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Review Paper

Advances and limitations of visual conditioning protocols in harnessed bees

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ABSTRACT

Bees are excellent invertebrate models for studying visual learning and memory mechanisms, because of their sophisticated visual system and impressive cognitive capacities associated with a relatively simple brain. Visual learning in free-flying bees has been traditionally studied using an operant conditioning paradigm. This well-established protocol, however, can hardly be combined with invasive procedures for studying the neurobiological basis of visual learning. Different efforts have been made to develop protocols in which harnessed honey bees could associate visual cues with reinforcement, though learning performances remain poorer than those obtained with free-flying animals. Especially in the last decade, the intention of improving visual learning performances of harnessed bees led many authors to adopt distinct visual conditioning protocols, altering parameters like harnessing method, nature and duration of visual stimulation, number of trials, inter-trial intervals, among others. As a result, the literature provides data hardly comparable and sometimes contradictory. In the present review, we provide an extensive analysis of the literature available on visual conditioning of harnessed bees, with special emphasis on the comparison of diverse conditioning parameters adopted by different authors. Together with this comparative overview, we discuss how these diverse conditioning parameters could modulate visual learning performances of harnessed bees.

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1. Introduction

The honey bee *Apis mellifera* has been used as a traditional model in the study of visual processing, learning and memory (Giurfa, 2007; Srinivasan, 2010; Avarguès-Weber et al., 2011; Dyer, 2012; Giurfa and Sandoz, 2012; Menzel, 2012). Like primates, honey bees possess true color vision (von Frisch, 1914; Daumer, 1956; Menzel, 1968; Backhaus, 1992; Vorobyev et al., 2001), shape and pattern vision (von Frisch, 1914; Srinivasan and Lehrer, 1988; Lehrer et al., 1995; Zhang et al., 1995; Srinivasan, 2006), motion vision (Srinivasan et al., 1999), and extensive visual learning capabilities (Giurfa et al., 2003; Dyer et al., 2005, 2008a; Srinivasan, 2010; Avarguès-Weber et al., 2011, 2012; Zhang et al., 2012). Furthermore, honey bees use landmarks and celestial cues such as azimuthal position of the sun and polarized light pattern of the sky (e-vector) to ensure efficient navigation in a complex environment (Rossel and Wehner, 1986; Collett and Baron, 1994). In this rich visual scenario, honey bees are able to acquire simple associative links between a visual stimulus and its outcome, as well as non-elemental visual associations (Schubert et al., 2002; Giurfa et al., 2003). They can extract common features among a set of rewarded stimuli to define categories or even manipulate abstract relational concepts as a rule (Benard et al., 2006; Zhang, 2006; Avarguès-Weber et al., 2011; Avarguès-Weber and Giurfa, 2013). Therefore, the honey bee represents an attractive insect model for studying the neural basis of visual processing and learning, in order to discover commonalities and differences among animals in the neural mechanisms underlying visual cognitive functions.

For more than a century, the main protocol used for studying visual cognition in honey bees has been the operant conditioning of free-flying animals (Avarguès-Weber et al., 2010, 2012). In this learning paradigm, free-flying bees are individually marked and pre-trained to fly towards an experimental place, in which they are subsequently trained to associate a visual target with a sucrose solution reward. Afterwards, bees can be tested at the same experimental place by performing unrewarded presentation of the visual target and/or alternative visual stimuli, in order to analyze visual memory, discrimination and generalization (Menzel, 1967; Zhang et al., 1992, 1999, 2005, 2012; Giurfa, 2004; Avarguès-Weber et al., 2011; Dyer et al., 2011; Dyer, 2012). Free-flying honey bees can be trained to a large variety of visual attributes such as colors, shapes and patterns, depth, motion, light intensity, contrast and complex configurations (Menzel, 1967; Zhang et al., 1995; Giurfa and Menzel, 1997; Horridge, 2000; Srinivasan, 2010; Avarguès-Weber et al., 2011). Experiments with free-flying bees present, however, the limitation of allowing neither a precise temporal control of visual stimulation nor a simultaneous access to the animal's brain, thus precluding the study of the neural bases of visual learning and perception (Avarguès-Weber et al., 2012). In this context, immobilizing bees constitutes a *sine qua non* condition for a successful achievement of these goals.

The existence of a conditioning protocol for training immobilized honey bees to associate an olfactory stimulus with sucrose reward (the olfactory conditioning of the proboscis extension reflex - PER; Bitterman et al., 1983; Matsumoto et al., 2012) has allowed a huge advance in our knowledge about the neural bases of olfactory learning and memory (Menzel, 1999; Menzel and Giurfa, 2001; Giurfa, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012). Honey bees reflexively extend their proboscis when their antennae are touched with sucrose solution (unconditioned stimulus – US). Pairing an odor (conditioned stimulus – CS) with the US leads to the quick acquisition of CS-US association and subsequent extension of the proboscis to the odor alone (conditioned response). This Pavlovian conditioning protocol has been combined to a large variety of invasive methods such as electrophysiology (Hammer, 1993; Mauelshagen, 1993; Abel and Menzel, 2001; Müller et al., 2002; Okada et al., 2007; Denker et al., 2010), calcium imaging (Faber et al., 1999; Galizia et al., 1999; Faber and Menzel, 2001; Sachse and Galizia, 2003; Sandoz et al., 2003; Guerrieri et al., 2005; Fernandez et al., 2009; Hourcade et al., 2009; Rath et al., 2011), pharmacology (Müller, 1996; Grünbaum and Müller, 1998; Hammer and Menzel, 1998; Müller, 2000; Lozono et al., 2001; Devaud et al., 2007, 2015; Boitard et al., 2015) and RNA interference (Farooqui et al., 2003, 2004). Therefore, olfactory conditioning of PER has allowed studying olfactory perception in honey bees at the cognitive, neurobiological and molecular levels (Menzel, 1999; Menzel and Giurfa, 2001; Giurfa, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012).

In contrast, the study of visual learning and memory in bees has rarely surpassed the behavioral level. The difficulty of training harnessed bees with visual cues when compared with free-flying bees has been the main limitation in that sense (Avarguès-Weber et al., 2012). Since the fifties, researchers have been reporting different complications related to visual conditioning of restrained honey bees, which include necessity of antennae amputation, low acquisition levels, poor discrimination between stimuli, necessity of numerous conditioning trials, varying learning performances depending on the spectral properties of the stimuli, among others (Kuwabara, 1957; Masuhr and Menzel, 1972; Gerber and Smith, 1998; Hori et al., 2006, 2007; Letzkus et al., 2008; Niggebrügge et al., 2009; Mota et al., 2011a; Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Balamurali et al., 2015). Probably with the aim of solving these complications and improving visual-PER learning performances, each of these authors has developed a distinct protocol, changing parameters like the harnessing method, number of trials, inter-trial interval, nature and duration of visual stimulation (Fig. 1 and Table 1). Therefore, the literature provides results obtained with different visual conditioning protocols that are rarely comparable and sometimes conflictive.

Here we provide an overview of the literature about associative visual-PER conditioning in bees. In order to analyze commonalities and differences in the methodological approaches developed by each author, we performed a systematic comparison between diverse conditioning parameters used in visual-PER studies (Table 1). By confronting these methodological parameters with the learning performances obtained, we discuss the advances and limitations of visual-PER conditioning protocols.

2. The modulatory effect of antennae in visual-PER conditioning

The first work reporting successful learning in visual conditioning of the honey bee PER was published by Kuwabara (1957), who described antennae amputation as a necessary procedure to allow the acquisition of color-reward associations and consequent color-dependent PER response. Kuwabara (1957) decided to remove the antennae of bees, because restrained bees with intact antennae apparently developed unspecific PER responses to the water vapor from the small spoon used to deliver sucrose solution as reward. Fifteen years after, Masuhr and Menzel (1972) reported results in visual-PER conditioning of honey bees after numerous training trials (up to 110 associative trials). These authors did not comment about the necessity of antennae amputation for the occurrence of associative visual learning and they apparently used bees with intact antennae. In 1998, Gerber and Smith used classical conditioning of the honey bee PER in a bimodal blocking paradigm with a visual and an olfactory stimulus. These authors reported no significant acquisition of visual-induced PER in bees with intact antennae during a 6-trial pre-training phase in which bees had

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