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Brief Communication

Higher-order trace conditioning in newborn rabbits

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Abstract

Temporal contingency is a key factor in associative learning but remains weakly investigated early in life. Few data suggest simultaneous presentation is required for young to associate different stimuli, whereas adults can learn them sequentially. Here, we investigated the ability of newborn rabbits to perform sensory preconditioning and second-order conditioning using trace intervals between odor presentations. Strikingly, pups are able to associate odor stimuli with 10- and 30-sec intervals in sensory preconditioning and second-order conditioning, respectively. The effectiveness of higher-order trace conditioning in newborn rabbits reveals that very young animals can display complex learning despite their relative immaturity.

Text

Temporal contiguity between stimuli is usually considered essential for associative learning in adult individuals (Pavlov 1927). However, first-order conditioning remains effective when a trace interval (TI), usually 10 to 30 sec, is inserted between the conditioned stimulus (CS) and unconditioned stimulus (US) (e.g., Kamin 1954; Ost et al. 1968; Kirkpatrick and Balsam 2016). In higher-order conditioning such as sensory preconditioning and second-order conditioning, two CSs, CS1 and CS2, are paired before or after the CS1 is paired with a US, respectively (Brogden 1939; Rescorla 1980a). Here again, conditioning is possible in adult rats when the CS1 and CS2 are separated by a short TI, and this is true for both sensory preconditioning (Silver and Meyer 1954; Lavin 1976; Lyn and Capaldi 1994; Holmes and Westbrook 2017) and second-order conditioning (Rescorla 1982, Stout et al. 2004).

Very young individuals exhibit robust first- and higher-order conditioning when pairings involved simultaneous presentations of the to-be-conditioned stimuli (Cheslock et al. 2003; for review: Cuevas and Giles 2016). However, a few studies suggest that they might be particularly sensitive to the insertion of a TI between stimulus presentation in training (e.g.,

first-order conditioning: Moye and Rudy 1987; Ivkovich et al. 2000; Barnet and Hunt 2005; higher-order conditioning: Cheattle and Rudy 1979; Cheslock et al. 2003). Indeed, sequential conditioning becomes more efficient with age in human infants (Cuevas and Giles 2016) and young animals (Chen et al. 1991).

In the European rabbit, newborns can learn new odorants through first- and higher-order conditioning. The mammary pheromone (MP) emitted by lactating rabbit females in their milk not only triggers stereotyped orocephalic movements allowing for pups to search and grasp the maternal nipples (Coureaud 2001; Schaal et al. 2003) but also remarkably promotes associative odor learning. Indeed, the MP acts as a US inducing appetitive conditioning to an odorant (CS1) by simultaneous and brief pairing: 24h later, the CS1 triggers a conditioned orocephalic searching-grasping response similar to the response triggered by the US (Coureaud et al. 2006, 2009). Rabbit pups are also able to respond to a second odorant (CS2) when CS1+MP pairing is followed by simultaneous CS1+CS2 pairing, demonstrating second-order conditioning (Coureaud et al. 2011), but also when simultaneous exposure to CS1+CS2 preceded CS1+MP pairing, demonstrating sensory preconditioning (Coureaud et al. 2013). These higher-order conditionings seem to be associative in nature as unpaired presentations of either CS1 and US or CS1 and CS2 impede CS2 responding (Coureaud et al. 2011, 2013). In particular, sensory preconditioning and second-order conditioning do not occur if CS1 and CS2 are separated by a 1-min TI (Coureaud et al. 2011, 2013). However, the effectiveness of shorter TIs in supporting higher-order conditioning remains to be evaluated in newborn rabbits. The present study provides this evaluation.

To that goal we used 305 newborn rabbits (from 64 litters) and odorants A, B and MP (see below) purchased from Sigma-Aldrich (France). Only 4 or 5 pups per litter were used per experiment (they were included in a single group, i.e. not used in more than one condition). To avoid interference with the pups' prandial state (Montigny et al. 2006) experiments always

occurred at 10:30 a.m., one hour before the daily nursing. Statistics consisted in comparison of proportion of responding pups to the stimuli by the Cochran Q test and χ^2 test of McNemar when pups from a same group are tested to distinct stimuli, and χ^2 test of Pearson when pups from distinct groups are tested to the same stimulus.

First, in order to confirm that simultaneous pairing of CS1+CS2 promotes sensory preconditioning, 1-day old pups (n = 15) were exposed in a behavioral room (adjacent to the animal housing room) by sub-group of 5 neonates as a maximum (in order to optimize their exposure) to a mixture of odorants A (ethyl-isobutyrate, 10^{-5} g/ml) and B (ethyl maltol, 10^{-5} g/ml). The exposure happened in a plastic basin through a scented cotton glove positioned 1 cm above the litter for 5 min (timer-controlled by a second experimenter) then immediately retired. On day 2, the pups were conditioned to odorant A by simultaneous pairing with MP (2-methylbut-2-enal, 10^{-5} g/ml) for 5 min in a new basin and through a new glove (the basins were rinsed with alcohol, water and then dried after each use; the gloves were used only once per experiment). On day 3, the pups were individually tested in an oral activation test (e.g. Schaal et al. 2003; Coureaud et al. 2009, 2011, 2013) during which each pup was immobilized in one hand of the experimenter, its head being left free, and the test odor was presented for 10 s with a glass-rod 0.5 cm in front of the nares. The response was positive when the stimulus elicited vigorous scanning movements of the head during stretching toward the rod followed by oral grasping of the rod; non-responding pups displayed no response but sniffing. Under the present conditions, the pups responded to both odorants A and B as well as to MP (A vs. B vs. MP: Cochran's Q test = 2, $P = 0.37$). For second-order conditioning, 1-day-old pups (n = 9) were conditioned to odorant A (by simultaneous pairing with MP for 5 min) and exposed on day 2 to A+B for 5 min (here and hereafter: same procedure of exposure as above). On day 3, pups responded in a strong and similar manner to A, B and MP (A vs. B vs. MP: $Q = 2$, $P = 0.37$).

To determine if an TI between presentations of neutral odorants would affect sensory preconditioning, 1-day-old pups were exposed to odorants A then B for 2.5 min each with a 10-sec (n = 15) or 30-sec TI (n = 15); the exposure to each odorant was made in distinct basins separated by more than 1 meter in a permanently ventilated room, to avoid any contamination between the different odorants. On day 2, they were conditioned to odorant A (by simultaneous pairing with MP for 5 min). Strikingly, pups responded strongly and similarly on day 3 to A, B and MP after 10-sec TI (A vs. B vs. MP: $Q = 3$, $P = 0.22$). However, pups responded to A and MP but not to B after 30-sec TI (A vs. B vs. MP: $Q = 18$, $P < 0.001$; 2 x 2 comparisons by χ^2 test of McNemar: B vs. MP or A: $\chi^2 \geq 7.1$, $P < 0.01$) (Fig. 1A-B). These results support the presence of sensory preconditioning in the 10- but not the 30-sec condition. Indeed, the proportions of responders to A after 10- and 30-sec TI were strong and similar ($\chi^2 < 0.5$, $P > 0.05$) while they dropped dramatically to B after 30- compared to 10-sec TI ($\chi^2 = 13.5$, $P < 0.001$).

Similar results were obtained when preconditioning to A then B was followed by conditioning to B (Fig. 1C-D) and after reverse exposure to the odorants (B then A) during preconditioning then conditioning to odorant A or B (Fig. 1E-H): pups responded to A, B and MP after 10-sec TI but only to the conditioned odorant after 30-sec TI (n = 10 per group; $Q = 2$, $P > 0.05$ with 10 sec of TI; $Q > 12$, $\chi^2 \geq 7.1$, and $P < 0.05$ in comparisons between A and B or MP with 30 sec of TI; drop in responsiveness to A in Fig. C vs. D and G vs. H: $\chi^2 \geq 7.2$, $P < 0.05$, and in responsiveness to B between the 10- and 30-sec condition in Fig. E-F: $\chi^2 = 9.8$, $P < 0.01$). Therefore, two main results emerged from these data: 1) sensory preconditioning was still effective when a short TI (10-sec) takes place between the odorants during initial exposure, and 2) it was present and equivalent in the forward (CS2//CS1, CS1+US) and backward (CS1//CS2, CS1+US) protocols.

To assess whether second-order conditioning would also tolerate TIs, 1-day-old pups were conditioned to odorant A (by simultaneous pairing with MP for 5 min) and exposed the day after to odorants A then B for 2.5 min each with a 10-sec ($n = 10$) or 30-sec TI ($n = 10$). On day 3, pups responded highly and similarly to A, B and MP after the 10-sec TI (A vs. B vs. MP: $Q = 2$, $P = 0.37$) but also after the 30-sec TI (A vs. B vs. MP: $Q = 4$, $P = 0.14$) (Fig. 2A-B). When the same experiment was conducted with a 60-sec TI between A and B, pups significantly responded to A and MP but not to B ($n = 10$; A vs. B vs. MP: $Q = 11$, $P < 0.01$; B vs. MP: $\chi^2 = 5.1$, $P < 0.05$; A vs. MP: $\chi^2 = 1.3$, $P = 0.25$; drop in responsiveness to B between 30- and 60-sec: $\chi^2 = 7.9$, $P < 0.01$) (Fig. 2C). Similar results were obtained after conditioning to odorant A and reverse exposure to the odorants (B then A) (Fig. D-F) and conditioning to B followed by exposure to B then A (Fig. G-H): pups responded to A, B and MP after the 10- and 30-sec TI but only to the conditioned stimulus after 60-sec TI ($n = 10$ per group; $Q \leq 4$, $P > 0.05$ with 10- and 30-sec TI; $Q > 15$, $\chi^2 \geq 5.1$ and $P < 0.05$ in comparisons between A and B or MP with 60-sec TI; responsiveness to odorant B in Fig. E vs. F: $\chi^2 = 7.9$, $P < 0.01$, and to odorant A in Fig. G vs. H: $\chi^2 = 5.2$, $P < 0.05$). Thus, second-order trace conditioning was functional in the forward (CS1+US, CS2//CS1) and backward (CS1+US, CS1//CS2) protocols in newborn rabbits with longer TI (30-sec instead of 10-sec) than sensory preconditioning.

To determine if this difference was related to the age of the pups at A and B exposure, i.e. day 1 for sensory preconditioning *but* day 2 for second-order conditioning, 12 pups were exposed on day 2 to odorants A then B with 30-sec TI, and conditioned to A on day 3. On day 4, pups responded to A and MP but not to B (A vs. B vs. MP: $Q = 17$, $P < 0.001$; B vs. MP or A: $\chi^2 \geq 6.1$, $P < 0.05$; data not shown) indicating sensory preconditioning was ineffective with a 30-sec TI. Thus, the difference of TI tolerated by the two procedures does not depend on the age of the newborns.

Natural environment of animals is extremely complex chemically: animals are exposed to numerous odor molecules and mixtures of molecules. We thus investigated higher-order trace conditioning in newborn rabbits using more than two odorants, i.e. three odorants. We first demonstrated that pups responded strongly and similarly to A, B and C (ethyl acetoacetate; Sigma-Aldrich) after simultaneous exposure to the 3 odorants in both preconditioning and second-order conditioning procedures ($n = 10$ per group; A vs. B vs. C vs. MP: $Q < 2$, $P > 0.05$) (Fig. 3A,D). Thus, rabbit pups exhibit robust higher-order conditioning to three odorants after simultaneous presentation.

To assess whether sensory preconditioning was effective with sequential exposure to 3 odorants, 1-day-old pups were exposed to odorants A then B then C for 2.5 min each with a 10-sec or 30-sec TI ($n = 10$ per group). Pups were then conditioned to A on day 2 and tested for their responsiveness to the distinct odorants on day 3. After the 10-sec TI, pups responded highly and similarly to A, B and MP, but not to C (A vs. B vs. C vs. MP: $Q = 23$, $P < 0.001$; C vs. MP, A or B: $\chi^2 \geq 6.1$, $P < 0.05$) (Fig. 3B), whereas pups responded only to A and MP after the 30-sec TI (A vs. B vs. C vs. MP: $Q = 26$, $P < 0.001$; B or C vs. A or MP: $\chi^2 \geq 7.1$, $P < 0.01$; drop in responsiveness to B between 30- and 60-sec: $\chi^2 = 12.9$, $P < 0.001$) (Fig. 3C). Thus, backward sensory preconditioning tolerated the same TI with two or three odorants in newborn rabbits, i.e. 10-sec but not 30-sec. However, the effect was only partial after 10-sec TI as behavioral response extended only to the odorant (B here) that directly followed the odorant that was conditioned (odorant A).

Regarding second-order conditioning with sequential exposure to three odorants, pups were conditioned to odorant A on day 1 before being exposed on day 2 to odorants A then B then C for 2.5 min each with a 10-sec, 30-sec or 60-sec TI ($n = 10$ per group). On day 3, pups responded strongly and similarly to A, B and MP but not to C with 10-sec (A vs. B vs. C vs. MP: $Q = 18$, $P < 0.001$; C vs. MP or B: $\chi^2 = 6.1$, $P < 0.05$; C vs. A: $\chi^2 = 3.1$, $P = 0.08$) and 30-

sec TI (A vs. B vs. C vs. MP: $Q = 25$, $P < 0.001$; C vs. MP, A or B: $\chi^2 \geq 7.1$, $P < 0.01$) (Fig. E-F) whereas pups only responded to A and MP with the 60-sec TI (A vs. B vs. C vs. MP: $Q = 30$, $P < 0.001$; B or C vs. A or MP: $\chi^2 \geq 10$, $P < 0.005$, drop in responsiveness to B between 30- and 60-sec: $\chi^2 = 12.9$, $P < 0.001$) (Fig. 3G). Thus, backward second-order conditioning tolerated the same 30-sec TI with two or three odorants in rabbit pups but, as for sensory preconditioning, with a partial effect as soon as 10-sec TI with three odorants; pups indeed responded only to the odorant that directly followed the presentation of the conditioned one but not to the third odorant (odorant C here).

According to previous literature in rat pups and human babies, trace conditioning would be non-functional in early life, as newborns do not tolerate any delay between stimuli to be associated, whereas older animals do (Cheatle and Rudy 1979; Moye and Rudy 1987; Chen et al. 1991; Ivkovich et al. 2000; Barnet and Hunt 2005; Cuevas and Giles 2016). Similarly, previous studies indicate rabbit pups cannot display first-order trace conditioning with 0-sec TI (Coureaud et al. 2006, 2011, 2013). Regarding the importance of hippocampus in first-order trace conditioning with discrete cues (Goldsberry et al. 2015; Kitamura et al. 2015), the failure of this first-order trace conditioning in rabbit neonates might result from the late maturation of the hippocampus.

The present study strikingly shows that rabbit pups are however able to form higher-order trace conditioning, and that different TI between odor presentations are tolerated: 10-sec TI for sensory preconditioning and 30-sec TI for second-order conditioning, regardless of the order of CSs presentation (forward or backward CS1-CS2 pairing). Similar greater tolerance for longer TI in second-order conditioning over sensory preconditioning was previously demonstrated in newborn rats, as immediate-sequential (0-sec TI) presentation of odor CSs impedes sensory preconditioning but not second-order conditioning; the latter being not effective with a 30-sec TI (Cheslock et al. 2003).

Sequential higher-order conditioning with three odorants provided similar but also novel information compared to two odorants in newborn rabbits. As with two odorants, backward sensory preconditioning was effective with 10-sec TI and backward second-order conditioning with 30-sec TI. However, these procedures were only partially effective since the pups did only respond to the odorant that directly followed the conditioned one (and not to the third one in the sequence). It would now be interesting to investigate what would be the response of the newborns when the conditioned odorant is the second or the third of the sequence (allowing to compare forward and backward pairings) to further understand how rabbit pups efficiently learn associative information about their environment.

Higher-order trace conditioning is thus functional in newborn rabbits, highlighting their remarkable cognitive capabilities despite their relative immaturity. An important and unexpected result was the equivalent backward and forward higher-order trace conditioning. Indeed, it is classically reported that forward conditioning is more effective than backward conditioning, even for higher-order conditioning (see for instance Rescorla 1980b; Hussaini et al., 2007), highlighting that the manner in which stimuli are presented influences their integration (see for review Holmes et al., 2022). This effective backward conditioning in rabbit pups is unlikely to be related to odor contamination according to the well-controlled experimental conditions (distinct recipients separated by 1 meter for each odorant in ventilated room) and the fact that first-order trace conditioning was ineffective (excluding any overlap between odorants). This could rather reflect a rabbit singularity compared to rodent pups (or even babies) due to the ecology of the species (for review: Cuevas and Giles 2016). Notably, the rabbit is to date the only species in which a pheromone (MP) emitted by lactating females and involved in newborns adaptation (nipple location/grasping and milk intake) has been described (Schaal et al. 2003; Coureaud et al. 2010). The use of the MP to induce conditioning might promote specific learning abilities, since this molecule is an extremely

powerful biological signal and reinforcer. These suggestions would require further comparative studies with other species such as rats, mice or humans, to investigate neonatal learning abilities in different conditioning procedures and their potential species-specificity. The difference of TI - 10-sec versus 30-sec - tolerated between the two procedures used here could be related to distinct levels of arousal (Kikas et al. 2021) and/or types of association involved. Indeed, in sensory preconditioning the two odorants are completely neutral during their presentation, therefore triggering low levels of arousal, whereas one odorant has already been reinforced in second-order conditioning, which enhances levels of arousal during subsequent CS1 and CS2 presentations. Therefore, stimulus-stimulus association supports sensory preconditioning, whereas stimulus-reward and/or stimulus-response association participates in second-order conditioning (Sharpe et al. 2017). Interestingly, these different types of association may relate on different brain structures. Indeed, several experiments have shown in adult rodents that higher-order conditioning depends on a wide brain network and that neural circuits underlying sensory preconditioning and second-order conditioning differ. For instance, manipulation of the perirhinal or the retrosplenial cortices affects stimulus-stimulus association during sensory preconditioning but not second-order conditioning in rats (Nicholson and Freeman 2000; Holmes et al. 2013; Robinson et al. 2014; Todd et al. 2016). On the contrary, the basolateral amygdala is involved in second-order conditioning but not in sensory preconditioning (Hatfield et al. 1996; Blundell et al. 2003; Dwyer and Killcross 2006; Parkes and Westbrook 2010). The selective recruitment of the amygdala during stimulus-reward and/or stimulus-response association may therefore help tolerating longer TI compared to sensory preconditioning.

However, much less is known about neural mechanisms that mediate sensory preconditioning and second-order conditioning early in life, and how these mechanisms evolve during development. Different neural circuits have been shown to support first-order

aversive learning in young and adult rats, with gradual involvement of the amygdala when the animal is getting older (Shionoya et al. 2006; Raineki et al. 2009). Moreover, noradrenaline depletion in the forebrain alters sensory preconditioning but not first-order aversive conditioning in both preweanling and adult rats (Archer et al. 1986; Chen et al. 1993). Since only very limited data are available in newborns and according to age-related differences in higher-order conditioning, it would be valuable to investigate the neural mechanisms and in particular the neurotransmitters (using pharmacological and molecular approaches) underlying these higher-order conditioning procedures in rabbit pups.

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Figure captions

Figure 1. Sensory preconditioning with two odorants. *(A,B,E,F) Case of conditioning to the odorant A.* Newborn rabbits were sequentially exposed to *(A,B)* odorants A then B or *(E,F)* odorants B then A with a 10-sec *(A,E)* or 30-sec trace interval (TI) *(B,F)* on day 1 (d1), then conditioned to odorant A by association with the mammary pheromone (MP) on day 2 (d2). Their behavioral response to A and B, and to the MP as a control, was tested 24 h later (d3). Sensory preconditioning tolerated a 10-sec TI during the preexposure phase since pups responded later to A and B, but not a 30-sec TI. *(C,D,G,H) Case of conditioning to the odorant B.* Rabbit pups were sequentially exposed to *(C,D)* odorants A then B or *(G,H)* odorants B then A with a 10-sec *(C,G)* or 30-sec TI *(D,H)* on d1, then conditioned to odorant B by association with the MP on d2, and their behavioral response to B, A and MP was tested on d3. Again, sensory preconditioning tolerated a 10-sec but not a 30-sec TI during the preexposure phase. (*) $P < 0.05$; (***) $P < 0.001$.

Figure 2. Second-order conditioning with two odorants. *(A,B,C) Case of conditioning to the odorant A and recall of A then B.* Newborn rabbits were conditioned to odorant A by pairing with the MP on d1, then sequentially exposed to A then B with *(A)* a 10-sec, *(B)* a 30-sec or *(C)* a 60-sec TI on d2, before testing of their behavior to A, B, MP on day 3 (d3). Second-order conditioning tolerated a 10- and 30-sec TI during the second conditioning phase since pups responded to B in addition to A, but not a 60-sec TI. *(D,E,F) Case of conditioning to the odorant A and recall of B then A.* Rabbit pups were conditioned to A by pairing with the MP on d1, then sequentially exposed to B then A with *(D)* a 10-sec, *(E)* a 30-sec or *(F)* a 60-sec TI on d2, and tested behaviorally to A, B, MP on d3. Second-order conditioning tolerated the 10- and 30-sec TI but not the 60-sec TI during the second conditioning phase. *(G,H) Case of*

conditioning to the odorant B and recall of B then A. Rabbit pups were conditioned to B by pairing with the MP on d1, then sequentially exposed to B then A with (G) a 30-sec or (H) a 60-sec TI on d2, and tested behaviorally to B, A, MP on d3. Second-order conditioning tolerated the 30-sec TI during the second conditioning phase since pups responded to A in addition to B, but not the 60-sec TI. (*) $P < 0.05$; (***) $P < 0.001$.

Figure 3. Sensory preconditioning and second-order conditioning with three odorants. (A,B,C) *Case of sensory preconditioning.* Newborn rabbits were exposed on d1 to the odorants A, B and C (A) simultaneously, or sequentially with (B) a 10-sec or (C) a 30-sec TI between the odorants, then conditioned to odorant A by pairing with the MP on d2, and tested behaviorally to A, B, C and MP on d3. Sensory preconditioning occurred simultaneously and with the 10-sec TI during the preexposure phase since the pups responded to B and C in addition to A, but not with the 30-sec TI. (D,E,F,G) *Case of second-order conditioning.* Pups were conditioned to A by pairing with the MP on d1, then exposed to A, B and C (D) simultaneously, or with (E) a 10-sec, (F) a 30-sec or (G) a 60-sec TI on d2, before behavioral testing to A, B, C, MP on d3. Second-order conditioning occurred simultaneously and with the 10-sec TI, but not with the 30-sec TI during the second conditioning phase. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Figure 1 – Coureaud, Colombel, Duchamp-Viret, Ferreira

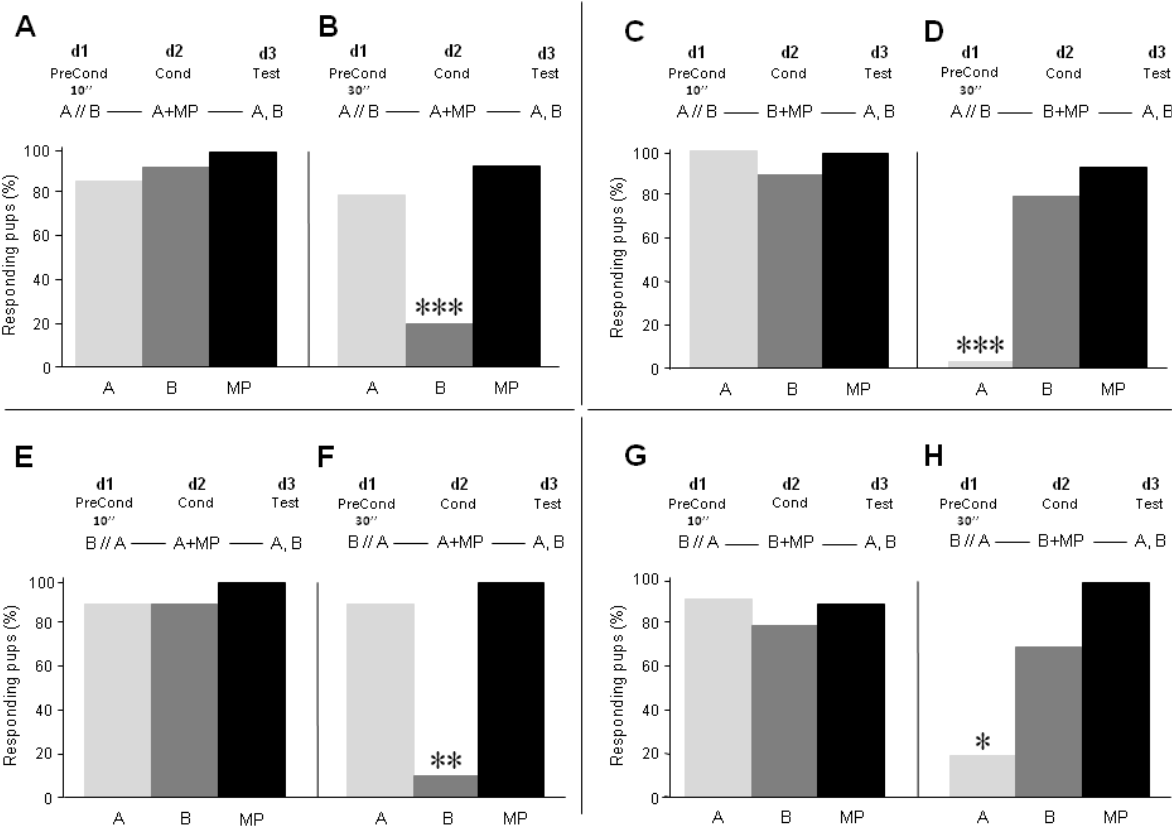
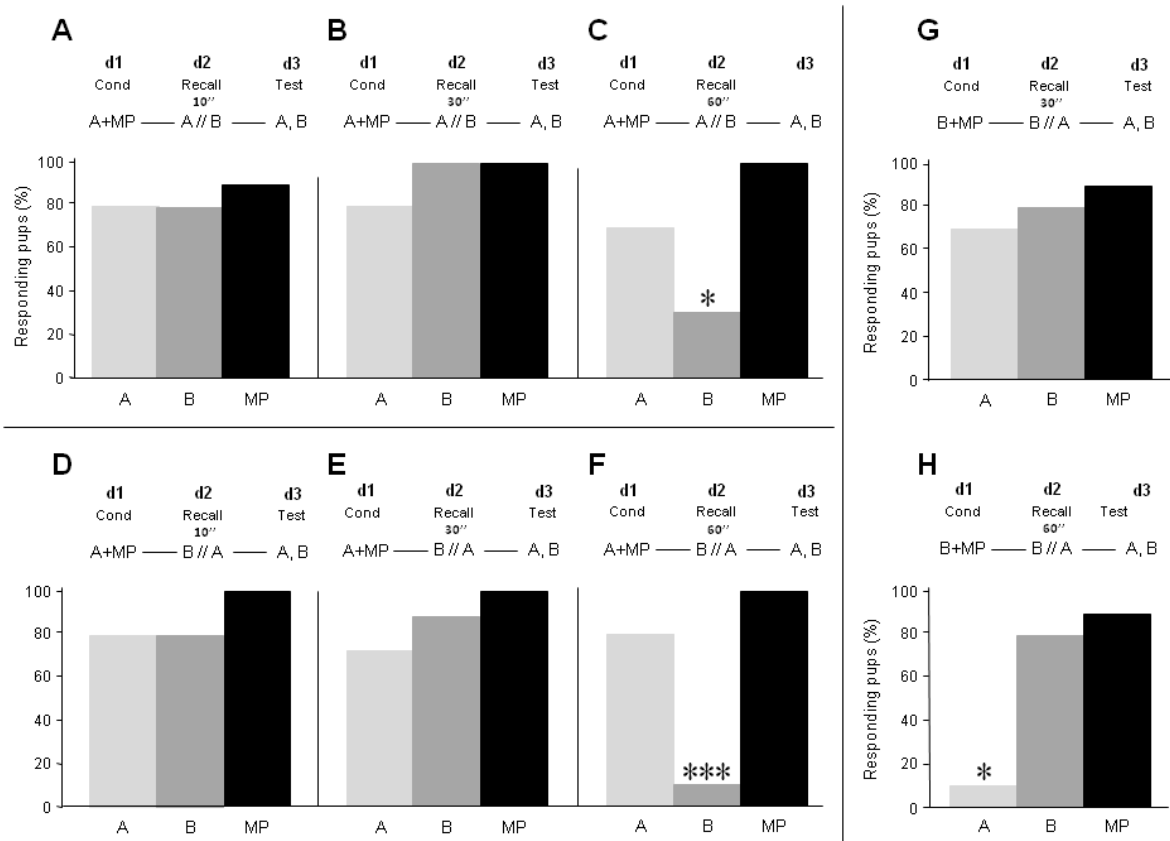


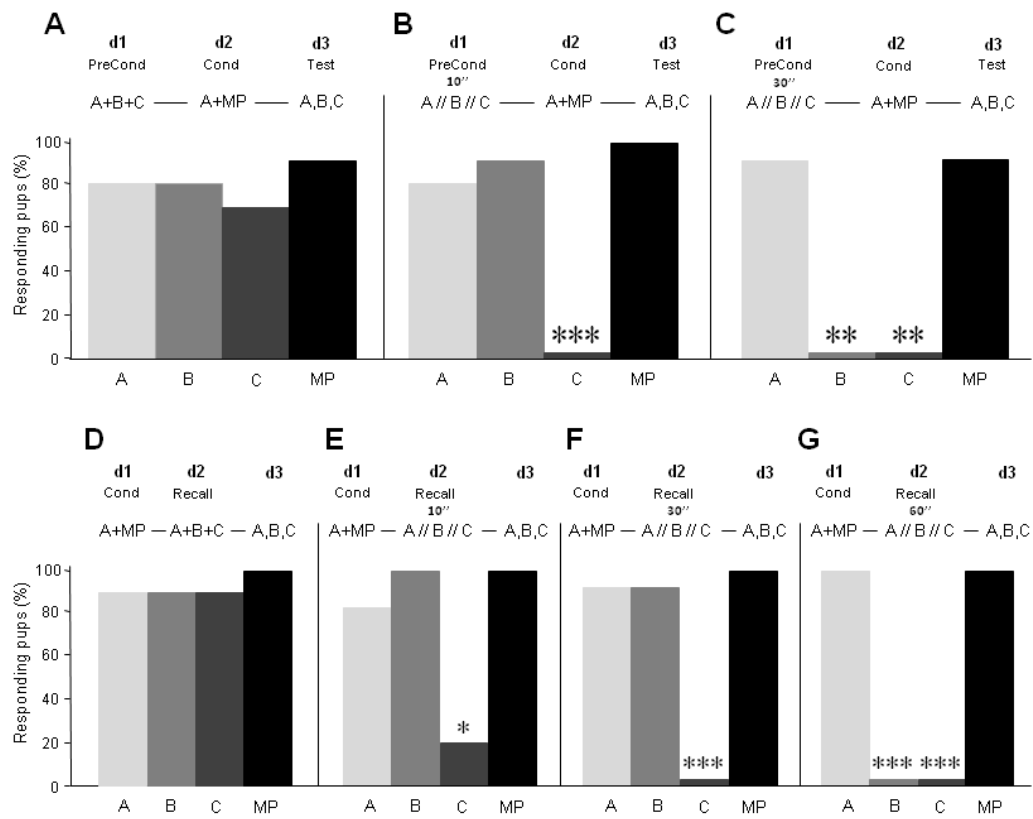
Figure 2– Coureaud, Colombel, Duchamp-Viret, Ferreira



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Figure 3 – Coureaud, Colombel, Duchamp-Viret, Ferreira



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