagaki *et al*., 2012). Moreover, food deprivation itself enhances food intake levels (Riemensperger *et al*., 2011). Taken together, these results underline the pleiotropic role of dopamine signaling in regulating feeding behaviour, suggesting that our results about diapause in flies with a manipulated dopamine signaling can be rather unlikely explained through local effects within the brain. Moreover, the marked increase of dopamine content in flies exposed to diapause-promoting conditions would remain difficult to explain considering dopamine as a neurotransmitter. Like for serotonin signaling, also for the dopamine one there is no information about the precise environmental stimulus triggering the release of this amine and in turn diapause. Further studies could be addressed towards potential alteration of

dopamine release/content in flies with mutations in genes encoding Transient Receptor Potential (TRP) channels involved in cold perception.

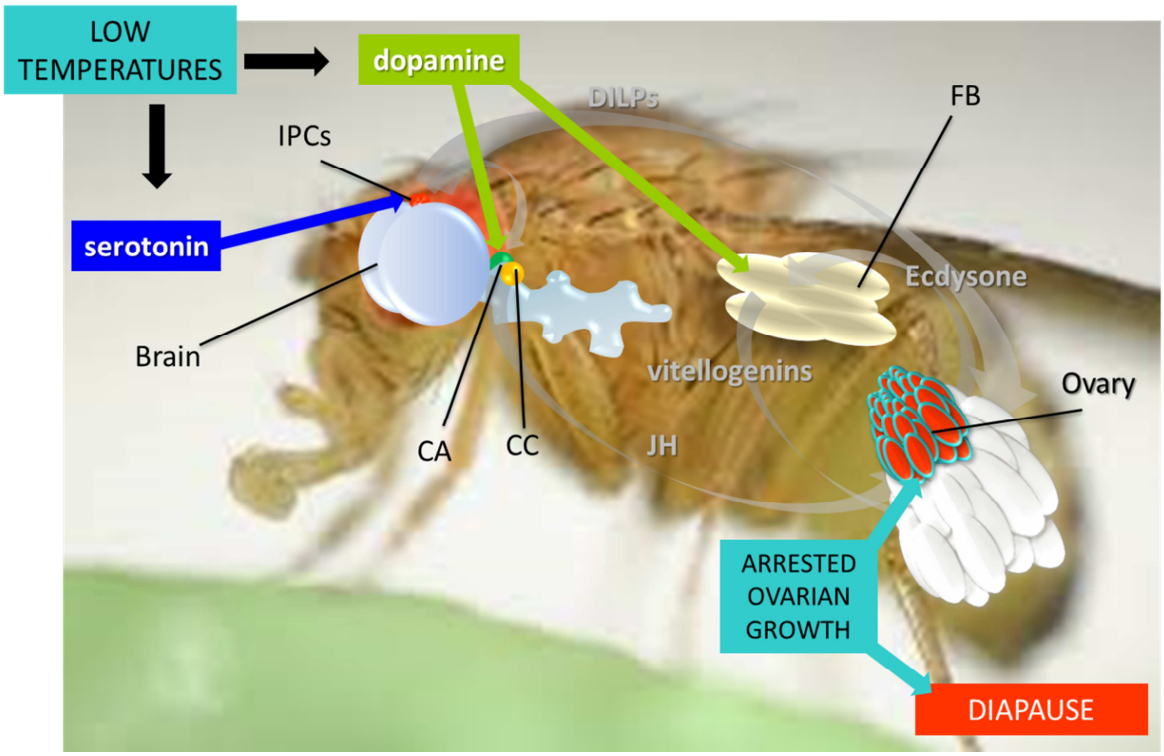

Figure 19. Proposed model for a Drosophila double level aminergic control of diapause. IPCs=Insulin Producing Cells; CA=*Corpus allatum*; CC=*Corpus cardium*; FB=fat bodies; DILPs=Drosophila Insulin-like peptides; JH=juvenile hormone.

4. Additional experiments

During my PhD, I carried out also some additional preliminary experiments, not strictly related to the main project characterizing the experimental work which I have described in the previous part of this thesis. Nevertheless, these experiments have been performed with the aim of understanding other aspects of the complex molecular mechanisms underlying insect diapause. Here I summarize the most relevant results which I have obtained investigating these additional features of diapause.

The long ticking of the clock: a natural variant at the *timeless* locus affects development and reproduction in *Drosophila melanogaster*

In millions of years, evolution shaped biological clocks allowing organisms to regulate the timing of physiological processes according to environmental cues. These molecular oscillators provide a reliable mechanism to anticipate both positive and negative external conditions, and their ability to be entrained by specific environmental stimuli can be fundamental in a changing environment like a seasonal one. As exposed in the chapter on insect diapause, temperature and day length (photoperiod), both capable of entraining endogenous clocks (Peschel and Helfrich-Förster, 2011), represent very often key information to induce changes in growth and development. Although a potential connection between the circadian clock and putative endogenous oscillators able to regulate longer-than-a-day phenomena (like development and diapause) has been proposed in the last decades (see Saunders and Bertossa, 2011), evidences underpinning the involvement of gears of the circadian clock machinery in life-history setting are lacking. *Period* (*per*) mutants, characterized by arrhythmic circadian behaviour, have been reported to discriminate between long and short days in a diapause assay (12°C) (Saunders *et al*., 1989); nevertheless, their critical day length (the day-length at which 50% of diapause response is elicited, Kurota and Shimada, 2003; Wang *et al*., 2012; Paolucci *et al*., 2013) was 2 light-hours shorter, when compared with the wild-type one (Saunders *et al*., 1989). Kyriacou *et al*. (1990) demonstrated how artificially induced mutations at the *per* locus, which alter the period of circadian rhythmicity (without causing arrhythmicity), affect developmental time. *perS* flies, characterized by having a shorter free running circadian period in locomotor activity (19 h instead of 24 h) and eclosion pattern (Konopka and Benzer, 1971), showed also a shorter developmental time, whereas *perL* flies, which free-running period settled around 28 h, had a longer developmental time. In 2007, Kyriacou and Costa labs published two papers dealing with a natural polymorphism at the *timeless* (*tim*) locus that affects Drosophila reproductive diapause. The authors characterized the role of the two alleles involved and found that the short isoform (*timS*) exhibits lower proportion of dormant females, whereas individuals harboring the long isoform (*timL*) are more prone to enter diapause (Tauber *et al*., 2007). To explain the different diapause propensity of the two *tim* variants, a molecular mechanism has

been proposed, showing how TIM-L binding with the inhibitory partner CRYPTOCHROME (CRY) is more labile with respect to TIM S-CRY interaction (Sandrelli *et al*., 2007). Although scarce, the evidences mentioned above suggest that components of the circadian clock machinery could be causally involved in regulating processes, which need more than a day to be completed. To investigate whether natural genetic variation at circadian clock *loci* can provide the molecular substrate for life-history setting, allowing organismal adaptation to seasonal environments, we focused our attention on the *ls-s tim* polymorphism previously described. First, we analyzed diapause response in Houten (Hu, Netherland) wild-type flies homozygous for *ls* or *s tim* alleles (already employed in Tauber *et al*., 2007 and Sandrelli *et al*., 2007) in our experimental conditions (12°C and Maize medium). Our results not only confirmed the previously published ones, but showed also more pronounced differences in the proportion of dormant females between *ls* and *s* individuals, in both short and long days (*Hu ls* 8:16, 87,8% ± 5,1; *Hu s* 8:16, 16,6% ± 4,9; *Hu ls* 16:8, 79,2% ± 7,0; *Hu s* 16:8, 11,9% ± 6,0) (Fig. 20a). Life history traits, such as diapause, developmental time, fecundity and aging, are deeply linked each other, relying on the same neuroendocrine mechanisms. Thus, it is not surprising to observe that populations of *D. melanogaster* from northern latitudes, which individuals are more prone to enter diapause, show also reduced early-life fecundity and increased lifespan (Schmidt *et al*., 2005a; Schmidt *et al*., 2005b; Schmidt and Paaby, 2008). To demonstrate a potential involvement of *ls-s tim* polymorphism in shaping other biological phenomena linked to insect dormancy we assayed developmental time of *Hu ls* and *s* flies. To avoid over-crowding effects, twenty first-instar larvae were placed in each vial (10 vials and 2 replicates for each genotype) containing Drosophila food medium, and their development was monitored till pupariation (see McBrayer *et al*., 2007) under LD 12:12 and 24°C. *Hu ls* flies exhibited a longer developmental time, with an average increase of about 12 h with respect to *Hu s* individuals (data not shown). To test whether a decrease in temperature (mimicking autumnal-like conditions) can exacerbate this phenotypic difference, we repeated this assay maintaining the same experimental conditions (including day length, LD 12:12), but at 15°C (a lower temperature which does not cause a significant pupal mortality, see David and Clavel, 1966). We found the same general pattern, with *Hu-ls* flies characterized by a slower development, but the average difference between pupariation curves settled around 48 h (Fig. 20b). In Drosophila, the genetic-induced lengthening of developmental time, observed down-regulating TOR signaling within the prothoracic gland, is transduced into an increase in body weight (Layalle *et al*., 2008). For this reason, we weighted *timeless-ls* and *-s* homozygous flies reared at 15°C, finding a statistically significant increase in body mass in both *ls* females and *ls* males with respect to the their *s* counterparts (Fig. 20c). Finally, we measured also female fecundity scoring every day the number of eggs laid by 11 females for 12 days (females were allowed to mate for two days after eclosion); as shown in Fig. 20d, *Hu-s* females laid more eggs per day in the interval considered if compared with *Hu-ls*. Taken together, these evidences demonstrate that *ls-s tim* natural polymorphism affects not solely diapause propensity, but also other life-history traits linked to insect dormancy. Thus, natural variations within *loci* encoding for circadian clock components could concur to shape complex developmental processes providing genetic basis for organismal adaptation to

temperate environments. Sandrelli *et al*. (2007) interpreted the weaker interaction between the L-TIM isoform and its photosensitive binding partner CRY compared to the S-TIM/CRY one, as a light-buffering system which dampens photoperiod potential effects on diapause onset or termination in regions at high latitudes. Indeed, in these regions, even in the presence of diapause-promoting or diapause-suppressing photoperiodic conditions, flies can often experience "hazardous" temperatures (Pittendrigh and Takamura, 1989; Pittendrigh *et al*.,

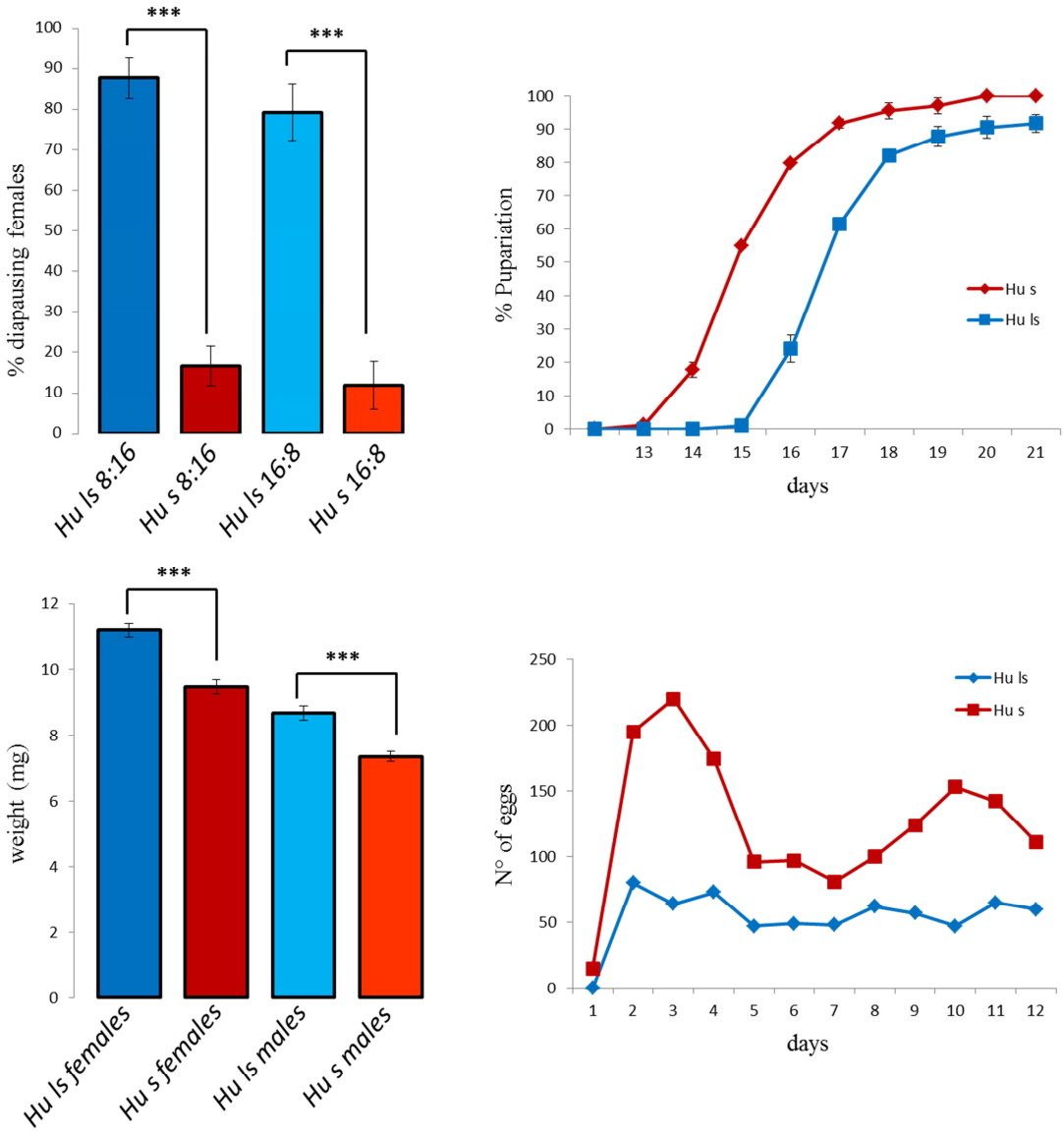

Figure 20. a) Diapause levels of *Hu s* and *Hu ls* flies under long and short photoperiods. Data are shown as Mean ± SD, 5 rep. n≥60. 2-Way-ANOVA-Interaction: *p<0.05; **p<0.01; ***p<0.001; b) developmental time of *Hu s* and *Hu ls* flies at 15°C, LD12:12. Data are shown as Mean±SE, 2 rep. n=200 c) body weight of Hu s and Hu ls females reared under 15°C, LD12:12. Data are shown as Mean ± SE, 10 rep. n≥10. Student t-test: *p<0.05; **p<0.01; ***p<0.001; d) estimated early-life fecundity of Hu s and Hu ls females along the first 12 days of adult life; n=10.

1991). The causal connection between the proposed mechanism and its effect on diapause remains still elusive and needs further studies to be identified. The results reported in Tauber

et al. (2007) coupled with our preliminary evidences, are also consistent with the possibility that the *ls-tim* allele could cause a slow-down of the ticking of those endogenous clocks potentially able to scan developmental and metabolic tempo, affecting in this way multiple life-history traits. However, further experiments are required to validate this hypothesis.

The impairment in thermosensation promotes diapause termination in *Drosophila melanogaster*

In all living beings, the ion channels Transient Receptor Potential (TRPs) provide the interface between the external and the internal environment. In Drosophila, for instance, they have been found to mediate responses to several stimuli, like temperature (Lee *et al*., 2005; Hamada *et al*., 2008; Rosenzweig *et al*., 2008; Kwon *et al*., 2010; Gallio *et al*., 2011), light (Wong *et al*., 1989; Niemeyer *et al*., 1996; Reuss *et al*., 1997), humidity (Liu *et al*., 2007), sounds (Kim *et al*., 2003; Gong *et al*., 2004; Zhang *et al*., 2013), gravity (Kamikouchi *et al*., 2009) and mechano-stimuli, like pain (Tracey, *et al*., 2003) (for review see Fowler and Montell, 2013). TRP channels can elicit changes in cellular physiology and membrane excitability perceiving directly the external stimulus, or indirectly, being activated by an upstream signaling (Christensen and Corey, 2007; Kwon *et al*., 2008; Shen *et al*., 2012; Delgado *et al*., 2014). Surprisingly, there is just one example in literature demonstrating whether an impairment in specific TRP signaling, and in turn in specific sensitive processes, could affect adaptive events relying on these stimuli like diapause. Recently, Sato *et al*. (2014) have shown that the down-regulation of *TRPA1* in *B. mori*, and the consequent impaired thermosensitivity, affects diapause hormone (DH) release and in turn diapause. In Drosophila, low temperatures seem to be the major cues in promoting the switch into diapause fate; for this reason we focused our attention on those TRP channels which are involved in low temperature perception. In 2011, Gallio *et al*. identified the first cold-responsive (19°C – 11°C) TRPs in adult fruit flies, called brivido 1 (brv1), brv2 and brv3, all of them located on Drosophila antennae. To determine whether an impairment in cold perception could rescue the shut-down in ovarian maturation which characterizes diapause response, we started testing in diapause-promoting conditions (12°C, LD 8:16) an hypomorphic mutant for *brv1* (*NP4486*, called *brv1 hyp*) and a null mutant for the same gene ($brv1^{-/-}$). As shown in Fig. 21a, *brv1 hyp* flies exhibit very low levels of dormant females (11,5% ± 3,1) with respect to control flies ($UAS\text{-}PI3K^{CA}$/+, 50,5% ± 3,9 and *DJ634*>+ 62,8% ± 5,6). Focusing on the $brv^{-/-}$ null mutant, we observed a consistent reduced proportion of diapausing females (26,4% ± 1,4) compared to the phenotypically similar $w^{1118}ls$ (57,6% ± 6,0), but not lower than that observed in flies with the same genetic background used as controls ($bw^{1};st^{1}$, 2,0% ± 0,7). It has to be considered that these control flies showed extremely low levels of diapause response if compared to all the controls that we have used in our experiments. It has been demonstrated that *brown*^{1} (bw^{1}) null mutant flies as well as the *scarlet*^{1} (st^{1}) ones are characterized by lower levels of serotonin and dopamine (as well as histamine) within the brain (Borycz *et al*., 2008), two biogenic amines which increase we

have shown to be sufficient to promote diapause induction/maintenance. Moreover, these two mutants showed a decrease in biogenic amine content comparable with that characterizing *white* null mutants ($w^{-/-}$), which mimic phenotypically $bw^{1};st^{1}$ individuals. The gene *w* encodes for a co-transporter that concurs in both drosopterins and ommochromes precursors trafficking, coupled with BROWN and SCARLET transporters, respectively (Reaume *et al*., 1991; Tearle *et al*., 1991). Drosopterins and ommochromes are both eye pigments, and the contemporary impairment of their trafficking within photoreceptor cells, due to the $w^{-/-}$ mutation or the $bw^{1};st^{1}$ combination, results in a similar absence of eye pigmentation (white phenotype). Thus, although $w^{-/-}$ and $bw^{1};st^{1}$ mutations cause similar phenotypic effects on eye pigmentation, they can lead to different effects on aminergic metabolism, and the comparison between diapause percentages of $w^{-/-}$ and $bw^{1};st^{1}$ flies supports this hypothesis Fig. 21a . Under this scenario it is plausible to envisage a potential synergistic effect between bw^{1} and st^{1} mutations in suppressing serotonin and dopamine synthesis, affecting strongly diapause response. What is anyway remarkable is the ovarian development of $brv1^{-/-}$ flies after being shifted (5 h after eclosion) for 11 days at 12°C. Although characterized by low fecundity (Gallio, personal communication), these females have well developed vitellogenic oocytes (also stages 14, the last and ready-to-be-laid stage) see Fig. 21c , with respect to the controls, even under very restrictive temperatures. In the future, it will be interesting to create a $brv1^{-/-}$ line with a wild-type background, in order to exclude potential effects due to other mutations, and test again the effects of the *brv1* null mutation on cold-induced insect diapause. However, taken together, our preliminary evidences suggest brv1 signaling as a candidate TRP signaling affecting complex developmental processes like insect dormancy.

In Drosophila larvae, mutants defective for another TRP channel, *Inactive* (*Iav*), located on chordotonal organs (thermosensory and mechanosensory structures) showed a compromised avoidance behaviour, with individuals unable to select the preferred temperature (17,5°C) over 14-16°C (Kwon *et al*., 2010). Furthermore, two TRP channels involved in phototransduction, TRP and TRPL, have been reported to mediate cool avoidance (cold temperatures ranged from 18°C to 10°C) in larvae, through a distinct downstream signaling (the phototransduction regulators NORPA and INAF were in fact dispensable) (Rosenzweig *et al*., 2008). Interestingly, these TRP channels are expressed in larval photoreceptors but not in the terminal organ (the larval thermosensitive organ). TRP and TRPL show the same photoreceptor-specific expression pattern in adult flies, where they retain their function in phototransduction (but also in hearing, see Senthilan *et al*., 2012). Although direct evidences are lacking in adult individuals, it is plausible to hypothesize an analogous involvement of TRP and TRPL in cold-temperature perception. Thus, we analyzed diapause response in *trp* loss of function mutants. As shown in Fig. 21b, fly lines carrying these mutations are characterized by a marked decrease in the proportion of dormant females (trp^{P343}, 7,8% ± 3,0; trp^{P365}, 14,1% ± 7,0) with respect to the controls ($w^{1118}s$, 39,2% ± 2,2; $UAS\text{-}PI3K^{CA}$/+ , 38,1% ± 3,4). Lower levels of diapause response were observed also in a null mutant for the other TRP channel, TRPL ($trpl^{302}$, 8,2% ± 4,6; controls were: $w^{1118}ls$, 57,6% ± 6,0 and *DJ634*>+ 62,8% ± 5,6). Surprisingly, *norpA* mutants phenocopied the drop in diapause incidence that characterizes *trp* and *trpl* mutants ($norpA^{P41}$, 10,3% ± 6,9), opposite to what Rosenzweig *et*

al. (2008) have found about cold avoidance. The latter result could reflect a potential difference between TRP and TRPL signaling between larval and adult stages, but we did not provide sufficient evidences to delineate the actual mechanism. Extremely low percentages of diapausing females were found also in trp^{1} flies (6,8% ± 4,9), characterized by a thermo-sensitive loss of function mutation. From electrophysiological analysis, TRP functionality of trp^{1} mutants results impaired under warm/high temperatures (25°C) (Minke, 1983), as well as *trp* expression in the eye (Pollock *et al*., 1995). The suppression of both functionality and expression are temperature-dependent, with robust effects at 25°C which decrease progressively with the lowering of temperature. Thus, our results concerning diapause response in trp^{1} mutants could suggest possible developmental effects of *trp* and *trpl* mutations which can cause an abnormal development of sensory structures (see Stortkuhl *et al*., 1999). Nevertheless, although wild-type cool avoidance has been observed in trp^{1} flies reared under permissive temperature (18°C) (Rosenzweig *et al*., 2008), the wave of receptor potential recorded in trp^{1} mutants grown at 19°C (permissive temperature) still shows a "non-wild-type" pattern, with a trend comparable to the one of mutants grown at 25°C (restrictive temperature which generates the loss of function). This raises the possibility that also at temperatures lower than the permissive ones, TRP functionality could be sufficiently compromised to impair its signaling. If true, our results should be considered evidences of TRP channels involvement in triggering diapause. It has to be mentioned that, unlike Rosenzweig *et al*. (2008), Kwon *et al*. (2010) did not find differences in trp^{P343} mutants for larval cold avoidance when individuals were exposed to 14°C (they preferred 17.5 °C against 14°C) from wild-type larvae. Furthermore, Kwon *et al*. (2008) demonstrated that trp^{1} larvae, as well as $norpA^{P41}$ ones, preferred the optimal larval temperature (18°C) against temperatures ≤ 16°C (again contrarily to what Rosenzweig *et al*. found regarding trp^{1}). Contradictions in literature underline the complexity of sensory systems; the difficult understanding of the molecular mechanisms underlying environment perception is emphasized by the promiscuity and pleiotropy of some TRP channels which can be activated by different external stimuli, likely thanks to different upstream or downstream signaling pathways. Problems in identifying whether the thermo-TRP signaling can affect complex developmental processes derive from the redundancy characterizing temperature sensing (as previously hinted), which can provide an effective compensatory mechanism if deficits in this sensory system occur. Taken together, our results provide a first preliminary evidence about a potential role of cold temperature-perceiving TRP channels in triggering the developmental switch into diapause fate. However, further experiments are necessary to go into depth about the molecular relationships between the environmental perception via TRP channels and adaptive physiological phenomena.

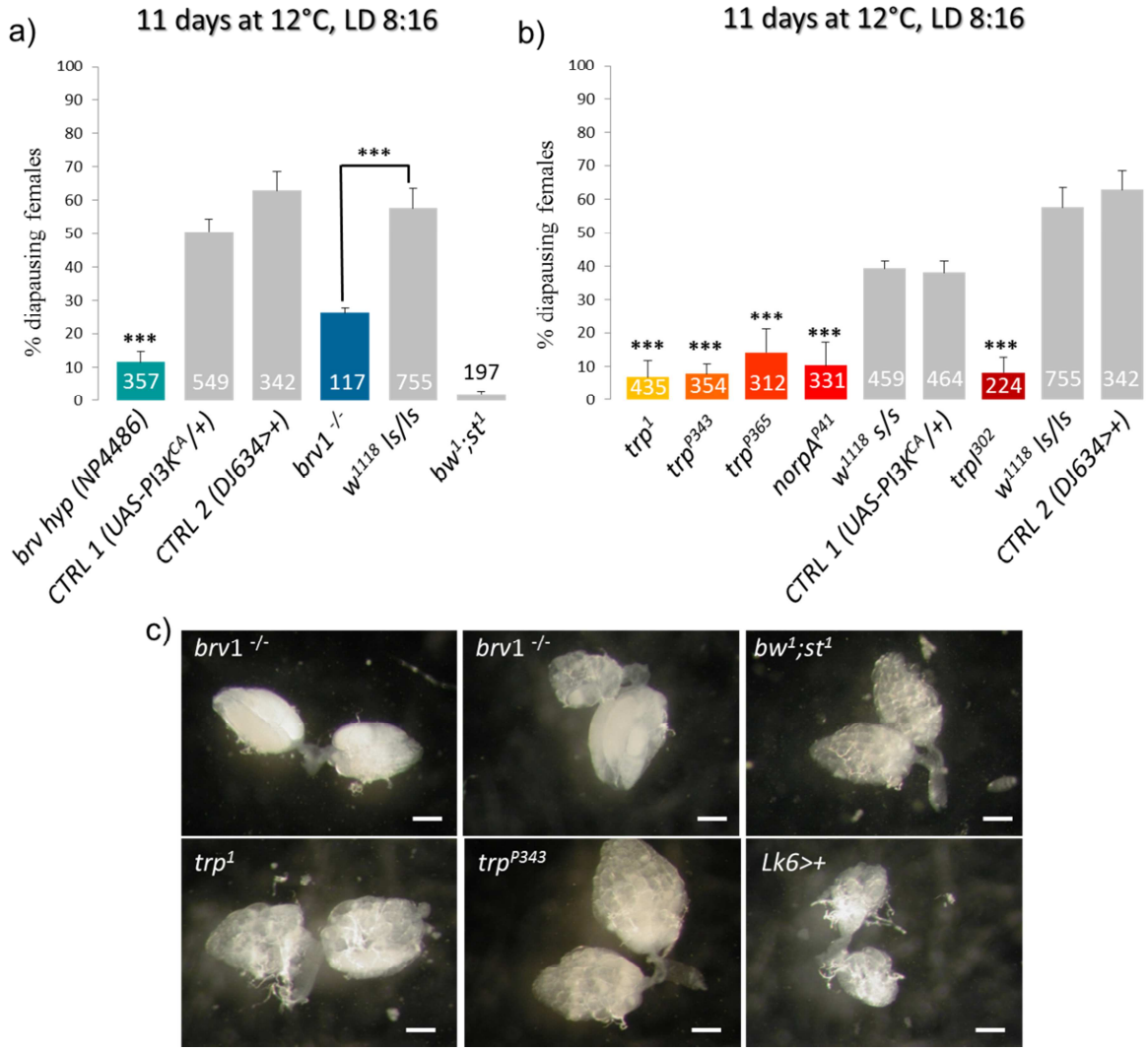

Figure 21. a and b) Diapause proportions of mutants for some TRP channels involved in cold temperatures perception with corrisponding controls. Numbers within bars refer to sample size. Data are shown as Mean ± SD, 2-7 rep. n≥60. 2-Way-ANOVA-Interaction: *p<0.05; ** *p*<0.01; ****p*<0.001; c) vitellogenic levels of brv1 and trp mutants. White bars represent 0.2 mm.

5. Materials and Methods

Fly stocks

The following fly strains were used: *dilp2(p)-Gal4* (gift from Erik Rulifson) and *dilp2-Gal4* (gift from Linda Partridge), *Gad1-Gal4* (gift from Pierre Leopold), *ppl-Gal4* (gift from Michael Pankratz), *UAS-Na^{+}ChBac* (gift from Michael O'Connor), *UAS-TSC1-2* (gift from Nicolas Tapon), *UAS-Upd2* (gift from Norbert Perrimon), *UAS-PKAR33* (gift from Françoís Rouyer), *w(tim-s)* (gift from Charlotte Helfrich-Foster), *NP4486* (called *brv1 hyp*) and *brv1$^{-/-}$* (gift from Charles S. Zucker), wild-type flies from Houten (called Hu-s and Hu-ls) (gift from Charalambos Kyriacou). *e^{1}*, *ple^{4}* (called *TH$^{-/-}$*), *Ddc^{DE1}* (called *Ddc hyp*), *dumb^{3}* (*DopR1* hypomorphic mutant, called *DopR1 hyp*), *Aug21-Gal4*, *Trh-Gal4*, *ple-Gal4* (called *TH-Gal4*), *Ddc-Gal4*, *cg-Gal4*, *Lk6^{DJ634}-Gal4* (called *DJ634-Gal4*), *Tdc-Gal4*, *UAS-Tor^{TED}* (called *UAS-Tor^{DN}*), *UAS-S6K^{STDETE}* (called *UAS-S6K^{CA}*), *bw^{1};st^{1}*, *trp^{1}*, *trp^{P343}*, *trp^{P365}*, *trpl^{302}*, *norpA^{P41}* were from Bloomington Drosophila Stock Center. *UAS-5HT-1A-RNAi*, *UAS-DopR1-RNAi*, *UAS-GBR-RNAi* (1); *UAS-GBR-RNAi* (2); *UAS-OAMB-RNAi* (1), were from VDRC and *w(tim-ls)*. Drosophila strains were maintained at 23°C under LD 12:12 on standard cornmeal food, (1 liter: 50 g inactivated yeast powder, 8,5 g agar, 72 g cornmeal, 79,3 g sucrose, 13,5 mL Nipagin in 75% EtOH).

Diapause assay

Larvae were maintained at 23°C under LD 12:12 and in the same density conditions until eclosion. Newly-eclosed adults (males and females) were collected in tubes (about 60 flies each) within 5 h since eclosion. Samples were quickly exposed to 12°C at two different photoperiods, LD 8:16 (short photoperiod) or LD 16:8 (long photoperiod). After 11 days, individuals were anesthetized and killed in EtOH 70%, females dissected in PBS and ovaries observed at 40X zoom with a LeicaMZ6 stereomicroscope. Diapause was scored according to Saunders *et al*. (1989) (females scored as diapausing if no vitellogenic stages were present, in other words before stage 8). Diapause levels are presented as the percentage of diapausing females ± Standard deviation (SD). At least 5 replicates of n≥60 flies were dissected for each genotype, unless stated. Ovary pictures show a representative situation of the ovarian growth for each genotype of interest. Bars represent 0.2 mm.

***ls-s timeless* genotyping**

10 females per strain were sampled, and individually stored in eppendorf vials at -20°C. For each specimen, 50 µL of Tris HCl pH 8.2 10 mM, EDTA 2 mM, NaCl 25 mM was added.

Afterwards flies were homogenized and 1 µL of proteinase K (10mg/mL) were added to each sample. Samples were incubated at 37°C for 45 min and subsequently at 100°C for 3 min. Samples were centrifuged 3 min at maximum speed, supernatant collected and stored at -20°C.

Fruit flies are characterized by a single nucleotide polymorphism at the *timeless* (*tim*) locus and this polymorphism affects diapause response. To determine the *tim* allelic variant characterizing each line/stock examined in the present study, an ARMS (Amplification Refractory Mutation System) PCR analysis was used according to Tauber *et al*. (2007). Two reactions were performed, that differed each other just for one forward primer, specific for the different polymorphic nucleotide. Another *tim* region was also amplified as an internal control of reaction efficiency.

The primers used were:

- GA: 5'-TGGAATAATCAGAACTTT**GA**-3' (forward primer specific for *ls-tim* allele)
- AT: 5'-TGGAATAATCAGAACTTT**AT**-3' (forward primer specific for *s-tim* allele)
- *tim3*: 5'-AGATTCCACAAGATCGTGTT-3' (reverse primer)
- C3: 5'-TATTCATGAACTTGTGAATC-3' (forward primer for internal control)
- C5: 5'-CATTCATTCCAAGCAGTATC-3' (reverse primer for internal control)

quantitative PCR

All the adult females analyzed for qPCR were reared at 25°C during larval-pupal life and shifted for 11 days at 12°C under LD 8:16 within 5 h post-eclosion. For each analyzed genotype, we sampled 3 biological replicates of 14 flies each and subsequently we divided heads and bodies, collecting only the latters. RNA was extracted using the Trizol (Invitrogen)-Chloroform extraction method and reverse-transcribed to cDNA using the SuperScript II Kit (Invitrogen). Transcript levels were assayed using GoTaq qPCR Master Mix (Promega) and normalized to *rp49* expression. Primers are listed in Table 4. Standard curves were generated using three serial dilutions of total RNA extracted from 10 adult females.

Table 4

Gene	forward primer	reverse primer
Obp99b	5'-CGAGCACGGATTCGATGT-3'	5'-CGATTCGTGCACCTCAACT-3'
Jon25Bii	5'-CAGGCTCAGTACACCCACAC-3'	5'-TGGTGTTGTAGTCCGAGTGC-3'
Kr-h1	5'-CAGAAAACATTCGCCGTACC-3'	5'-ATGGCCGTTCACCAGTGT-3'
InR	5'-GCAAACTCTGCCAGACGAA-3'	5'-CGCATCCACCCAAACAAT-3'
4E-BP	5'-CACCACTCCTGGAGGCACCAA-3'	5'-GAAGGGAGTACGCGGAGTTC-3'
yp1	5'-CATTGAGCGTCTGGAGAACA-3'	5'-GGATCTGCGACAGGTGGTA-3'
yp2	5'-ACGCTGTTGGACAAGCTCTAC-3'	5'-GGTGTAATCGGGCTTGAAGA-3'
yp3	5'-GACTGAAGCCGACCAAGTG-3'	5'-TGATTTGGCCAACGTGGTA-3'

Dopamine quantification

We reared larvae at 23°C (LD 12:12) until pupal hatching. After 5 h post-eclosion we collected adult flies and transferred them into three different conditions (12°C, LD 8:16; 12°C, LD 12:12; 23°C, LD 12:12) for 11 days in the same density conditions. Afterwards, we sampled 3 replicates of 500 females (1500 flies) for each condition and we froze them at -80°C. Subsequently, flies were homogenized in ice-cold $HClO_4$ 0.1M, samples centrifuged at 13.000 rcf (relative centrifugal force) for 10 min and supernatant filtered through Minisart® 0,45 µm filters. A commercial kit for catecholamines determination (Chromsystems) was used. This kit requires a preparation phase of samples before chromatographic analysis. 100 µL of Internal Standard and 6 mL of Neutralization Buffer were added to 3 mL of sample to reach an adequate pH. Samples have been purified through a Sample Clean Up Column (which retains catecholamines), subsequently washed first with water and subsequently with Elution Buffer (all these reagents were provided by Chromsystems). Dopamine levels were measured by HPLC-ECD. The HPLC system consisted of a CLC330 Solvent delivery system coupled with a programmable autosampler (CLC200), a column thermostat and an electrochemical detector (CLC100) (Chromsystems). 20 µL of eluted sample were injected in a C18 reverse-phase column (Chromsystems). Geminyx software was used for data acquisition (Chromsystems).

Statistical analysis

Data from diapause assay were converted in *arcsen* values prior to be statistically analyzed. One way ANOVA was performed using the R statistical software version 2.15.1. HPLC and expression data were analyzed by means of Student t-test (Excel).

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