Definition of bee health and set of key health indicators for each of the three model bee species

Deliverable D8.1

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PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Summary

In this deliverable we present a practical, working definition of 'health' from the perspective of bees and the parameters important for their well-being and survival, and identify a set of key indicators for measuring and evaluating this health status in bees, for practical use in designing, implementing and evaluating management practices to optimize the health, well-being and survival of bees in different environments. In this, we have taken advantage of the much more advanced developments in human health systems, definitions and indicators and adapted the concepts, criteria and methodology to the particularities of bees, their lives, needs and environments. In doing so, we simultaneously synchronize the conceptualization of bee health, its dimensions and its indicators to that of other systems, environments and organisms within the current One-Health concept, where the health of any one organism is contingent on the health of the environment it depends on for survival and well-being. By adapting the current WHO definition of 'health' in humans:

"A state of complete physical, mental and social well-being,
and not merely the absence of disease or infirmity"

to bees, we have conceptualized bee health as a hierarchical set of interdependent homeostatic layers, or systems (molecular, cellular, organismal, individual, social, ecological, evolutionary), that individually and together protect the bee (i.e., provide resilience) against short-, medium- and long-term fluctuations in its environment. These homeostatic systems are dynamic, flexible resources, to be used and replenished in the service of the life of the bee. We subsequently identified several major dimensions (molecular, microbial, parasitic, nutritional, social-behavioural, environmental and population-genetic) that are key to managing bee life and health at different scales. For each of these dimensions we then identified a number of key indicators for measuring the status of the health dimension at any one time, using the following definition of what constitutes a 'health indicator':

"An estimate of a given health dimension in a target population"

Health indicators are quantitative estimates of a particular trait in the bee or its environment that can be predictably linked to a certain aspect of health. Although indicators can be estimated on individual bees or their individual environments, they are primarily population-level statistics used to define and manage the health status of a group of organisms (bees) in a particular environment or context. The global properties, uses and limitations of these indicators are described and explained. These include context-dependence of the value, plasticity with respect to biological improvement, and duality, in the sense that an indicator value represents both the environment that the organism lives in (challenge) and the response of the organism to this environment (response). The indicators for the various bee health dimensions were chosen with respect to twelve desirable attributes that together describe the usefulness of the attribute for practical bee health management. Most particularly, the indicators and/or estimation methodology were chosen to be applicable to as many bee species as possible, for cross-species comparison and validation. Where possible, alternative methods for use in different time-frames or conditions were given. For each health dimension, the most relevant, accurate and robust indicators were chosen, rather than an exhaustive list of all possible potential indicators.

1. Definition of Bee Health

1.1. A basic definition of health

'Health' is a simple, intuitive term to describe the well-being or optimal biological status of an organism in its environment. The term has progressively broadened from its initial narrow, disease-based medical context ("…freedom from the risk of disease and untimely death"; Stokes et al., 1982), through the incorporation of ecosystem hierarchies, concepts and models (Morgenstern, 1995; VanLeeuwen et al., 1999; Barton and Grant 2006; Figure 1), to the current One-Health approach, where the health of the individual is contextual to, and dependent on, the health of its (environmental) support systems (Gibbs, 2014; Figure 2).

The current WHO definition of 'health' is:

> *"A state of complete physical, mental and social well-being, and not merely the absence of disease or infirmity"*

Although the definition developed in relation to the human experience, the underlying concepts are universal and thus suitable for all organisms and biological systems. A basic definition of "Bee Health" therefore simply transposes these same concepts to bees, incorporating their life cycles, needs, living environments and behaviour.

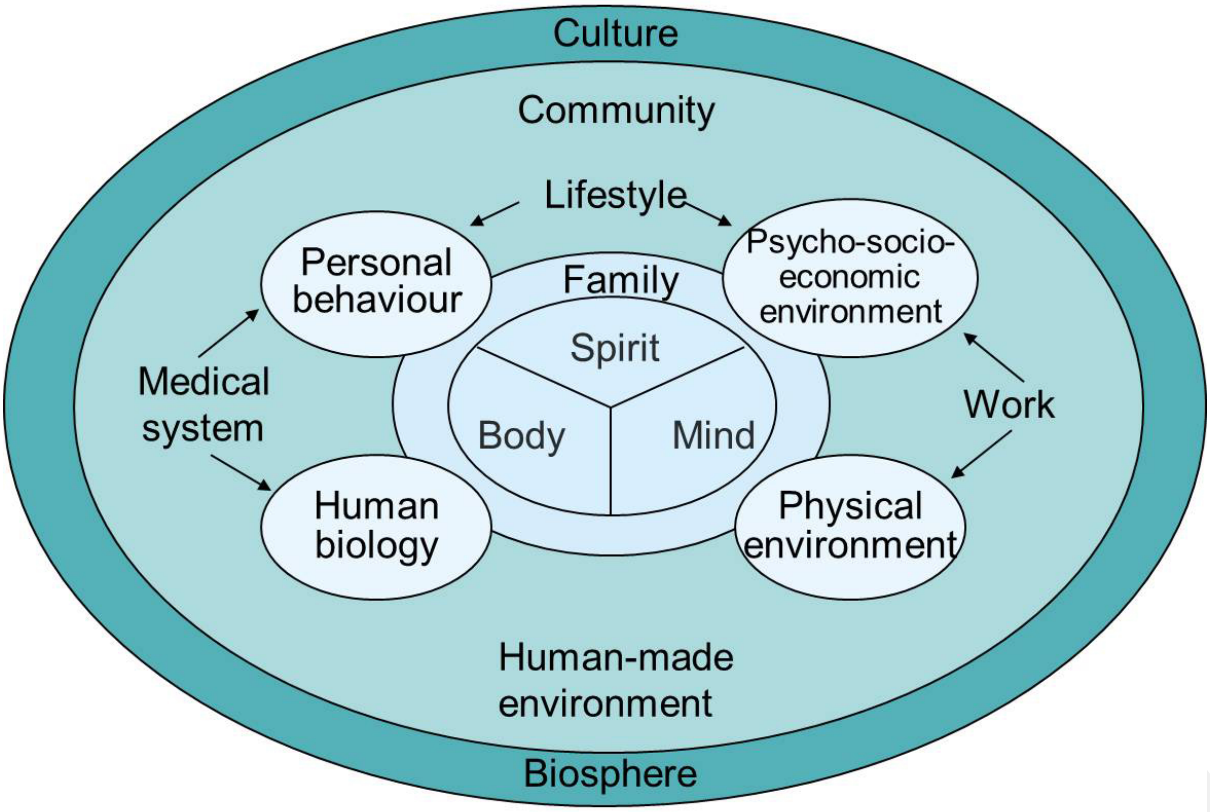

Figure 1 Example of a hierarchical conceptual model of human health. After VanLeeuwen et al. 1999.

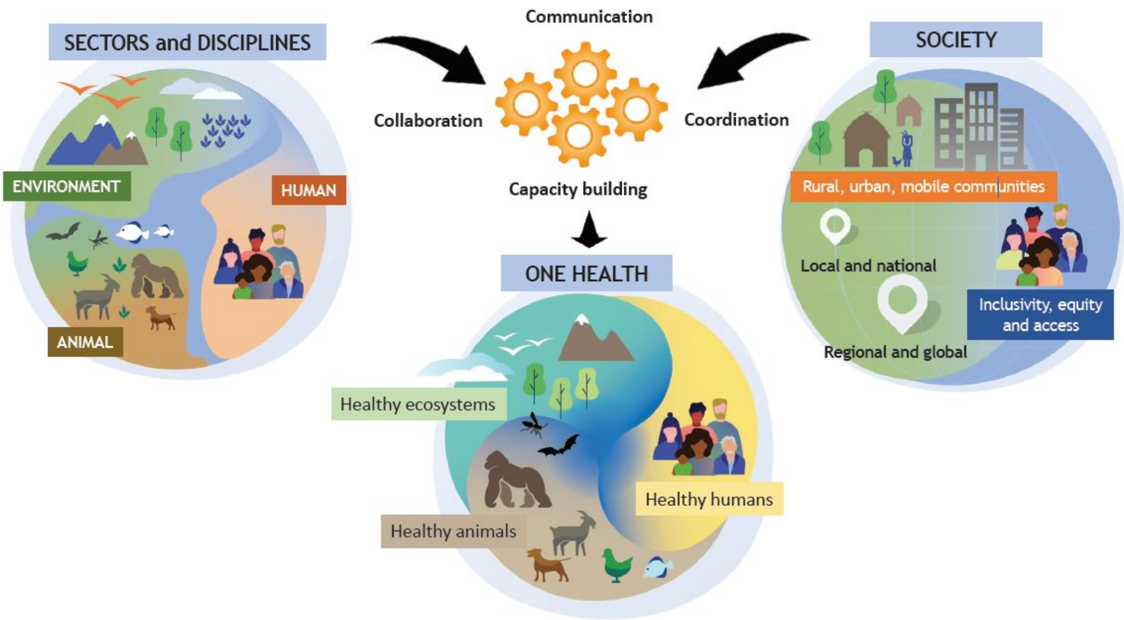

Figure 2 Schematic representation of the One-Health concept where human health is contingent on the health of its environments. From the World Health Organization (WHO) 2006; www.who.int.

1.2. Health as a resource

Another useful concept borrowed from human health systems, and adopted by the WHO, is to consider 'health' as a **resource** rather than a **status** or a goal. A resource is something that stands in service of a goal (life, living): to be used, abused, depleted, repaired, optimized and replenished. This captures much better the dynamism, responsiveness and resilience of health than defining health as a status or goal, which is a much more rigid and inflexible concept. It also aligns much better with the ecosystems and One-Health modelling approaches, and indeed to how (health) decisions and compromises are made in real life.

1.3. The diverse dimensions of health

'Health' is a holistic concept, encompassing the entire organism (López-Uribe et al. 2020). However, an organism is a complex, multi-faceted entity interacting at multiple levels with a complex, dynamic environment. Consequently, its health is equally multi-faceted, interactive and complex. In PoshBee's holistic conceptual model of bee health (Figure 3), this complexity is represented as a hierarchical series of interconnected layers of bee-life organization, from molecular, through cellular and organ/tissue level organization to whole organisms, in both individual and social context, and subsequently to higher order population-genetic level organization set within an ecological/environmental and evolutionary context. Each of these layers of bee-life organization responds differently to the environmental challenges that bees face. Although this complexity makes it difficult to superimpose a rigid experimental, analytical or predictive framework to the study of bee health and its main determinants and drivers, it is still possible to identify several major subcomponents, or 'dimensions', to overall health that facilitate addressing the practical challenges of optimizing bee health. The ones we have identified for this report as most relevant to the aims of PoshBee are: molecular health, microbial health, parasitic health, nutritional health, social-behavioural health, environmental health and genetic health. Below we briefly describe each of these in turn, with particular reference to the three model bee species used in PoshBee: *Apis mellifera*, *Bombus terrestris*, and *Osmia bicornis*.

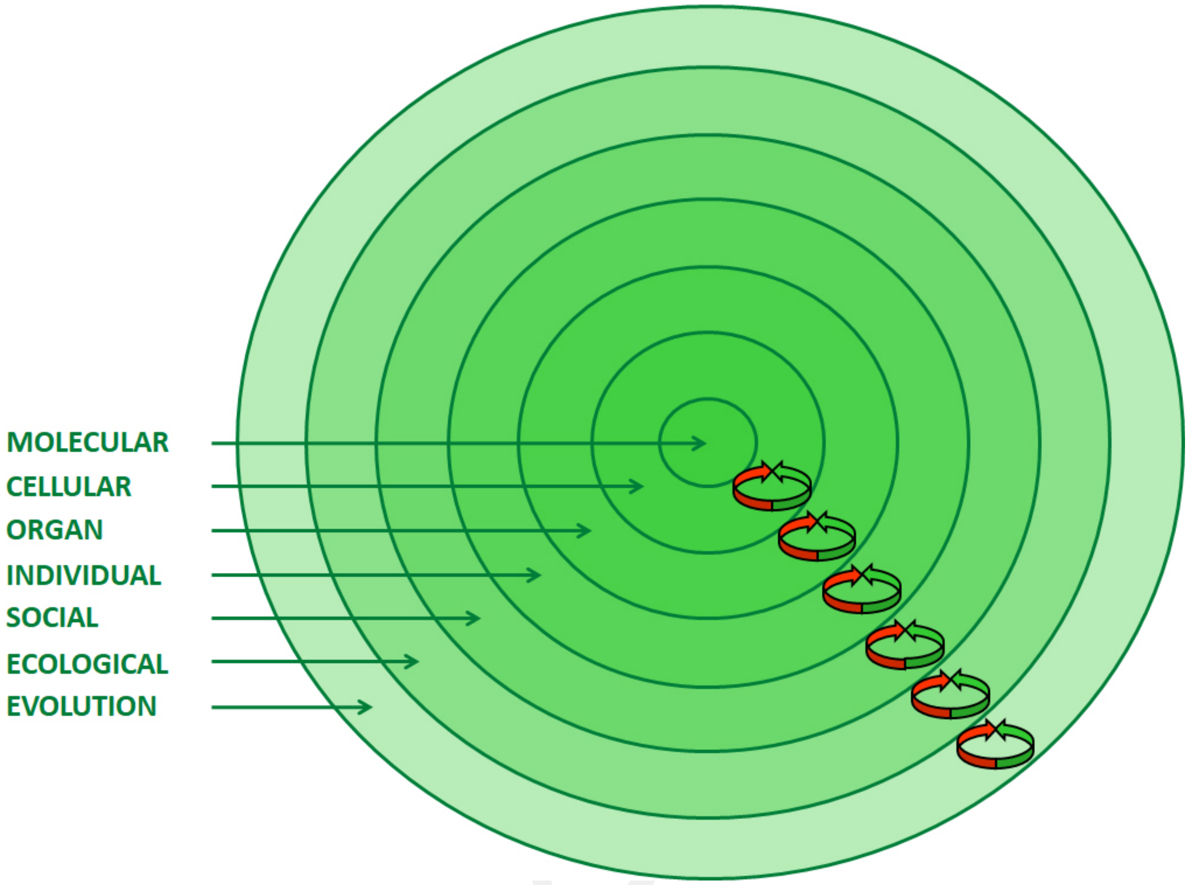

Figure 3 Schematic representation of the hierarchy of interconnected homeostatic layers of bee life and organization, in the PoshBee holistic conceptual model of bee health.

1.3.1. Molecular Health

Molecular health concerns the primary cellular and molecular response mechanisms, i.e., the transcriptome, the proteome, and the metabolome (Manzoni et al. 2018; Pinu et al. 2019). An (environmental) challenge is detected by the organism's sensory systems and, through a chain of molecular interactions, this sensory input activates or de-activates relevant gene pathways (transcriptome), to produce the enzymes and proteins (proteome) and metabolic compounds (metabolome) which then address the environmental challenge (Wang et al. 2019). Included in molecular health are the innate and adaptive immune systems (Doublet et al. 2017; Nazzi and Pennacchio 2014; 2018; Grassl et al. 2018; Annoscia et al. 2020), specific inducible response mechanisms to specific challenges (Tsvetkov and Zayed 2021; Weaver et al. 2021), metabolite and xenobiotic detoxification mechanisms (Gong and Diao 2017; du Rand et al. 2017), antimicrobial peptides (Danihlík et al. 2015), and the natural and universal antiviral mechanisms inherent in the molecular mechanisms and complexes used for managing the destruction and turnover of mRNA (McMenamin et al. 2018). The transcriptome is usually highly responsive with fast response times, in the order of minutes-hours, after which the signal dissipates quickly through the mRNA turnover management system (Haasnoot et al. 2007). The proteome and metabolome changes are generally more persistent, although they are also subject to activation, de-activation, and turnover (Manzoni et al. 2018; Pinu et al. 2019).

1.3.2. Microbial Health

The microbiome is a collective term for all the symbiotic microorganisms that are intimately associated with a host organism, primarily those that inhabit the gut environment (Engel et al. 2016; Kwong and Moran 2016). The bee bacterial microbiome is dominated by a core group of about 8-10 taxa that together comprise about 98% of the bacterial microbiome, supplemented by a large diversity of minor taxa comprising the remaining 1-2% (Sabree et al. 2012; Kwong and Moran 2016; Romero et al. 2019). The major species of bacteria are *Snodgrassella alvi*, *Gilliamella apicola*, *Frischella perrara*, *Bartonella apis*, *Bombella apis* and bacteria belonging to *Lactobacillaceae* and *Bifidobacteriaceae* (Martinson et al. 2012; Kešnerová et al. 2020). *Lactobacillaceae* and *Bifidobacteriaceae* are lactic acid fermenting bacteria that reside primarily in the honeycrop, where they contribute to the curation of nectar and the inhibition of bacterial pathogens (Vásquez et al. 2012). *Bartonella apis* is part of a genus of common insect symbionts whose role is still unclear, but is possibly related to protein and nitrogen metabolism (Segers et al. 2017). *Bombella apis* is part of the mouth microbiome and probably involved in curing the glandular secretions fed to larvae and queens (Dalenberg et al. 2020; Smith et al. 2021). *Gilliamella apicola* and *Snodgrassella alvi* are the main bacterial species of the mid- and hindgut, whose principal roles include food metabolism, neutralization of dietary toxins and protection against gut parasites and pathogens (Kwong and Moran, 2016; Romero et al. 2019). *Frischella perrara* is a facultative pathogen associated with scabbing and melanization in the pylorus region connecting the mid- and hindgut (Engel et al. 2015). By volume the microbiome consists mostly of beneficial bacteria whose primary role is to convert ingested food, most significantly pollen in the case of bees, into nutrients to be absorbed and distributed to the cells and tissues for powering the energetic and growth requirements of the host (Engel et al. 2016). It is also directly involved in health and immunity through metabolism and detoxification of xenobiotic compounds (Zheng et al. 2016, 2017; Emery et al. 2017; Kešnerová et al. 2017; Kwong et al. 2017; Atashgahi et al. 2018; Chmiel et al. 2019; Wu et al. 2020) as well as direct interaction with the host immune system (Janashia and Alaux 2016) and inhibition of bee pathogens (Vazqúez et al. 2012; Näpflin and Schmid-Hempel 2018). The microbiome is therefore an important component of health, since it links the external environment through food consumption and nutrition to the internal environment. The composition of the microbiome is dynamic and responsive to changes in the environmental input (Yun et al. 2018; Donkersley et al. 2018; Kešnerová et al. 2020; Thaduri et al. 2021), through selection and differential growth of different bacterial species and strains in relation to the functional needs of the moment. Although most of the bacterial taxa in the microbiome are primarily beneficial or neutral with respect to bee health and performance (Engel *et al.* 2016; Praet et al. 2018; Beaurepaire *et al.* 2020), a few, particularly from the minor taxa, can become pathogenic when their absolute or relative levels exceed homeostatic limits, or their organizational context changes (Raymann et al. 2017, 2018; Motta et al. 2018; Raymann & Moran 2018).

Apart from symbiotic bacteria, the bee microbiome also contains a number of pathogenic organisms, principally fungi, microsporidians, neogregarines, trypanosomatids, and a large diversity of viruses (Engel et al. 2016; Galbraith et al. 2018; Beaurepaire et al. 2020) that are shed into the gut lumen and are transmitted between bees as part of the microbiome. Their effects on the host are individual, host-specific and largely additive.

Since the turnover in the microbiome is dependent on selective processes within the gut involving independent organisms (bacteria), the time-scale of these changes is days-weeks-months (Yun et al. 2018; Kešnerová et al. 2020; Thaduri et al. 2021), i.e., an order slower than the host transcriptome-proteome response.

In bees, the microbiome can be shared within and between bee species through direct social interaction and through communal foraging on overlapping floral networks (Adler et al. 2018; Bodden et al. 2019; Keller et al. 2021), principally by brushing trace faecal material encountered in or around flowers into the pollen pellets (Figueroa et al. 2019). Thus, both through sharing and because of similar functional roles, the microbiomes of most bee species are similar in composition and function (Engel et al. 2016; Cariveau et al. 2014; Kwong and Moran 2016; Kwong et al. 2014).

1.3.3. Parasitic Health

Bees are also host to a number of larger parasites, principally mites, which affect their health directly and indirectly. The best known of these is the ectoparasitic mite *Varroa destructor*, which primarily infests *Apis mellifera* and occasionally *A. cerana*, but neither bumblebees nor solitary bees. However, varroa can still affect bumblebees and solitary bees indirectly. Varroa is a very efficient vector of a number of generalist, multi-host bee viruses, resulting in the epidemic transmission, propagation and accumulation of these viruses in individual honeybees and honeybee colonies. These then serve as a source of virus infection to other bee species, through direct contact, communal foraging networks and water sources, or through robbing activities when floral resources have ceased. Bumblebees and solitary bees also have their own assemblages of mites, both of the bee and of the nest (Goulson 2010). Some of these are largely commensals while others are parasitic.

1.3.4. Nutritional Health

A regular and balanced diet is a major component of health in all organisms. Too little or inappropriately balanced food leads to malnutrition, which is a contributing factor to many illnesses and ailments, and accentuates the negative consequences of environmental stressors (De Grandi-Hoffman et al. 2015; Conroy et al. 2016; Bordier et al. 2017; Dolezal and Toth 2018; Linguadoca et al. 2021), while appropriately balanced nutrition can reduce or abolish the negative consequences of environmental stressors (Alaux et al. 2010; Annoscia et al. 2017; Rundlöf and Lundin 2019; McNeil et al. 2020; Barraud et al. 2020; Barascou et al. 2021b). The bee diet consists of floral nectar, as the main source of carbohydrates for energetic needs, and pollen, as the principal source of proteins, lipids, and micronutrients. Different plants produce pollen with different nutritional composition, principally the relative amounts of protein and lipids (Vaudo et al. 2020), while different bee species also have different nutritional requirements (Wood et al. 2021). This means that certain types of pollen are more suited to some bees than others, depending on the match between pollen composition and the nutritional requirements of the bee (Roger et al. 2017a; 2017b). Both nectar and pollen can also contain compounds that can cause indigestion in certain bees (Zheng et al. 2016), while a monotonous diet can lead to malnutrition, even if available in sufficient quantity, if the pollen profile does not match sufficiently well with the nutritional requirements of the bee (Vaudo et al. 2016; Roger et al. 2017a). Furthermore, toxic compounds can be present in pollen, nectar, or the honeydew collected by bees which requires detoxification (e.g., nicotine, melizitose; du Rand et al. 2017; Zheng et al. 2016) or can help defend the bee against diseases or parasites (Giacomini et al. 2018). A minority of bee species are oligolectic, foraging on a narrow range of plants whose pollen matches well with the nutritional requirements of the bee (Cerceau et al. 2019), while the majority of bee species are polylectic, with their nutritional needs satisfied by the diversity of pollen with different nutritional compositions. This means that the nutritional needs of the majority of bees are best served by a spatial and temporal diversity of flowering plants within their foraging landscapes (Andersson et al. 2013; Wray and Elle 2015; Senapathi et al. 2016; Roger 2017b; Rundlöf et al. 2018).

1.3.5. Social-Behavioural Health

Bees have well developed sensory, learning, memory, and behavioural systems for navigating the complexities of life. Decades of neurological experiments have proven the high degree of sophistication, adaptability, and ingenuity of the bee brain with respect to learning, remembering, and sharing behaviours and skills. Foraging long distances around a fixed central nest requires excellent reading, mapping, and orientation in the landscape; different floral resources require different techniques for collecting pollen and nectar; nesting materials need to be collected, assembled, and organized, all of which is learned, optimized, remembered, and shared. In social bees, communication and social behaviour is also learned behaviour, subject to improvement and optimization through corrective positive and negative feedback loops (Camazine and Sneyd 1991; Bonabeau et al. 1997; Camazine et al. 1997). The importance of learned behaviours to bee life simultaneously emphasizes the importance of neurological, social, and behavioural health to overall bee health. This neurological and behavioural adaptability and flexibility of bees is also important for plant-pollinator relationships. Plants use visual and olfactory cues to attract bees to their flowers and encourage fidelity (and thus pollination) with nectar and pollen, but occasionally also with neuroactive metabolites (e.g., caffeine, nicotine) that improve neurological acuity (sensation, training, memory), much like they do in humans, ensuring greater fidelity and better pollination for fewer costs or rewards (Köhler et al. 2012; Wright et al. 2013). At low levels such metabolites can help bees find and exploit nectar and pollen that might otherwise go undetected (Köhler et al. 2012; Wright et al. 2013). However, in excess they can instil dependency to draw bees away from more profitable plant resources, which may be an evolutionary strategy on the part of the plant. Synthetic analogues of such natural neuroactive metabolites, such as those used in agricultural insecticides, have similar beneficial or detrimental effects on bee behaviour, depending on the dosage (Cutler and Rix 2015; Kessler et al. 2015; Arce et al. 2018; Siviter et al. 2019). At excessive levels such neuroactive chemicals can seriously compromise the ability of the bee to function normally, with negative consequences for all aspects of bee life and survival (Tosi et al. 2017; Wu-Smart and Spivak 2018; Tong et al. 2019; Straub et al. 2019, 2020; Coulon et al. 2020; Tamburini et al. 2021a; 2021b). The bee brain is also a target for several bee viruses that affect bee behaviour, including learning, memory, orientation, aggression, sensation, division of labour, hygienic behaviour, foraging preferences, and flight performance. The extent to which this may be beneficial or detrimental to the bee depends in part on the ecological context of the bee, not least the transmission of these viruses to rival bee species with perhaps less tolerance.

1.3.6. Environmental Health

Environmental health concerns the ability of the immediate environment of the organism to provide the organism with all the resources needed to sustain a healthy, fulfilling, and disease-free life. This includes minimum exposure to physical, chemical and biological stress factors in the environment as well as the availability of resources to overcome or compensate for stress-inducing factors. For bees, whose foraging landscape is restricted by flight distance from a central nesting location, this mostly involves sufficient floral diversity and heterogeneity in time and space during their active season (Alaux et al. 2017), which varies from a few weeks for most solitary bee species to 2-6 months (depending on species) for the semi-social bumblebees, to half a year or more for the eusocial honeybees. Physical, chemical and biological stress factors have always been part of the bee environment, since bees have developed a range of homeostatic mechanisms to avoid, tolerate or neutralize such stressors (Maebe et al. 2021a; 2021b). Anthropogenic changes in the bee landscape, principally through the agricultural concentration and homogenization of floral resources in time and space, have also concentrated and changed the exposure of bees to these stressors, and have exposed bees to additional anthropogenic chemical stressors in bee-attractive flowering crops (Rundlöf et al. 2015; Balfour et al. 2017; Wintermantel et al. 2018; Osterman et al. 2019). The extent of this additional chemical stress depends on the timing and duration of the bee active season in relation to the peak flowering period of the bee-attractive crops, the manner of application (topical or systemic), and the timing and duration of chemical pest control agents, as well as the climatic conditions during and after treatment that affect the availability, persistence, decay, and residual activity of the chemical agents within the field of application and their dissemination through groundwater or air to other fields and areas outside the field of application, including to other floral resources that can then be accessed by the bees (Di Noi et al. 2021).

1.3.7. Population-Genetic Health

Compared to most insect species, bees have relatively low reproductive rates: a handful of off-spring per reproductive female per year for most solitary bee species and for the eusocial honeybees as little as one new queen every 1.5 years. However, bees have two mechanisms that help maximize the adaptive potential of the relatively small bee genome. The first is a very high rate of genetic recombination (Wallberg et al. 2015; Kawakami et al. 2019; Jones et al. 2019) that leverages the combinatorial potential of the available genetic variation. The second is a haplo-diploid mating system that exposes this variation directly to natural selection in the drone offspring, thus ensuring rapid adaptation of the genome to new environmental challenges. Eusocial honeybees furthermore also have a polyandrous mating system, usually involving 10-50 drones (Tarpy et al. 2012; Wallberg et al. 2014), that increases the within-colony genetic diversity, which is associated with multiple health benefits (Mattila and Seeley 2007; Tarpy et al. 2012). These mechanisms for maximizing genetic diversity are essential for the long-term adaptation and survival of bee species in a rapidly changing environment, and therefore a very important aspect of health over multigenerational time-scales (Grozinger and Robinson 2015; Wallberg et al. 2014, 2015, 2017; Carvell et al. 2017; Blacher et al. 2017; Jones et al. 2019; Kawakami et al. 2019).

1.4. Resilience and Health

A very particular aspect of holistic health is the concept of 'resilience', which describes an organism's capacity to overcome (temporary) suboptimal changes in its external environment with minimal consequences for its long-term health and survival (Ulgezen et al. 2021)). Resilience is therefore an extremely important aspect of health, since it describes the various homeostatic and buffering mechanisms available to an organism to continually adapt to, and survive in, a dynamic and ever-changing environment. Most of these adaptive mechanisms involve plastic responses of the individual organism to its immediate environment. However, if the environmental change is persistent and long-term, this can also involve genetic and genomic changes, through Darwinian selection. This concept as applied to bees and their health has been described in detail in PoshBee MileStone 17. In PoshBee's holistic conceptual model of bee health (Figure 3), the individual and combined boundaries to these homeostatic mechanisms define the depth of this buffering capacity, and thus the robustness of the organism's overall resilience. It also identifies 'sociality' as an additional, distinct, and very potent homeostatic resilience mechanism available just to social bees and not to solitary bees.

1.5. Interpreting health system responses: context, shifting baselines and dual meaning

Implicit in the concept of 'health' as a resource comprising a series of dynamic, replenishable homeostatic response mechanisms is the logical corollary that this resource is meant to be **used**, to benefit the life and well-being of the organism. In other words, its very purpose is to **respond** to environmental and life challenges. Such responses can be measured and quantified, summarized and interpreted. For instance, cardiac exercise (an environmental challenge) increases the blood pressure, heart rate, breathing frequency, oxygen uptake, and lactic acid build-up in humans, while during recovery afterwards these measures all slowly return to their pre-exercise levels. **Repeated** cardiac exercise adapts the musculature, anatomy, physiology, lung capacity and metabolic processes to make these responses more efficient, such that the same exercise induces less change in these indicators during exercise and faster recovery afterwards. These two observations illustrate three very important principles of health, resilience and health systems, especially with respect to the analysis and interpretation of health indicator data.

- The first principle is that the absolute value of any health indicator is dependent on the **context** in which it was obtained (e.g., hearth rate while resting or exercising).
- The second principle is that the adaptive response mechanisms themselves are subject to optimization **towards** the challenge (e.g., regular exercise limits heart rate increase during exercise), which is furthermore also reversible (e.g., loss of fitness with lack of exercise). In other words, environmental challenge can **improve** the **efficiency, speed** and **strength** of the health response mechanisms corresponding to that challenge.
- The third, and perhaps most difficult, is that a change in the value of an indicator reflects BOTH that the (health) system has been challenged (i.e., it reflects an aspect of the **environment**) AND simultaneously that the system has **responded** to this challenge (i.e., it reflects aspects of the organism's homeostatic response mechanisms) (e.g., an increase in heart rate implies both that the organism has been challenged by the exercise and that the organism is reacting to a challenging exercise). Conversely, a known, measured change in the environment without a corresponding change in indicator value means that either the health system in question is not affected by the environmental challenge, or is defective and can no longer respond, even though it should. In other words, change is not necessarily bad, nor is the absence of change necessarily good, when interpreting health system responses to environmental challenges.

Although we used a simple example, human exercise, to illustrate these points, these principles apply to most, if not all, aspects and dimensions of (bee) health. Well established examples include mRNA transcription, translation and RISC-based mRNA turnover and anti-viral defense mechanisms, innate and humoral immune system components, the adaptive flux in microbiome composition and function in relation to environmental input, communication and social organization in response to environmental cues in social bees, including social hygienic behaviour, and epigenetic and genomic responses to persistent environmental challenge. For example, following a microbial infection, an increased expression of the genes encoding antimicrobial peptides is often observed (Doublet et al.

2017), but this condition is indicative both of the infection in progress and of the innate immune response of the organisms to the infecting microbes, through the peptides synthesized after gene expression (Evans et al. 2006).

2. Key bee health indicators

As is clear from the previous section, and particularly section 1.5, great care should be taken with the selection, measurement, analysis and interpretation of any markers or indicators of various aspects and dimensions of health in whatever organism we study. Particularly important is a very clear awareness of the **limitations** of any indicator, both technically and with respect to how it represents an organism's health. This is particularly important for non-human organisms, whose life contexts are not as intuitively accessible, or perhaps even knowable, as for humans. For many organisms health research also lags far behind human and veterinary health research, especially when it comes to reliable health indicators. This is especially true for non-model organisms or those not directly linked to human progress, which in the case of bee health concerns pretty much all bee species except the honeybee.

2.1. A simple definition of a health indicator

The term 'health indicator' also derives originally from human medicine, and in particular public health where health indicators are used to measure, quantify, and summarize different attributes of individual health at the population-level (Breslow 2006). A simple definition of a health indicator is therefore:

"An estimate of a given health dimension in a target population"

A key attribute of a health indicator is that it can be measured and quantified (Parrish 2010). Another key attribute is that they are population-level estimates, rather than individual estimates. This means that they are generally represented as a mean within a range of acceptable values. A third feature of health indicators is that their value lies mostly in the summary statistics, which can reveal a health situation that may not be immediately obvious from the individual values (Etches et al., 2006). In other words, it may reveal underlying contexts that are only extractable from aggregated data. The multifaceted nature of health means that there are many ways to measure and quantify it and thus many potential health indicators. As before, the superior development of human (public) health monitoring can serve as a useful reference point for how this can be approached in bee health. Human health indicators range from simple physical estimates (e.g., length, weight, temperature) through more complex biochemical profiling (e.g., metabolic panels) to genomic screening for genetic markers linked to a range of genetic diseases or predispositions to certain health outcomes and societal (public) health indicators affecting groups of individuals. Health indicators can also be aggregated to assess the performance, or 'health' of entire public health systems. This information can then be used to inform interventions and adjustments to improve overall health, well-being and survival for individuals and populations (Brownson et al. 1999). Similar types of health indicators can also be produced for bees, or indeed for any other organism. The accuracy, range and practical utility of any candidate health indicator then has to be established and confirmed through research, before it can be used to inform bee health management decisions. The purpose of this part of D8.1 is to deliver a set of reliable, robust bee health indicators whose performance is supported by extensive research, both within and outside PoshBee. We have taken a conservative and stringent approach to this task, considering a shorter list of superior indicators of greater practical value than a more exhaustive list of less reliable indicators.

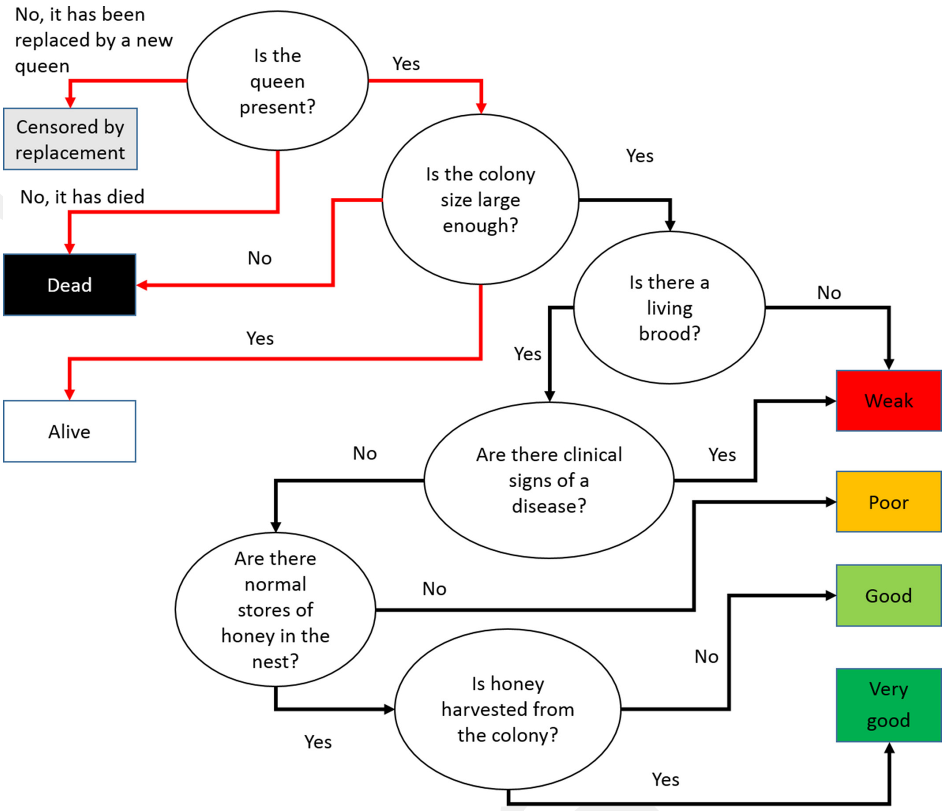

Figure 4 Decision tree for assessing honeybee colony-level health status using the HEALTHY-B toolbox (AHAW 2016).

2.2. Types of health indicators

There are several ways to classify health indicators which can facilitate how and where they are employed for bee health management decisions. An obvious one is which **dimension** of bee health the indicator addresses, as described in section 1. Another is whether the indicator estimates the health status of the **bee** (intrinsic), the status of its **environment** (external) or concerns a **global** attribute that does not have an analogue at the individual level (e.g., population density, environmental indices). For social bees this can also include whether the individual bee or its colony is considered the operational unit, for either the indicator measurement or the associated management options. The EFSA Healthy-B toolbox (EFSA Panel on Animal Health and Welfare 2016) is a good example of colony-level health indicators with matching management decisions (Figure 4), as is the Integrated Biological Response Index for honeybee ecotoxicology status (Caliani et al. 2021). Finally, indicators can be classified as either **positive** (health promoting) or **negative** (health decreasing). Furthermore, positive and negative indicators are also subject to the concept of **hormesis**, where the positive or negative outcome changes with the size of the estimate (e.g., many biochemical agents, medicines), or where the optimum outcome is an **intermediate** value due to negative consequences for values that are either too low or too high (e.g., the size of an organism). For bees this can be particularly relevant for a range of biological, chemical, and physical stress-inducing conditions and agents. See MS17 for further explanation.

2.3. Attributes of a good health indicator

Different health indicators measure different aspects of health with varying levels of efficiency and effectiveness in different situations and environments. The following are some attributes to help evaluate the usefulness of alternative indicators for specific situations (Pan American Health Organization 2018):

- **Variability:** The indicator values should vary over a reasonable range of detection
- **Measurability:** It should be possible to measure the indicator reliably and accurately
- **Feasibility:** It should be feasible to measure the indicator in practical situations
- **Validity:** The indicator should measure what it intends to measure
- **Timeliness:** The results of the measurement should be available fast enough to allow for useful intervention
- **Robustness:** The indicator should match the health status stably under all conditions
- **Replicability:** The indicator measurement should be similar between technical operators
- **Sustainability:** The indicator should be useful over a long period of time
- **Relevance and importance:** The indicator should ideally be associated with an actionable mitigating intervention and not just of academic interest
- **Comprehensibility:** The indicator should be understood by practitioners so that they can use the information correctly and profitably
- **Independence:** The indicator should ideally be minimally affected by other indicators
- **Universality:** The indicator should be applicable to as many bee species as possible

2.4. A few key bee health indicators

With reference to the above criteria for what constitutes a meaningful, reliable, and practical health indicator, we have identified a few potential health indicators in each of the main bee health domains for consideration, together with suggestions as to how best to access or measure the indicator. The list has been compiled from the perspective of analysing live, adult bees caught in natural environments, where all the necessary information for the indicator must come from either the bee itself or the environment in which the bee was caught. This broad perspective automatically gears the indicator strategy towards **all** bees, not just those from experimental settings. Also taken into consideration was the stability of the indicator with respect to sample collection and preservation strategy, i.e., where a choice was available, the more stable indicator, metabolite or technology was chosen.

2.4.1. Morphological indicators

One of the most basic health indicators is the **size** of the bee. All bees display some degree of size variation that can be linked to health or its absence, whether from disease, malnutrition, or other environmental stressors. For instance, although the mean weight and length of honeybee worker bees is fairly constant across subspecies, it can be significantly affected by biological (e.g., varroa parasitism and virus infection), chemical and physical (e.g., temperature) stressors (Yang and Cox-Foster 2005; Annoscia et al. 2012; Azpiazu et al. 2019). Bumblebees in particular have great capacity for size plasticity that is affected by all sorts of conditions, primarily nutritional quality and quantity during the larval phase, but also chemical and physical stress (Maebe et al. 2021a; 2021b), and this plasticity can linked to differences in flight range, foraging efficiency, and colony performance (Greenleaf et al. 2007). This size polymorphism and plasticity also extends to bumblebee queens (Owen 1988), whose average size has gradually increased during the last century (Gérard et al.

2020a, 2021), where queen size is positively associated with superior diapause survival, nest initiation, foraging, dominance, fecundity, and reproductive success (Beekman et al. 1998) and negatively affected by a range of biological, chemical, and physical stress (Baron et al. 2017). There are a number of ways to measure bee size, e.g., weight or some measure of length, width, or volume. One of the most reliable and practical is the **Inter-Tegular Distance** (**ITD**), which measures the width of the thorax between the wing entry points using a calliper (Cane 1987). ITD is furthermore also a non-destructive method that can be performed on live, stunned bees and, unlike weight, is not affected by the defecation status of the bee. In both honeybee and bumblebee queens, ovary activation and fecundity is reversibly affected by the social environment (Sarro et al. 2019), and in the case of honeybee queens involves a sizeable expansion of the abdomen during the active season. For honeybees therefore, the size (length, width) of the queen abdomen is also frequently used as an indirect marker for the size of the ovaries and the fecundity of the queen. In bumblebees, ovary development and fecundity is more plastic. The bee **fat body** is the major repository of vitellogenin and other important immune system proteins, lipids, and carbohydrates (Vanderplanck et al. 2021). The size and composition of the fat body may therefore be a good indirect bee health indicator encompassing a number of important resources. In bumblebees, wing size and shape are also potentially useful morphological health indicators, since they are predictably responsive to a number of stressful conditions, particularly disease and temperature. Wing asymmetry by contrast is less reliable as a morphological stress marker (Gérard et al. 2018).

2.4.2. Molecular indicators

Vitellogenin is an egg yolk protein found in practically all oviparous animals, including most invertebrates. In bees it is stored in fat bodies in the abdomen and brain to provide for the first eggs after emerging from diapause (Münch & Amdam 2010; Treanore *et al.* 2020). However, in honeybees and to a lesser degree bumblebees, vitellogenin has acquired a more plastic profile to also include roles in division of labour, brood care and ageing (Guidugli *et al.* 2005; Salmela *et al.* 2015; Alaux *et al.* 2017; Winkler *et al.* 2018; Harwood et al. 2019). In essence, vitellogenin links pollen foraging to reproduction and winter survival. It also has important immune functions. As such, vitellogenin is the single most important protein for health and survival in all bees and could be an excellent prognostic marker for the actual and future status of any wild caught bee. **Antimicrobial peptides** (AMPs) are also a ubiquitous class of compounds found across the animal kingdom that directly or indirectly target microbial agents and are a major part of the host's disease protection mechanisms (Danihlík et al. 2015; Mahlapuu et al. 2016; Wu et al. 2020a). Other potential molecular indicators include **heat-shock proteins**, which are a frequent generic stress response in many organisms (Feder and Hofmann 1999; Taylor et al. 2014; McKinstry et al 2017) and linked to antiviral activity in bees (McMenamin et al. 2020). Vitellogenin, AMP, and heat-shock proteins are basal to many health functions and processes and as such are good broad indicators for health or health problems in general, but less good for identifying the specific cause of the health problem. The opposite is true for molecular indicators towards the end of a metabolic process, such as metabolic biomarkers for various conditions (Wang et al. 2019), which are good at identifying specific individual ailments, but not others, and are thus less suitable for a generic assessment of the overall health status of the organism. A second main choice concerns the type of metabolite analysed. In experimental studies, short-term changes in the synthesis of these proteins or their precursors can be accessed through changes in mRNA synthesis, i.e., the transcriptome (Nazzi et al. 2012; Di Prisco et al. 2013, 2016; Aufauvre et al. 2014; Brandt et al. 2016; Doublet et al. 2017; Annoscia et al. 2020;

Al Naggar and Paxton 2021). However, for actual status assessment of wild caught bees it is preferable to access the final active effector molecules (proteome, metabolome) or their functions (Barascou et al. 2021a; 2022) since the transcriptome is a transient short-term metabolite at the very start of the molecular response process, separated from the final function needed to deal with the environmental challenge by further buffers in the proteome (composition, activation) and metabolome (de Smet et al. 2017; Collison et al. 2018; Osterman et al. 2019). PoshBee WP9 is currently creating a database of protein and metabolite molecular markers and profiles in bee haemolymph that are diagnostic for a range of stress-inducing agents and conditions, although, unfortunately, the current methodology appears limited with respect to many of the indicator attributes (i.e., feasibility, timeliness, robustness, replicability, comprehensibility, relevance, sustainability, and universality).

2.4.3. Microbial indicators

There are a number of positive and negative health indicators associated with the bee microbiome. The obvious negative health indicators are the levels of pathogenic microorganisms, such as viruses, bacteria, microsporidians, neogregarines, trypanisomatids, and others. Independently of where in the host these microbes replicate, almost all are shed in large amounts into the gut lumen for release into the nest and/or external environment with the faeces. The relative and absolute abundance of these pathogens in the faeces is therefore both an indicator of the health status of the bee itself with respect to these pathogens, and the epidemic potential of these pathogens to the wider bee community, through various transmission networks involving environmental matrices (floral networks, pollen, nectar, water, soil, nesting material) contaminated with these faeces (Figueroa et al. 2019). The bee is also host to a suite of mostly beneficial bacteria (Engel et al. 2016; Kwong and Moran 2016), whose relative composition changes considerably during the season, in response to environmental cues, social status and the needs of the bee (Yun et al. 2018; Kešnerová et al. 2020; Thaduri et al. 2021). The intestinal microbiome is dominated by 8-10 bacterial species that account for ~98% of the bacterial mass, with a larger number of minor taxa accounting for the remaining 2%. Microbiome dysbiosis is often associated with a microbiome compositional imbalance, usually a relative excess of one of the minor species, which can be precipitated by a number of factors. Consequently, a useful non-destructive indicator for the health of the bee microbiome is the relative proportion of major and minor bacterial taxa in the bee faeces. The raw data is most easily obtained through amplicon sequencing and bioinformatics analyses of the bacterial 16S rRNA gene from total faecal (or abdomen) DNA. The microbiome compositional stability can then be analysed through a number of standard diversity indices (Thaduri et al., 2021).

2.4.4. Parasitic indicators

The single most important determinant of honeybee health is the level of infestation with *Varroa destructor* (Dietemann et al. 2012; Traynor et al. 2020; Mondet et al. 2020). Varroa infestation in honeybee colonies can be measured in a number of ways (Dietemann et al. 2013). However, the damage caused by varroa infestation is entirely due to the epidemic transmission by varroa of several lethal virus infections, principally deformed wing virus and acute bee paralysis virus (Traynor et al. 2020). These viruses are also infectious and damaging to bumblebees and other Hymenopteran insects (Beaurepaire et al. 2020). Due to the close, direct and causal relationship between varroa infestation and deformed wing virus infection levels (Nazzi et al. 2012; Yañez et al. 2020), the presence and varroa-status of managed or feral honeybee colonies in an environment can also be inferred indirectly from the presence and levels of deformed wing virus in flying or trapped honeybees. Since deformed wing virus is also of health significance to other bees, it can serve as a useful common indicator for virus health in all bees, as well as an indirect marker for the presence of varroa-infested honeybee colonies in the vicinity (Fürst et al. 2014; McMahon et al. 2015; Piot et al. 2022).

2.4.5. Nutritional indicators

Although for honeybees in particular, nectar foraging and storage is a major factor in colony survival, for most bee species the main nutritional requirement is for pollen. Different bee species have different requirements for protein and lipids while different plant species produce pollen with different protein and lipid composition (Roger et al. 2017a; Wood et al. 2021) with different levels of digestibility for different bee species (Vanderplanck et al. 2018). This means that a good generic indicator for nutritional health covering all bee species is the **protein**, **lipid**, and **carbohydrate** composition of different floral sources of pollen (Vaudo et al. 2020). The nutritional needs of different bee species can then be met by either a perfectly matched pollen source (as is the case for oligolectic bees; Burger et al. 2021) or a diverse blend of different pollen sources (as is the case for polylectic bees). Both cases argue for a floral conservation approach that maximizes a diversity of floral habitats within the flight range and dispersal rates of most bee species (Bartomeus et al. 2013; Roger et al. 2017b), to minimize disruption to plant-pollinator communities through climate change (Gérard et al. 2020b; Duchene et al. 2020) and agriculture (Baraud et al. 2020; Barascou et al. 2020; 2021b).

2.4.6. Social-Behavioural indicators

Bee behaviour is the highest level of functional health indicator associated with individual bees (Barrascou et al. 2021c; 2022) and key to how the bee explores and extracts maximum benefit from its environment (Prado et al. 2019). A behavioural indicator essentially reflects the bee’s neurological capacity, memory and decision making. There are two suitable indicators that can be applied to wild-caught bees of all species. The first is the **Proboscis Extension Reflex** (**PER**), which registers the neurological sensitivity to a substance and forms the basis of many laboratory learning and memory tests in many pollinating insects (Lunau et al 2018), particularly honeybees (Frost et al. 2012) and bumblebees (Laloi et al. 1999; Toda et al. 2018) and which can be adapted for use on wild caught bees in the field (Muth et al. 2018). The second is **orientation** which incorporates all aspects of cognition, learning, memory and decision making, and is readily affected by a number of biological and chemical agents and is similar for all bee species irrespective of foraging or nesting strategies.

2.4.7. Environmental indicators

There are two types of indicators to register the suitability of an environment to sustain healthy and diverse populations of different bee species. The first group concerns direct estimates of known negative biological, (agro)chemical and physical stressors or agents in different environmental matrices. These matrices can include the bees themselves, the nectar and pollen they carry, or environmental samples of air, water or soil. The second group concerns the composition of the landscape, particularly with respect to the availability of suitable nesting sites and foraging opportunity and diversity throughout the active season. This second category is usually represented in a **Landscape Heterogeneity Index** combining different landscape features and types, with different indices emphasizing different features (e.g. Li and Reynolds, 1995; Díaz-Varela et al. 2016; Sumasgutner et al. 2019; Balzarolo et al. 2019; Yoshioka et al. 2017; Tscharntke et al. 2012). Particularly important is the continuous availability of a great diversity of flowers throughout the season, to maximize the beneficial effects of balanced nutrition for bee health (see above) and the availability of suitable nesting sites in these landscapes for different types of bees (Senapathi et al. 2016; Thomson and Page 2020). Most landscape analyses approximate these needs indirectly, through assumptions based on global landscape types and (anthropogenic) land-use (Rundlöf et al. 2008; Andersson et al. 2013; Perovíc et al. 2015; Herbertsson et al. 2016, 2018; Milano et al. 2019; Gervais et al. 2020), while other analyses address this more directly and from a bee-centric view (e.g., Potts et al. 2003; Wray and Elle 2015; Thomson and Page 2020; Duchenne et al. 2020). Also, for these environmental indicators, it is important that can be of practical use to implement and monitor changes (Kremen et al. 2007; Kuchling et al. 2018).

2.4.8. Genetic indicators

Healthy bee populations need sufficient genetic diversity to adapt to changing environments and challenges. A simple indicator of genetic diversity that can be obtained from individual bees captured in the landscape is the proportion of heterozygous alleles, or **Single Nucleotide Polymorphisms** (**SNPs**) in a diploid (i.e., female) bee. This information is traditionally obtained through microsatellite assays and more recently (and directly) through sequencing all or part of the bee genome. At population level these estimates of genetic polymorphism are pooled into a population-level statistic, the **Fixation Index** (**F_{ST}** index), which measures the diversity among the SNPs of a local subpopulation relative to that of the larger population (Wallberg et al. 2014).

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