Environmental Pollution 243 (2018) 1588–1601

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Endocrine disruption and chronic effects of plant protection products in bees: Can we better protect our pollinators?☆

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ARTICLE INFO

Article history:
Received 9 April 2018
Received in revised form
21 September 2018
Accepted 22 September 2018
Available online 25 September 2018

Keywords:
Bees
Plant protection products
Pesticides
Biocides
Chronic toxicity
Endocrine disruption

ABSTRACT

Exposure to plant protection products (PPPs) is one of the causes for the population decline of pollinators. In addition to direct exposure, pollinators are exposed to PPPs by pollen, nectar and honey that often contain residues of multiple PPPs. While in legislation PPPs are regarded mainly for their acute toxicity in bees, other effects such as neurotoxicity, immunotoxicity, behavioural changes, stress responses and chronic effects that may harm different physiologically and ecologically relevant traits are much less or not regarded. Despite the fact that endocrine disruption by PPPs is among key effects weakening survival and thriving of populations, pollinators have been poorly investigated in this regard. Here we summarize known endocrine disruptive effects of PPPs in bees and compare them to other chronic effects. Endocrine disruption in honey bees comprise negative effects on reproductive success of queens and drones and behavioural transition of nurse bees to foragers. Among identified PPPs are insecticides, including neonicotinoids, fipronil, chlorantraniliprole and azadirachtin.

So far, there exists no OECD guideline to investigate possible endocrine effects of PPPs. Admittedly, investigation of effects on reproduction success of queens and drones is rarely possible under laboratory conditions. But the behavioural transition of nurse bees to foragers could be a possible endpoint to analyse endocrine effects of PPPs under laboratory conditions. We identified some genes, including vitellogenin, which regulate this transition and which may be used as biomarkers for endocrine disruptive PPPs. We plea for a better implementation of the adverse outcome pathway concept into bee's research and propose a procedure for extending and complementing current assessments, including OECD guidelines, with additional physiological and molecular endpoints. Consequently, assessing potential endocrine disruption in pollinators should receive much more relevance.

1. Introduction

The recent decline of honey bees, wild bumble bees and solitary bees is of concern for beekeepers and pollination in agricultural and natural ecosystems (Cameron et al., 2011; Goulson et al., 2015). Although not completely understood, the decline has multiple causes (Goulson et al., 2015), including lack of wildflowers for pollinators, decline of plant biodiversity associated with intensified farming in agriculture (e.g. monocultures), impacts of pathogens and parasites such as *Varroa destructor* (Nazzi et al., 2012), and unwanted effects of plant protection products (PPPs) (Sanchez-Bayo and Goka, 2014). Shortage of wildflowers, habitat loss and deterioration of landscape are known causes (Carvell et al., 2017), while intensive use of PPPs came into focus rather recently. Often, causes are multifactorial and they act in combination. As bees are the main pollinators in most landscapes, the decline of bee populations has important negative consequences for plant pollination, and consequently, lower yields of crops and fruits. While many causes are not much debated, the influence of PPPs seems controversial, partly because of economic and agricultural interests. Contrary to many situations where PPPs are important, and the belief in their power, reduced use may create environmental

☆ This paper has been recommended for acceptance by David Carpenter.

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https://doi.org/10.1016/j.envpol.2018.09.117
0269-7491/

benefits and does not necessarily result in decreases in productivity and profitability in agriculture (Lechenet et al., 2017). Furthermore, in light of the striking decline of insect biomass in Germany over the last decades (Hallmann et al., 2017), and the widespread contamination of honey by neonicotinoids (Mitchell et al., 2017), the question of potential chronic impacts of PPPs on environmental and human health becomes important.

Bees are exposed to different classes of insecticides including neonicotinoids, pyrethroids and organophosphates (Mullin et al., 2010; Sanchez-Bayo and Goka, 2014) and other PPPs such as fungicides. The most frequent residues of agrochemicals found in honey bees are organophosphate and pyrethroid insecticides followed by fungicides (Johnson et al., 2010; Tosi et al., 2018). Nowadays pyrethroids and organophosphates are less used for the favour of neonicotinoids, which are the most widely used class of insecticides. Multiple pesticide residues in 24% of living bees and in 85% of dead bees were detected with chlorpyrifos, boscalid, thiacloprid, acetamiprid and azoxystrobin in the low ng/g range (Kiljanek et al., 2017). In Greece, for instance, up to 39.9 ng/bee clothianidin, 5.74 ng/bee imidacloprid and 49.6 ng/bee thiamethoxam were found (Kasiotis et al., 2014), and in Poland, up to 4.1 ng/g thiamethoxam and 15 ng/g fenoxycarb (Barganska et al., 2014). Similarly, in France up to 11.1 μg/kg imidacloprid was found in honey bees (Chauzat et al., 2011) to name a few reports.

Chemical residue data demonstrate that bees are exposed to a variety of different PPPs via direct exposure, nectar, pollen and honey. In 198 honey samples collected all over the world, at least one of the neonicotinoids acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam occurred in 75% of the samples at an average of 1.8 ng/g honey, in 45% samples two or more neonicotinoids and in 10% all five neonicotinoids occurred (Mitchell et al., 2017). Often, pollen contain mixtures of many PPPs. For instance, honey bee colonies near cornfields were chronically exposed to 26 different agrochemicals via wildflower pollen (Tsvetkov et al., 2017). In honey bee colonies located in agricultural landscape honey and wax were contaminated by many sorts of PPPs (Krupke et al., 2012), including neonicotinoids (Mitchell et al., 2017).

There is increasing evidence from laboratory and field studies that insecticides such as neonicotinoids can lead to decline of honey and wild bee populations, thus they are posing a risk to pollinators (Woodcock et al., 2016) by reducing survival and homing capacity (Gill et al., 2012; Goulson, 2013; Sandrock et al., 2014a; b; Godfray et al., 2014), decreasing foraging activity, as well as resulting in population declines (Rundlöf et al., 2015; Woodcock et al., 2017) and loss of queens (Tsvetkov et al., 2017). Moreover, adverse effects may increase in combination with fungicides. Consequently, the neonicotinoids thiamethoxam, imidacloprid and clothianidin became restricted in Europe and Canada in 2013 and banned 2018 for outdoor use in Europe and Switzerland. Besides neonicotinoids, the organophosphates phosmet and chlorpyrifos are thought to pose high risks to honey bees on a global scale (van der Sluijs et al., 2013).

In addition to PPPs, heavy metals may also display a risk for pollinators. Negative effects of manganese on foraging activity (Søvik et al., 2015) and adverse effects of selenium on behaviour and survival (Hladun et al., 2012) were observed. In addition, the exposure of honey bee colonies to environmental relevant concentrations of cadmium, copper, lead and selenium had negative effects on colony weight gain and brood survival (Hladun et al., 2016).

The adverse effects of PPPs on pollinators are assessed by determining mortality (OECD TG 213, 214, 237, 246 and 247), which often takes place within the regulatory context (risk assessment), but also by assessing different behavioural effects such as homing, orientation, foraging activity, learning and memory formation (Aliouane et al., 2009; Decourtye et al., 2003; Yang et al., 2008; OECD TG 245). On the other hand, additional physiological endpoints, and particularly molecular effects, are poorly studied. There exist only a few molecular studies focusing on the modes of action of PPPs (Christen et al., 2016, 2017; Christen and Fent, 2017; De Smet et al., 2017; Wu et al., 2017; Christen et al., 2018). Furthermore, there is a lack of studies linking physiological with molecular effects, or research on the molecular foundation of population relevant traits. In addition, there is a lack of laboratory and field studies on potential chronic effects of PPPs on bees. However, ecologically relevant effects induced by PPPs, including colony weight gain, loss of queens and population declines on bumble bee colonies and on wild bees have been investigated recently (Rundlöf et al., 2015; Whitehorn et al., 2012; Williams et al., 2015; Woodcock et al., 2017).

Among the different adverse effects of PPPs, neurotoxicity, immunotoxicity, behavioural alterations, general stress and mortality, as well as reproductive effects were reported, but the underlying modes of action were poorly investigated. Many PPPs, including older organochlorine pesticides, fungicides (Christen et al., 2014), herbicides (Abarikwu et al., 2010; Friedmann, 2002), and insecticides (Li et al., 2017; Westlund and Yargeau, 2017) were shown in vertebrates and *in vitro* systems to induce endocrine activities. However, despite reports on adverse effects of PPPs in bees on the reproduction capacity, particularly in bumble and solitary wild bees, the underlying endocrine mechanisms for the reduced number of gametes or reduced fecundity of the queenwere not analysed.

These endocrine disrupters (EDs) are compounds that interfere with the hormonal regulation, and as a consequence, compromise the reproductive outcome (gamete production, fertility, fecundity, behaviour etc.). Additional effects consist of hormonal imbalances affecting other physiological outcomes (e.g. mating or courtship behaviour). Among PPPs are compounds that have an intended mode of action that specifically interferes with the endocrine system of insects including insect growth regulators such as juvenile hormone analogues or ecdysone as moulting hormone. However, generally little knowledge exists in pollinators on endocrine effects of PPPs. Furthermore, chronic toxicity studies including reproductive effect studies in queens and drones are not or only rarely performed. Nonetheless, reduced reproductive anatomy and physiology of honey bee queens experimentally exposed to neonicotinoids (Williams et al., 2015), or fertility impairment of drones by the insecticide fipronil (Kairo et al., 2016) have been reported.

The question whether or not endocrine related adverse effects by PPPs are of concern in bees and play a role in the population decline of pollinators has rarely and not yet sufficiently been addressed. In light of the increasing loss of insect species in European countries (Vogel, 2017; Hallmann et al., 2017) understanding of such effects has a high relevance. There is a need for analysing endocrine disruptive effects to pollinators to better understand adverse outcome pathways (AOP), a concept, which is currently little regarded in the research and effects assessment of PPPs in bees.

The scope and aim of our review is to tackle the question of endocrine disruption by PPPs in bees in more depth. While other adverse effects, particularly neurotoxicity (Belzunces et al., 2012) or effects of specific PPPs, such as neonicotinoids (Blacquiere et al., 2012; Fairbrother et al., 2014; Sanchez-Bayo & Goka, 2014; Goulson, 2013) have been reviewed, possible endocrine disruptive effects of PPPs towards pollinators are not well understood, nor reviewed, despite their ecological importance. Here we plea for an implementation of endocrine disruptive endpoints in the effects assessment of PPPs in future research and regulation for a better

understanding of their potential adverse effects to pollinator populations.

2. Endocrine system of bees

The endocrine system of bees consists of different hormones and glands. An anatomical overview with tissues and organs is depicted in http://carrsconsulting.com/honeybee/normal/anatomybee.htm. Important endocrine hormones in social hymenoptera are the juvenile hormone (JH), mainly synthesised in the *Corpora allata*, and ecdysteroids, which are synthesised in the ovary among others. Both hormones are involved in ovary activation, vitellogenin synthesis and/or oogenesis in adult females (Hartfelder and Emlen, 2012). Hormone synthesis is partly regulated by food and pheromones (Rachinsky et al., 1990). In addition, in the honey bee, JH, ecdysteroids and vitellogenin are important elements for behavioural regulation in the worker caste (Amdam et al., 2003, 2005). JH and ecdysteroids are the main inducers of vitellogenin synthesis and uptake in social hymenoptera (Raikhel et al., 2005). While in the late pupal stage JH acts as inducer of vitellogenin (Barchuk et al., 2002), vitellogenin synthesis is not up-regulated by these hormones in adult honey bees, however (Hartfelder and Engels, 1998).

In addition to its function in reproduction and behavioural transition from nurse bee to forager, vitellogenin plays a role in the synthesis of proteins that nurse bees feed to the larvae (Amdam et al., 2003). This function is based on the expression of vitellogenin receptors at the hypopharyngal glands (HPGs) which is the main royal jelly producer of worker bees (Amdam et al., 2003). In adult worker bees, elevated JH titers negatively affect the hemolymph vitellogenin level (Hartfelder and Engels, 1998; Rutz et al., 1976). In the hemolymph of workers younger than 3 days no vitellogenin is detectable, vitellogenin levels increase up to day 7–9, and during the transition from nurse bee to forager, vitellogenin level decrease rapidly (Pinto et al., 2000). Similarly, down-regulation of vitellogenin by RNAi leads to an increase in JH titers in worker bees (Guidugli et al., 2005). This specific interaction is called the "Double Repressor" hypothesis (Amdam and Omholt, 2003). Vitellogenin is the major zinc carrier in honey bee hemolymphe and plasma zinc concentration of forager is lower than of nurse bees (Amdam et al., 2004). As the endocrine system of animals show a highly sensitive response to changes in extracellular zinc levels (Baraldi et al., 1986), regulatory action of vitellogenin on JH might work through decreased zinc levels (Guidugli et al., 2005). Fig. 1 depicts a simplified summary of the endocrine regulation in bees.

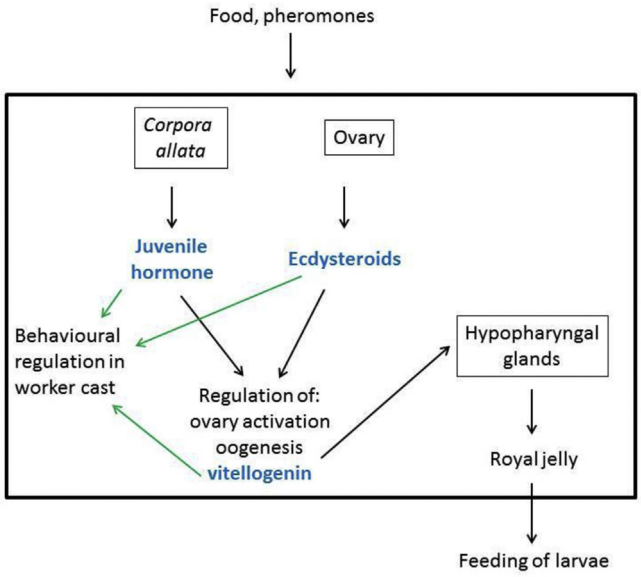

Fig. 1. Simplified overview of the endocrine regulation in bees. Glands are shown in squares, hormones and vitellogenin are shown in blue, general regulatory functions are shown as black arrows, regulatory functions concerning behavioural transition of nurse bees to foragers are shown as green arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.1. Queens of honey bees

Although queen and workers have the same genome, their reproductive functions exhibit significant differences. Queens have fully developed active ovaries, while they remain inactive in workers. The onset of ovary activation is tightly regulated in queens. Five to 10 days after emergence, queens reach sexual maturity and initiate mating flights, during which the queen will mate with an average of a dozen of drones (Tarpy et al., 2004). Once the queen completes the mating process, ovaries become fully activated and egg laying is initiated (Tanaka and Hartfelder, 2004). Virgin queens show high titres of JH and ecdysteroids, whereas both titres drop in egg-laying queens. This suggests that both hormones are involved in regulation of the reproductive process in honey bee queens (Wegener et al., 2013).

Insect ovaries are composed of functional units, called ovarioles, which contain sequentially developing egg chambers (Büning, 1994). Honey bee workers have 2–26 ovarioles, while queens contain 100- 180 ovarioles per ovary (Sakagami and Akahira, 1958; Jackson et al., 2011). The adult ovary phenotype difference becomes established during the final larval instar, when massive programmed cell death leads to the degeneration of 95–99% of the ovarioles in workers. The higher JH levels in queen larvae protect the ovaries against such degradation (Capella and Hartfelder, 1998). Activated ovarioles contain eggs in successive stages of development (upper part: multinucleate protoplasmic mass; lower part: defined oocytes (eggs), trophocytes (nurse cells) and follicle cells) (Snodgrass, 1956; King and Büning, 1985). In contrast, inactivated ovaries are composed of thin ovarioles that lack mature eggs (Snodgrass, 1956).

During the transition from virgin queens to fully mated queens, behavioural and physiological changes as well as large scale transcriptional changes occur in the brain and ovaries. In comparison to virgin queens, egg-laying queens do not show any flight attempts anymore, have fully developed ovaries, show higher levels of vitellogenin (mRNA and protein) and show a different pheromone profile (Kocher et al., 2008; Nino et al., 2011). Transcriptional changes include, among others changes, profiles of genes linked to protein folding, protein catabolism, cellular metabolism, oxidative phosphorylation, ATP metabolic processes and response to stress (Kocher et al., 2008; Nino et al., 2011).

By RNA sequencing a total of 1615 differently expressed genes (DEGs) between ovaries of virgin and mated queens were detected, and more than 5300 DEGs between inactivated and activated worker ovaries (Niu et al., 2014). All major royal jelly protein encoding genes (MRJPs) were down-regulated in activated worker ovaries and most of them were not detectable in ovaries of egg-laying queens (Niu et al., 2014). Genes encoding for JH biosynthesis were down-regulated in activated ovaries, whereas genes encoding for JH degrading enzymes were down-regulated. Low levels of JH are associated with ovary development and egg laying behaviour in honey bee queens and queenless workers (Robinson et al., 1991, 1992). Cytochrome P450 enzymes responsible for the synthesis of 20-hydroxyecdysone (20E), the active form of ecdysteroids, are up-regulated in activated ovaries (Niu et al., 2014).

Ecdysteroid titres are higher in egg-laying queens and workers than in non-reproductive honey bees (Robinson et al., 1991).

Besides the capability to reproduce, honey bee queens are long-living. It is suggested that JH, vitellogenin (VTG) and insulin-IGF1 signalling (IIS) play important roles in regulating queen longevity (Corona et al., 2007). In emerging virgin queens, JH and VTG titres are high, whereas in older, reproductive queens, JH titres drop and VTG titres stay high (Corona et al., 2007).

The development of the morphological and life history differences between queens and worker bees can also be linked to differences in mitochondrial structure and dynamics. In the queen larval fat body, higher densities of mitochondria were found and queen larvae have higher maximum capacities of ATP production at lower physiological demand. And the expression of mitogenesis-related factors such as TFB1 and TFB2 homologs is higher in queen larvae than in worker larvae (Santos et al., 2016). Thus, differential nutrition of queen and worker larvae affects mitochondrial dynamics and functionality (Santos et al., 2016).

2.2. The transition from nurse to forager bees

Honey bees, like many species of social insects, display a division in labor among colony members that is based on behavioural specialization associated with age. Adult worker bees perform a series of in-hive tasks and shift in the age of 2–3 weeks to foraging for nectar and pollen outside the hive. The transition to foraging involves changes in the endocrine activity, metabolism, circadian clock activity, brain chemistry, brain structure and brain gene expression (Robinson, 2002). Important regulators during this process are JH, ecdysteroids such as 20-hydroxyecdysone and vitellogenin (Amdam et al., 2003, 2005). The transition to foraging is also dependent on the environment and can be accelerated, delayed or even reversed depending on the needs of the colony (Robinson, 2002).

Young bees, not yet competent to forage, show extensive changes in gene expression and brain structures completed by day 8 of age. Subsequent differences in onset age of foraging were correlated with differences in juvenile hormone (JH) titres, JH-target gene expression, ecdysteroids titres and ecdysteroids target gene expression suggesting that these endocrine systems mediate the genetic differences (Whitfield et al., 2006; Amdam and Page, 2010; Paul et al., 2005). Ecdysteroid receptor genes and some ecdysteroid-regulated genes, *Mblk-1*/*AmE93*, *AmBroad*-Complex and *AmE75* are selectively expressed in the honey bee mushroom bodies (Paul et al., 2005, 2006) and it is suggested that these ecdysteroid-signalling in the mushroom bodies is involved in regulating honey bee social behaviour, including worker-age polyethism (Yamazaki et al., 2006).

In addition to JH and ecdysteroids target genes, molecular pathways associated with *foraging* and *malvolio* genes are involved in honey bee behavioural maturation. The expression of *foraging* is increased in foragers (Ben-Shahar et al., 2002). *Malvolio* encodes for manganese transporters in brain cells. Manganese influences the responsiveness to sucrose, one component of honey bee division in labor. Sucrose responsiveness increases with age and is highest in foragers (Ben-Shahar et al., 2004).

The importance of alterations in gene expression during nurse-forager transition is demonstrated by the fact that 2663 genes are differently expressed in the *Pars intercerebralis* (PI) between nurse and forager honey bees (Wheeler et al., 2015). Among these differently expressed genes are 10 genes encoding for neuropeptides and 82 genes encoding for transcription factors. JH treatment revealed different expression of 365 genes in the PI, indicating that JH is not the only regulator of behavioural maturation.

The hypopharyngeal glands (HPGs), paired exocrine glands in the head of honey bees, are also involved in the behavioural transition. Hypopharyngeal glands consist of a pair of long glands coiled in the sides of the head. Their activity depends on the age of workers, their food and presence of larvae and is most active in young bees. In nurse bees, these glands are well developed and the main function is the synthesis of royal jelly proteins. In foragers, the HPGs shrink and the expression of carbohydrate-metabolizing enzymes such as α-amylase, α-glucosidase 3 and glucose oxidase, for the processing of nectar to honey increases (Wheeler et al., 2015). The shrinking of the HPGs and the increased expression of α-glucosidase are induced by JH (Sasgawa et al., 1989; Rutz et al., 1976). Besides JH, ecdysone signalling regulates the physiological state of the HPGs in association with behavioural maturation of honey bee workers. Related to gene expression, indicators of the behavioural state of the workers are increased expression of *buffy* and *mrjp2* (encodes major royal jelly proteins) in nurse HPGs and the increased expression of *hbg3* (encodes α-glucosidase 3) and *matrix metalloproteinase 1* in forager HPGs (Ueno et al., 2015). A simplified overview about the behavioural transition in adult workers is shown in Fig. 2.

The transition from nurse bee to forager is in addition regulated by changes in mitochondrial capacity. The differences between the different castes include alterations in the cellular redox state and in the differential expression of genes likely to be regulated by reactive oxygen species including the core genes of the hypoxia response (Cervoni et al., 2017).

2.3. Endocrine system in drones

Drones, male honey bees produced from unfertilised eggs, display only limited functions in a honey bee colony. Their tasks are producing sperms and mating with a queen. Thus, drones lack hypopharyngeal glands, wax glands and most of the structures to collect food (Hrassnigg and Crailsheim, 2005). In addition, the *Corpora allata,* the main producer of the juvenile hormone (JH), is smaller in adult drones than in adult workers (van Laere, 1971). No JH is synthesised in drone pupae, but after exclusion, JH synthesis increases to a maximum in 9 days old drones and decreases thereafter (Tozetto et al., 1995). Experimental application of JH promoted the flight activity of drones as it does in workers (Tozetto et al., 1997). It is suggested that JH exerts functions in the spermatogenesis and mucus production by the accessory glands (Tozetto et al., 1997). In contrast, ecdysteroids act as negative regulators in the maturation process of drones accessory glands (Colonello and Hartfelder, 2003). Injection of 20-hydroxyecdysone into newly emerged drones abolished the normal increase in protein content of accessory glands and prolonged the persistence of the protein pattern typical for immature glands.

Ecdysteroids hemolymph titer decline rapidly soon after emergence (Colonello and Hartfelder, 2003). At emergence, ecdysteroid hemolymph titers are elevated, followed by s steep decline during the first 24–32 h after emergence from brood cell. At day 8 of adult drones, there is a minor peak of ecdysteroids in the hemolymph. Thereafter, first orientation flights of drones start, suggesting that ecdysteroids play a role in this behavioural change of drones (Colonello and Hartfelder, 2003). Vitellogenin levels increase in the first 3 days after emergence, peak at day 3 and decrease afterwards (Trenczek et al., 1989). The absolute amount of vitellogenin in drones is much lower than in workers. Vitellogenin is present in the reproductive tract of adult drones, including the accessory glands, but not secreted (Colonello-Frattini et al., 2010). With age of the drones, expression of vitellogenin transcripts decrease, but the expression of vitellogenin receptor transcripts increases in fat body. This enhanced receptor expression may boost the transport of vitellogenin protein back into the fat body, thus gradually depleting

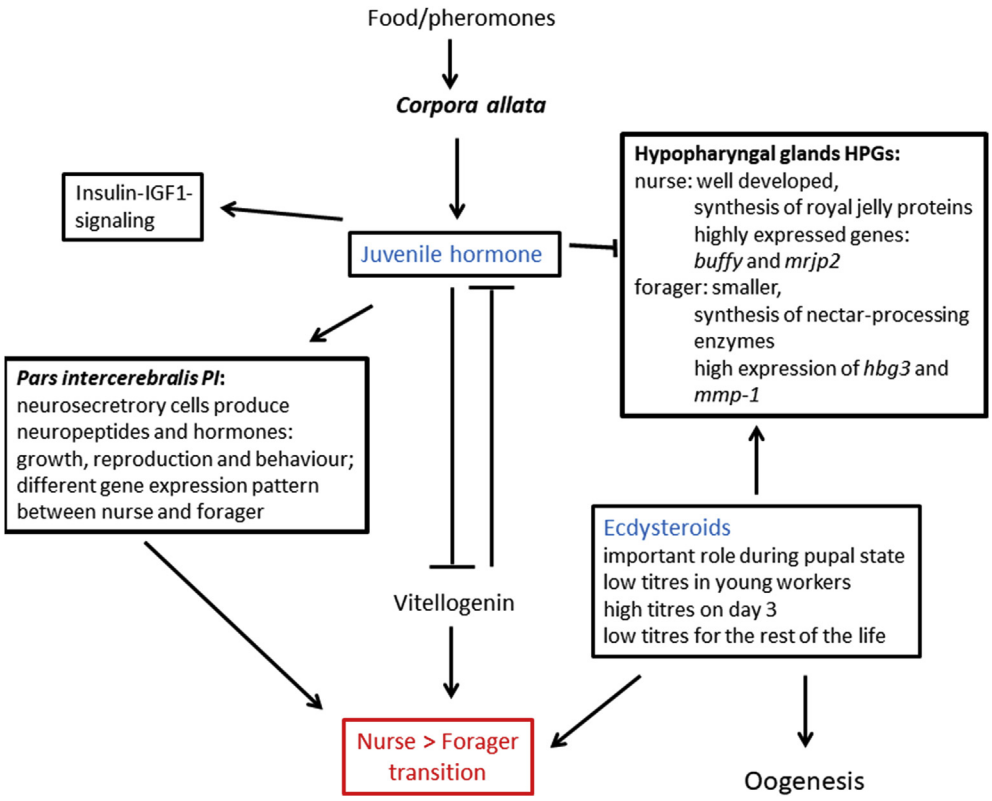

Fig. 2. A simplified overview about the behavioural transition in adult workers. ┤: inhibitory effects, arrows: activating effects.

the vitellogenin level circulation in the hemolymph (Colonello-Frattini et al., 2010). The function of vitellogenin in drones, as a precursor yolk protein in females, is discussed in the context of the transport of sugars, lipids, phosphates, vitamins and hormones (Piulachs et al., 2003), but its exact role remains unresolved.

3. Indications for endocrine effects in bees

Endocrine disruptive effects on honey bees can occur at different organisation levels of the bee colony. They may affect the queen, drones or worker bees. The following endocrine effects on queens may occur: negative effects on the development of the ovaries leading to low egg quality; bad or low mating rates leading to low rates of fertilised eggs; low return rates form mating flight and low egg laying rates. In drones, endocrine disruptive compounds may induce adverse effects on the development and number of sperms thus leading to lower sperm quality, and on lowering mating rates of drones with queens. Endocrine disruptive effects in honey bee workers could be the activation of the normally inactive ovaries and a disturbed behavioural transition of nurse bees to foragers.

3.1. Adverse effects on reproduction

Endocrine effects that negatively affect reproduction of honey bees are associated with effects in queens and drones, and may affect bee development. In queens, they may include mating success, reduced number of produced eggs, and reduced amount and viability of sperms stored in the spermathecal. In drones, reduced number and viability of sperms can be considered as endocrine effects. In addition, delay in larval development and effects on exclusion rates may be further endocrine effects.

Table 1 summarizes endocrine effects of PPPs on bees. Reduced production of eggs, decreased ovary size, decreased number of stored sperms and decreased viability of stored sperms was shown after exposure of honey bee queens to 1 ppb clothianidin and 4 ppb thiamethoxam (Williams et al., 2015). Adverse effects on egg laying and locomotor activity of queens were detected after exposure of bee colonies to 10 ppb imidacloprid (Wu-Smart and Spivak, 2016). Exposure of honey bee drones to a mixture of 4.5 ppb thiamethoxam and 1.5 ppb clothianidin reduced the life span of the drones and decreased the sperm viability and sperm quality (Straub et al. 2016). A reduced number of brood cells was demonstrated after 40 days exposure of honey bee colonies to 5 ppb imidacloprid (De Smet et al., 2017). 7 days exposure of honey bee queens to 0.02 ppm imidacloprid reduced the viability of stored sperms (Chaimanee et al., 2016). Exposure of honey bee drones under laboratory and semi-field conditions to 0.1 μg/L fipronil decreased spermatozoa quantity and increased spermatozoa mortality (Kairo et al., 2017). Decreased spermatozoa concentration and sperm viability were detected after exposure of drones to 0.1 μg/L fipronil (Kairo et al., 2016). Similar negative effects on reproduction were found after exposure of bumble bees and solitary bees to plant protection products. Negative effects on bumble bee reproduction were detected in bumble bee colonies placed adjacent to oilseed rape fields treated with clothianidin and imidacloprid. Measured concentrations in collected pollen and nectar were up to 4 ppb thiamethoxam (even not applied to the rape field) and up to 0.3 ppb clothianidin. Imidacloprid was neither detected in pollen nor in nectar (Goulson, 2015). A decrease in fecundity was observed in queen-less bumble bee micro-colonies after exposure to 1 μg/L imidacloprid (Laycock et al., 2012). Exposure of solitary bees to a mixture of 2.87 μg/kg thiamethoxam and 0.45 μg/kg clothianidin led to reduced number of produced off-spring and a male-biased off-spring rate (Sandrock et al., 2014a). Bumble bee colonies placed for two weeks close to raspberry crops treated with

Table 1
Summary of known endocrine effects of pesticides to bees.

Compound	Effects	Effect concentrations	References
Imidacloprid	- Weight gain of bumble bee colony over 8 weeks: reduced growth - Number of produced queens: reduced number of new queens	Pollen: 0.7 μg/kg Sugar water: 6 μg/kg	Whitehorn et al., 2012
Mixture Clothianidin with β-Cyfluthrin	- Honey bee colony growth and reproduction rate: both reduced - nesting of solitary bees: reduced		Rundlöf et al., 2015
Clothianidin (CLO), Imidacloprid (IMD)	- Negative effects on bumble bee colony weight gain and queen production - detection of thiamethoxam in nectar and pollen even not applied	- colonies placed adjacent to oilseed rape field treated with clothianidin or imidacloprid - analysis of neonicotinoids in pollen and nectar: up to 1.5 ppb TMX in pollen and up to 4 ppb in nectar, CLO: up to 0.3 ppb in nectar, IMD: not detected	Goulson 2015
Clothianidin, Thiamethoxam (TMX)	- Honey bee colony growth in spring after exposure the year before: smaller bee colonies - negative effects on reproduction of wild bees	Field study: application of up to 18.05 g/ha clothianidin and up to 11.14 g/ha thiamethoxam	Woodcock et al., 2017
Fipronil	- 20 days exposure of drones in tunnel: decreased number of sperms, decreased viability of sperms - unexposed queens inseminated with such sperms: reduced reproduction potential	0.1 μg/L fipronil	Kairo et al., 2016
Imidacloprid	- exposure of queen less bumble bee micro-colonies: decreased fecundity but no effects on ovary development - Imidacloprid reduced feeding activity - hypothesis: imidacloprid effects due to nutrient limitation	1 μg/L imidacloprid	Laycock et al., 2012
Mixture Thiamethoxam and Clothianidin	- Exposure of solitary bees - 50% reduced off-spring - male-biased off-spring rate	2.87 μg/kg thiamethoxam and 0.45 μg/kg clothianidin	Sandrock et al., 2014a
Mixture Thiamethoxam and Clothianidin	- Exposure of honey bee hive to binary mixture of clothianidin and thiamethoxam - more queen supersedure and less swarming	2.05 ppb clothianidin and 5.31 ppb thiamethoxam	Sandrock et al., 2014b
Mixture Thiamethoxam and Clothianidin	- Exposure of honey bee drones - drone lifespan: decreased - sperm viability: decreased - living sperm quantity: decreased	4.5 ppb thiamethoxam and 1.5 ppb clothianidin	Straub et al., 2016
Mixture Thiamethoxam and Clothianidin	- Exposure of developing honey bee queens - % of queens producing eggs: decreased - % of surviving queens producing diploid off-spring: decreased - ovary size: increased - number of stored sperms: decreased - number of stored, viable sperms: decreased	4 ppb thiamethoxam and 1 ppb clothianidin	Williams et al., 2015
Imidacloprid	- adverse effects on egg-laying and locomotor activity of honey bee queens - adverse effects on foraging and hygienic activity of honey bee workers	10 ppb imidacloprid	Wu-Smart and Spivak, 2016
Fipronil	- exposure of drones from emergence until sexual maturation under laboratory and under semi-field conditions - survival and maturation better under laboratory conditions - for both approaches: decreased spermatozoa quantity and increased spermatozoa mortality	0.1 μg/L fipronil	Kairo et al., 2017
Imidacloprid	- Chronic exposure (up to 40 days) in laboratory and under field conditions - exposed colonies: brood and population decline after first brood cycle - different data for gene expression between laboratory and field approach	5 ppb imidacloprid	De Smet et al., 2017
Mixture of 17 different pesticides	- Brood combs with contaminated wax and without - delayed larval development between day 4 and day 8		Wu et al., 2011
Imidacloprid	- Contact toxicity - Exposure of mated honey bee queens - after 7 days: sperm viability decreased	0.02 ppm imidacloprid	Chaimanee et al., 2016
Thiacloprid	- exposure of bumble bee colony's for 2 weeks close to thiacloprid treated raspberry field - after two weeks: placed back at unexposed site - reduced final weight - reduced production of reproductives	Up to 771 ppb thiacloprid in pollen and up to 561 ppb in nectar	Ellis et al., 2017
Chlorantraniliprole	- dietary exposure of bumble bees: suppression of reproduction in worker bumble bees	0.4 mg/L chlorantraniliprole	Smagghe et al., 2013
Methoprene	- Topical exposure of workers: earlier foraging	250 μg/bee	Robinson, 1985

(*continued on next page*)

Table 1 (*continued*)

Compound	Effects	Effect concentrations	References
Law Williams mixture (JH analogue)	- Exposure of workers: negative effects on hypopharyngeal glands and earlier foraging	10–200 μg/bee	Jaycox et al., 1974
Juvenile hormone III	- Exposure of workers: negative effects on hypopharyngeal glands	1 μg/bee	Rutz et al., 1974
Kinoprene	- Feeding of workers with contaminated pollen: negative effects on hypopharyngeal gland and reduced longevity		Gerig 1975
Diflubenzuron	- Topical exposure of larvae: negative effects on larval development	30 ng/larvae	Czoppelt and Rembold, 1981
Diflubenzuron	- Topical exposure of workers: negative effects on hypopharyngeal glands	50 μg/bee	Gupta and Chandel, 1995
Pyriproxyfen	- Exposure of young workers: impaired vitellogenin synthesis in hemolymph	1.25 μg/bee	Pinto et al., 2000
Diflubenzuron and Fenoxycarb	- Exposure of honey bee colonies to maximum application rates: negative effects on egg production	0.3 L/500 L diflubenzuron and 0.6 kg/200 L fenoxycarb	Thompson et al., 2005
Pyriproxyfen	- Oral exposure of larvae: negative effects on larval development	10 ppm	Chen et al., 2016
Fenoxycarb	- Exposure of honey bee colonies: higher brood mortality and higher variability in exclusion rates	20 mg/L	Milchreit et al., 2016
Azadirachtin	- 11 weeks exposure of bumble bees: less male off-spring and reduced egg production	0.64 and 3.2 mg/L	Barbosa et al., 2015

thiacloprid were less reproductive and had a lower final weight. Analysis of collected pollen and nectar revealed thiacloprid concentrations in pollen up to 771 ppb and in nectar up to 561 ppb (Ellis et al., 2017). The dietary exposure of bumble bees to 0.4 mg/L chlorantraniliprole reduced the number or produced drones (Smagghe et al., 2013).

3.2. Endocrine effects of insect growth regulators, a special class of plant protection products

Insect growth regulating insecticides (IGRs) have been developed due to their high activity and selectivity against insects with inherently low toxicity to non-target organisms. IGRs can act as juvenile hormones analogue such as fenoxycarb, methoprene, hydroprene and pyriproxyfen, as ecdysteroid synthesis inhibitor including azadirachtin or as ecdysteroid analogue such as tebufenozide. Additionally, chitin synthesis inhibitors such as diflubenzuron and triflumuron are used to prevent the synthesis of the exoskeleton. Due to their effects on molting these types of plant protection products are likely to pose a greater hazard to larval stages than adult insects.

Possible negative effects of insect growth regulators on the development and hormone system of honey bees were studied long ago (Table 1). Workers topically exposed to 250 μg of JH analogue methoprene started foraging earlier than controls (Robinson, 1985). Exposure of honey bees to 10–200 μg/bee to the Law-Williams mixture (a JH analogue) negatively affected development of hypopharyngal glands and induced earlier foraging (Jaycox et al., 1974). The development of the hypopharyngal glands was also disturbed after exposure of workers to 1 μg/bee of JH III (Rutz et al., 1974). Workers fed with kinoprene (JH analogue) contaminated pollen showed negative effects on the hypopharyngal glands and reduced longevity (Gerig, 1975). Topical application of 30 ng of the chitin synthesis inhibitor diflubenzuron of larvae induced negative effects on larval development and resulted in dead prepupae (Czoppelt and Rembold, 1981). Negative effects on the development of the hypopharyngal glands were observed after topical exposure of workers to 50 μg diflubenzuron (Gupta and Chandel, 1995). Exposure of young workers to concentrations higher than 1.25 mg of the JH analogue pyriproxifen impaired vitellogenin synthesis in the hemolymph (Pinto et al., 2000).

Negative effects on honey bee queen mating and egg production were seen after exposure of honey bee colonies to maximal application rates of diflubenzuron (0.3 L/500 L) and fenoxycarb (0.6 kg/200 L) (Thompson et al., 2005). Adverse effects on larval development and on exclusion rates were detected after exposure to pyriproxifen and fenoxycarb (Chen et al., 2016; Milchreit et al., 2016). Pyriproxifen displayed negative effects on larval development and on the production of royal jelly at 10 ppm (Chen et al., 2016). Exposure of honey bee colonies to 20 mg/L fenoxycarb induced a higher mortality of brood and a high variability of exclusion rates (Milchreit et al., 2016). Chronic exposure (11 weeks) of bumble bees to the ecdysone synthesis inhibitor azadirachtin decreased the egg production, decreased the production of male off-spring and decreased the weight of male off-spring. The effects on male off-spring occurred at 0.64 mg/L and reproductive effects at 3.2 mg/L (Barbosa et al., 2015).

3.3. Current pollinator testing: benefits and limitations of OECD guidelines

The current OECD guidelines (Table 2) focus on the acute toxicity of PPPs for honey bees and bumble bees. Besides acute oral and contact toxicity tests in adult honey bees (OECD guidelines TG 213, 214, 246 and 247), an acute larval toxicity test is also implemented (OECD guideline 237). These guidelines are useful for determining the acute toxicity of chemicals, an information needed for regulatory purposes. However, they do not provide information about chronic effects or the whole pattern of adverse effects associated with the compound. Information about long-term effects or chronic toxicity of PPPs is often lacking, as well as data on potential modes of action. This also holds true for adverse effects on reproduction, development, immune system activity and behaviour despite the fact that such endpoints are negatively affected by PPPs.

However, a novel chronic oral toxicity test (TG 245, 10 days feeding test in young honey bees in the laboratory) is proposed by the OECD and now in use. Abnormal behaviour and mortality is recorded each day and endpoints after 10 days are LC_{50} (median lethal concentration) and LDD_{50} (median lethal dietary dose). No observed effect concentration (NOEC) and no observed effect dietary dose (NOEDD) are derived based on these endpoints, but physiological, biochemical or molecular endpoints are not assessed.

Table 2
Current OECD guidelines to analyse the acute toxicity of PPPs in bees.

Guideline	Endpoints
OECD GD 75	**Guidance document on honey bee (*Apis mellifera*) brood test under semi-field conditions**
OECD TG 213	**Acute oral toxicity test with adult worker honey bees or bumblebees**
OECD TG 247	- Feeding diet with test substance and recording mortality after 24, 48 h and in some cases after 72 and 96 h
OECD TG 214	**Acute contact toxicity with adult worker honey bees or bumblebees**
OECD TG 246	- Direct application of test substance on thorax - Recording mortality after 24, 48 h and in some cases after 72 and 96 h
OECD TG 237	**Acute larval toxicity test** - Single exposure on larval day 4 to test substance and recording mortality at day 5, 6 and 7
OECD TG 245	**Honey bee (*Apis mellifera* L.), chronic oral toxicity test (10 day feeding)** - Exposure of young adult bees to treated food (sucrose solution) over a period of 10 days
OECD Draft GD	**Draft guidance document on honeybee (*Apis mellifera*) larval toxicity test, repeated exposure**
OECD Draft TG	**Honey Bee Larval Toxicity Test, Repeated Exposure**

This novel guideline will provide refined LC_{50} values but adverse effects on reproduction, development, immune system activity and behaviour will not be detected. In addition, a novel chronic larval test guideline is under investigation (https://www.ibacon.com/your-study-type/terrestrial-ecotoxicology/bees/oecd-draft-tg-honey-bee-larval-toxicity-test-repeated). Larvae are feed with a diet containing the test substance at day 3, 4, 5 and 6. Mortality is recorded at day 4, 5, 6, 7, 8, 15 and 22. At day 22, the number of emerged honey bees is determined. For obtaining better information on the toxicity potential of a PPP, investigations of the previous mentioned endpoints are recommended, particularly on reproduction. A novel OECD guideline on homing flight activity of honey bees after single exposure to sublethal doses to PPPs is planned. In the following, we outline potential endocrine endpoints that can be assessed in bees for such purposes.

3.4. Potential endpoints for assessing endocrine disrupting activities in honey bees

Endocrine disruption in honey bees can be defined as negative effects on reproductive success of queens and drones and behavioural transition of nurse bees to foragers, but no OECD guideline exists to analyse queens or drones for reproductive success and its underlying endocrine modes of action. Admittedly, this would not be possible under laboratory conditions and choice of best exposure time (adult stage or developing stages) would also be a challenge.

Thus, the most feasible approach would be to use adult workers of different age (nurse bees and foragers) to analyse the gene expression of selected target genes, their gene products (for instance vitellogenin) and hemolymph titers of selected hormones involved in behavioural transition of nurse bees to foragers after exposure to a test substance. Based on the present literature, expressional analysis of the following target genes in the brain of worker bees, such as *buffy*, *mrjp2*, *cyp302a1* and *vitellogenin*, which show all higher expression in nurse than forager bees (Ueno et al., 2015; Yamazaki et al., 2011, Christen et al., 2016, 2017), and *hbg3*, metalloproteinase 1, *foraging* and *ilp-1* (Ueno et al., 2015; Ben-Shahar et al., 2002; Corona et al., 2007), which all show higher expression in foragers, may be indicative to determine possible endocrine effects.

Another feasible approach would be to analyse the expression of certain genes in the ovaries of workers. Here candidate genes would be *nm-sro*, which is higher expressed in the ovaries of nurse bees, and *cyp314a1*, which is higher expressed in foragers ovaries (Yamazaki et al., 2011). Changes in the expression pattern of these genes may be indicative of an endocrine mode of action. A summary of all possible identified target genes is shown in Table 3. In addition, we hypothesize that hemolymph titers of juvenile hormone and vitellogenin may be good biomarkers for endocrine effects, as nurse bees show low juvenile hormone titers and high vitellogenin titers and foragers the opposite (Fig. 3) (Guidugli et al., 2005).

Based on identified target genes regulating the behavioural transition of nurse bees to foragers and on the different hemolymph hormone titers, we propose the following test protocol. Workers younger than 35 days (nurse bees) and older than 42 days (foragers) are exposed for 48 h to test substances followed by gene expression analysis in the brain and ovaries. In parallel, hormone titers of juvenile hormone and vitellogenin in the hemolymph are analysed. Changes in some of these end points may be indicative for endocrine effects and further testing would be needed. An overview of the proposed test scheme is shown in Fig. 4.

We propose further test strategies that may be included in a larval toxicity test, based on OECD guideline 237, with gene expression analysis as end point in addition to mortality only. Additionally, chronic tests with nurse bees would be desirable. First instar synchronized larvae (day 1) are transferred to 48 well plates and fed with artificial diet. At day 4, larvae are fed with artificial diet spiked with different concentrations of the test substance and after 5, 6 and 7 days, the ovaries of the larvae are dissected and the expression of *hsp60*, *hsp90* and *hex-70b*, regulated by juvenile hormone- and therefore good candidates to analyse endocrine effects (Table 3)- is analysed. Changes in gene expression indicate endocrine effects of the test chemical. In addition, chronic tests with nurse bees could be performed. Nurse bees (age 35 days) are exposed to the test substance for 7 days (age bees at the end of the test 42 days: young foragers). With this approach, bees are exposed during the transition time of nurses to foragers. After the 7 days exposure, juvenile hormone and vitellogenin titers in the hemolymph are analysed and the expression of genes mainly expressed in the brain of foragers (*foraging*, *malvolio*, *hbg-3* and *metalloproteinase 1*, Table 3) is analysed. In addition, the expression of *cyp314a1* and *nm-sro* in the ovaries is analysed. These two genes are differently expressed between nurse bees and foragers (Table 2). Changes in hormone titers or gene expression indicate endocrine effects of the test substance.

4. A short overview of other chronic effects of PPPs to bees

Most insecticides are neurotoxicants. Pyrethroids exert their insecticidal effect by prolonging the open phase of the sodium channel (Naumann, 1990), organophosphates inhibit the enzyme acetylcholinesterase, which mediates the transmission of nerve signals (Fukuto, 1990) and neonicotinoids act as specific agonists on insect acetylcholine receptors. Due to the mode of action, such insecticides primarily display neurotoxic effects on bees, including induction of acetylcholine esterase activity in honey bee larvae

Table 3
Potential candidate genes to investigate endocrine effects in honey bees.

Gene	Function/Effect	Reference
CYP314A1	- codes for enzymes that catalyse the conversion of ecdysone to 20E - up-regulated in activated ovaries of queens and workers	Niu et al., 2014
CYP9Q1 and *CYP9Q2*	- detoxification enzymes - down-regulated in activated ovaries	
Yellow-g and *yellow-g2*	- expressed in ovaries - induced in activated ovaries	
JH epoxidase hydrolase (*JHEH*)	- degradation of JH - down-regulated in worker ovaries	
Juvenile hormone esterase (*JHE*)	- degradation of JH - down-regulated in activated ovaries of queens and workers	
HSP90	- important for cross-talk between JH and ecdysteroid signalling pathway - induced by JH in larvae ovary	Lago et al., 2016
HSP60	- inhibited by JH in larvae ovary	
Hex70b	- storage protein and function as JH binding protein - inhibited by JH in larvae ovary	
Short chain dehydrogenase reductase (*sdr*)	- in different honey bee castes differently expressed - down-regulated by ecdysteroids and up-regulated by JH in worker larvae ovaries	
Foraging and *malvolio*	- involved in behavioural maturation - increased in foragers - encodes a cGMP-dependent protein kinase (PKG) - higher expressed in the brain of foragers than of nurse bees - treatment with cGMP induced precocious foraging	Ben-Shahar et al., 2002, 2004
Buffy and *mrjp2*	- up-regulated in HPGs of nurse bees	Ueno et al., 2015
Hbg3 and *metalloproteinase 1*	- up-regulated in HPGs of forager bees	
Insulin like peptide 1 (*ILP-1*) and *insulin receptor 1* (*InR-1*)	- higher expressed in foragers than in nurse bees - exposure to JH-analogue methoprene: induction of *ilp-1* in workers	Corona et al., 2007
ultraspiracle	- transcription factor (insect homolog to retinoid X receptor) - RNAi knock down: delayed transition from nurse to forager - binds to cis motive: cis motive found in JH responsive genes - up-regulated in response to JH in fat body	Ament et al., 2012
Non-molting glossy/Shroud (*nm-sro*)	- involved in the biosynthesis of 20E - higher expressed in the ovaries of nurse bees than foragers	Yamazaki et al., 2011
CYP302A1	- involved in the biosynthesis of 20E - higher expressed in the brain of nurse bees than in foragers	Yamazaki et al., 2011
CYP314A1	- involved in biosynthesis of E20 - higher expressed in ovaries of foragers than in nurse bees	Yamazaki et al., 2011
vitellogenin	- higher expressed in nurse bees - plant protection products such as neonicotinoids up-regulate vitellogenin in the brain	Christen et al., 2016, 2017
Amsima (HIF-1a) *Amfatiga (PHD)*	- higher expressed in the brain and thorax of nurse bees compared to foragers - higher expressed in the abdomen in foragers compared to nurse bees	Cervoni et al., 2017

Fig. 3. Hormone titers found in the hemolymph of queens, nurse bees and foragers.

after exposure to thiamethoxam (Tavares et al., 2015), induction of acetylcholine esterase mRNA in bumble bee foragers of colonies placed in areas with corn plating operations by clothianidin and thiamethoxam (Samson-Robert et al., 2015), negative effects on learning performance after imidacloprid exposure (Decourtye et al., 2003), negative effects on olfactory learning, motor sensory and cognitive functions after exposure to fipronil or thiamethoxam (Aliouane et al., 2009) and the failure in homing of foragers exposed to thiamethoxam (Henry et al., 2012).

Table S1 summarizes observed effects and effects concentrations. In addition, these PPPs also negatively affect the immune system of bees. Feeding of bees with a diet containing imidacloprid for 5 days had negative effects on the activity of the phenoloxidase, one important regulator of bee immunity in foragers (Wegener et al., 2016). 24 h exposure of bees to clothianidin, imidacloprid and thiacloprid caused a reduction in haemocyte density, in encapsulation response and in antimicrobial activity of the hemolymph (Brandt et al., 2016). Bees exposed to clothianidin and imidacloprid showed a reduced activity of the immune response causing an increased replication of deforming wing virus (Di Prisco et al., 2013). Interestingly, clothianidin also negatively effected immune signalling in human immune cell suggesting a potential risk of immunotoxicity that neonicotinoids may have on vertebrates (Di Prisco et al., 2017). A decrease in glucose oxidase activity, important to sterilize the colony and brood, was detected in *Nosema* infected bees exposed to imidacloprid (Alaux et al., 2010). Co-exposure of bees to boscalid and pyraclostrobin in cage experiments and in a field study led to higher virus titers such as black queen cell virus and deforming wing virus (Degrandi-Hoffman et al., 2015). Detailed information about the above mentioned effects and exposure concentrations are summarized in Table S2.

Additional chronic effects found with clothianidin spiked pollen were increased worker mortality, decreased social immunity and increased queenlessness (Tsvetkov et al., 2017). Negative effects on foraging activity, homing success, navigation and social communication were observed in bee colonies exposed for several weeks to thiacloprid (Tison et al., 2016). 12 days exposure of winter bees to clothianidin had negative effects on long-term memory (Alkassab and Kirchner, 2016) and 24 days exposure of bumble bees to thiamethoxam impaired learning and memory formation (Stanley et al., 2015). 22 days exposure of *Nosema* infected bees to fipronil increased protein oxidation and oxidative damage (Paris et al., 2017). A

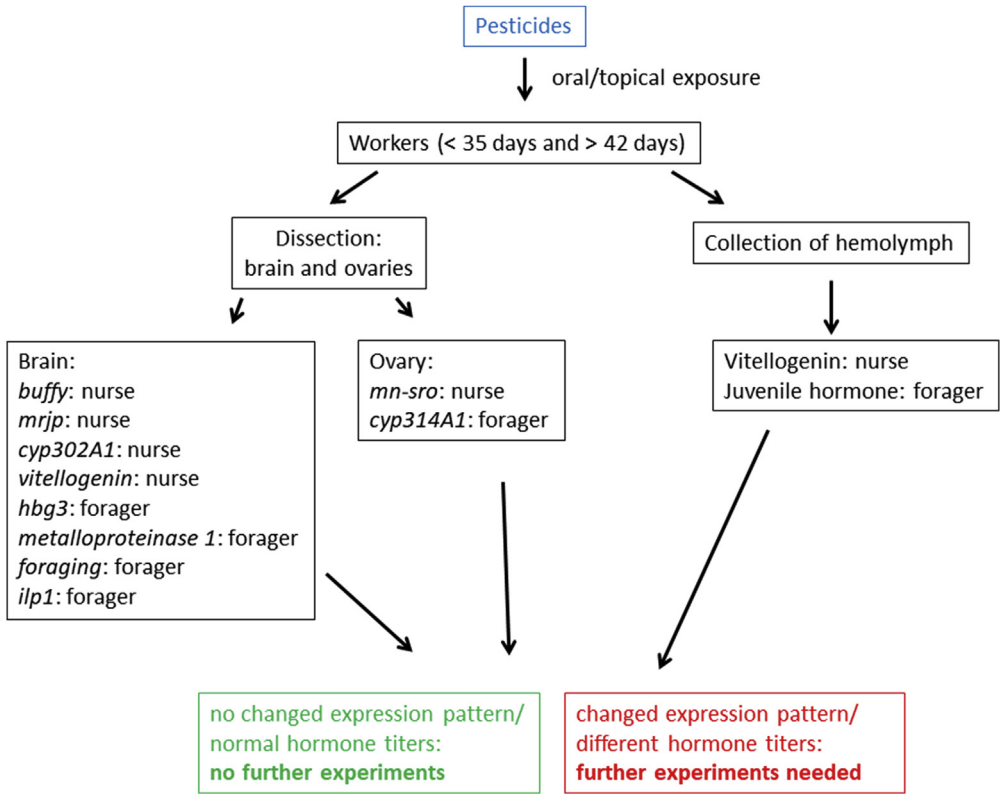

Fig. 4. Proposed procedure to analyse potential endocrine effects of substances in honey bees.

summary with exposure concentrations is given in Table S3.

Alterations in gene expression was also found upon exposure of honey bees to neonicotinoids such as acetamiprid, clothianidin, imidacloprid and thiamethoxam. Differentially expressed genes in the brain included immune system genes, stress genes, genes linked to memory formation, *vitellogenin* and acetylcholine receptors (Christen et al., 2016). Exposure of honey bee larvae to imidacloprid altered the expression of 578 different genes in the head of newly emerged adults. Among them were repression of genes encoding major royal jelly proteins, which play an important role in the development of the bee colony (Wu et al., 2017). In addition to major royal jelly proteins, genes of various biological processes including carbohydrate and lipid metabolism were differentially expressed in adult honey bees exposed to clothianidin, imidacloprid and thiamethoxam (Christen et al., 2018). The expression of cytochrome P450 genes was altered in honey bee queens after exposure to imidacloprid and coumaphos (Chaimanee et al., 2016). Similar effects on the expression of cytochrome P450 genes were found in honey bee workers after imidacloprid exposure (De Smet et al., 2017; Christen et al., 2017). Oral exposure of caged worker bees to chlorpyrifos, malathion, cypermethrin and chlorantraniliprole changed the expression of immune system genes, cytochrome P450 genes, *vitellogenin* and ER stress related genes (Christen and Fent, 2017). The exposure of bee larvae to chlorothalonil, coumaphos, fluvalinate, imidacloprid and myclobutanil altered the expression of immune system genes and stress genes (Gregorc et al., 2012). Detailed information including exposure time and concentrations is given in Table S4. A generalized overview of all discussed adverse effects of PPPs on bees is given in Table 4.

5. Conclusions

Endocrine disruption as adverse effect of PPPs in bees has largely been neglected, although its consequences are ecologically relevant. Due to the population decline of honey bees and wild bees, compromised fertility and reproduction becomes one of the key parameters in the PPP's adverse effects in addition to neurotoxic and immune system compromising effects or known effects including *Varroa* parasites and shortage of wildflowers. Our review sheds new lights into the potential endocrine activity of PPPs. The following endocrine effects were identified:

- reduced reproduction activity and success of queens
- reduced sperm quality and quantity in drones
- adverse interaction with behavioural transition of nurse bees to foragers

Current OECD guidelines do not consider endocrine disruption in bees. However, our review shows that endocrine disrupting effects occur in bees. Consequently, we plea for complementing the assessment of PPPs in research and in the regulatory context by considering potential endocrine endpoints in pollinator testing. Assessment of an endocrine mode of action and consequently adverse endocrine effects on reproduction of queens and drones is very challenging as is the effects assessment in whole bee populations. To better cover endocrine effects, we propose to include suitable endpoint for the analysis of adverse effects of PPPs, such as the behavioural transition of nurse bees to foragers. An indicative parameter is vitellogenin, which is easy to determine and has promises (Christen et al., 2016; Christen and Fent, 2017; Christen et al., 2017), which, after validation, may be considered in existing or new OECD test guidelines. Assessment of vitellogenin induction on the mRNA or protein level may potentially become a representative biomarker for adverse endocrine activity (Fig. 4). Further research should assess the validity of this proposed biomarker.

Table 4
General overview of all discussed adverse effects of PPPs on bees.

Compound	Neurotoxicity/ Behavioural effects	Immunotoxic effects	Chronic effects	Molecular effects	Effects on reproduction	Reference
Neonicotinoids						
Acetamiprid	–	–	–	d		d: Christen et al., 2016
Clothianidin	a	b	c	d	e	a: Samson-Robert et al., 2015 b: Brandt et al., 2016; Di Prisco et al., 2013 c: Alkassab and Kirchner 2016; Tsvetkov et al., 2017 d: Christen et al., 2016 e: Goulson 2015; Rundlöf et al., 2015; Sandrock et al., 2014a; Sandrock et al., 2014b; Straub et al. 2016; Williams et al., 2015; Woodcock et al., 2017
Imidacloprid	a	b	–	d	e	a: Decourtye et al., 2003 b: Alaux et al. 2010; Brandt et al., 2016; Di Prisco et al., 2013; Wegener et al., 2016 d: Chaimanee et al., 2016; Christen et al., 2016; De Smet et al., 2017; Gregorc et al., 2012; Wu et al., 2017 e: Chaimanee et al., 2016; De Smet et al., 2017; Goulson 2015; Laycock et al., 2012; Whitehorn et al., 2012; Wu-Smart and Spivak, 2016
Thiacloprid	–	b	c	–	e	b: Brandt et al., 2016 c: Tison et al., 2016 e: Ellis et al., 2017
Thiamethoxam	a	–	c	d	e	a: Aliouane et al., 2009; Henry et al., 2012; Samson-Robert et al., 2015, Tavares et al., 2015 c: Stanley et al., 2015 d: Christen et al., 2016 e: Sandrock et al., 2014a; Sandrock et al., 2014b; Straub et al. 2016; Williams et al., 2015; Woodcock et al., 2017
Pyrethroids						
β-Cyfluthrin	–	–	–	–	e	e: Rundlöf et al., 2015
Cypermethrin	–	–	–	d	–	d: Christen and Fent, 2017
Fluvalinate	–	–	–	d	–	d: Gregorc et al., 2012
Organophosphates						
Chlorpyriphos	–	–	–	d	–	d: Christen and Fent, 2017
Coumaphos	–	–	–	d	–	d: Chaimanee et al., 2016; Gregorc et al., 2012;
Malathion				d		d: Christen and Fent, 2017
Phenylpyrazole						
Fipronil	a	–	c	–	e	a: Aliouane et al., 2009 c: Paris et al., 2017; Renzi et al., 2016 e: Kairo et al., 2016; Kairo et al., 2017
Diamide						
Chloranthraniliprole	–	–	–	d	e	d: Christen and Fent, 2017 e: Smagghe et al., 2013
Fungicides						
Boscalid	–	b	–	–	–	b: Degrandi-Hoffman et al., 2015
Chlorthalonil	–	–	–	d	–	d: Gregorc et al., 2012
Myclobutanil	–	–	–	d	–	d: Gregorc et al., 2012
Pyraclostrobin	–	b	–	–	–	b: Degrandi-Hoffman et al., 2015
Ecdysone Blocker						
Azadirachtin	–	–	–	–	e	e: Barbosa et al., 2015
Chitin Synthesis Inhibitor						
Diflubenzuron	–	–	–	–	e	e: Czoppelt and Rembold, 1981; Gupta and Chandel, 1995; Thompson et al., 2005
Juvenile Hormone/JH-analogue						
Fenoxycarb	–	–	–	–	e	e: Chen et al., 2016; Milchreit et al., 2016; Thompson et al., 2005
Juvenile hormone III	–	–	–	–	e	e: Rutz et al., 1974
Kinoprene	–	–	–	–	e	e: Gerig, 1975
Law-Williams Mixture	–	–	–	–	e	e: Jayox et al., 1974
Methoprene	–	–	–	–	e	e: Robinson, 1985
Pyriproxifen	–	–	–	–	e	e: Chen et al., 2016; Milchreit et al., 2016; Pinto et al., 2000

Furthermore, there is a lack of studies on the mechanisms of action of PPPs. Molecular effect studies are important for understanding the molecular and physiological basis of observed AOPs. For the endocrine mode of action, biomarkers should be defined. By focusing on toxicologically relevant pathways considering AOPs, future research should yield a better understanding of adverse effects of PPPs in bees and may help to complement regulatory guidelines.

Acknowledgement

This work was supported by a grant (contract no. 16.0091.PJ/Q272-0747) from the Swiss Federal Office for the Environment, Bern, Switzerland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.09.117.

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