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Title: Combined predisposed preferences for colour and biological motion make robust development of social attachment through imprinting

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Abstract

To study how predisposed preferences shape the formation of social attachment through imprinting, newly-hatched domestic chicks (*Gallus gallus domesticus*) were simultaneously exposed to two animations composed of comparable light points in different colours (red and yellow), one for a walking motion and another for a linear motion. When a walking animation in red was combined with a linear one in yellow, chicks formed a learned preference for the former that represented biological motion (BM). When the motion-colour association was swapped, chicks failed to form a preference for a walking in yellow, indicating a bias to a specific association of motion and colour. Accordingly, experiments using realistic walking chicken videos revealed a preference for a red video over a yellow one, when the whole body or the head was coloured. On the other hand, when the BM preference had been pre-induced by using an artefact moving rigidly (non-BM), a clear preference for a yellow walking animation emerged after training by the swapped association. Even if the first-seen moving object was a nonbiological artefact such as the toy, the visual experience would induce a predisposed BM preference, making chicks selectively memorise the object with natural features. Imprinting causes a rapid inflow of thyroid hormone in the telencephalon leading to the induction of the BM preference, which would make the robust formation of social attachment selectively to the BM-associated object such as the mother hen.

Keywords

early social deprivation; sensitive period; thyroid hormone; developmental homeostasis; Conspec-Conlern mechanism; domestic chicks

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Introduction

How could cognitive processes develop toward adaptive socialization? The importance of early post-natal experiences has been recognized since the studies by M. and H. Harlow on isolated neonatal babies of the rhesus monkey (Harlow et al., 1965, Harlow and Suomi 1971). Lasting and irreversible behavioural and neurobiological effects of early social deprivation have been shown in rats (Tóth et al., 2008), mice (Makinodan et al., 2012) and humans (Carlson and Earls, 1995; Moulson et al., 2015; also see the recent epigenome study by Naumova et al., 2019). Early isolation escalates maladaptive aggressiveness in various fish species (blue gourami, Tooker and Miller, 1980; cichlid fish, Barki and Volpato, 1998; Siamese fighting fish, Ichihashi et al., 2004), indicating that the importance of social experiences in early life may be common among vertebrates.

Furthermore, these studies suggest that specific experiences are required and the way these experiences work is predisposed. In the case of the isolated monkey babies (Harlow and Suomi 1971) for example, even a limited physical contact with other infant monkeys and the subsequent predisposed interactions among subjects allowed sophisticated social behaviour to develop almost normally. We must understand how the predispositions interact with the post-natal learning, more specifically, how the innately predisposed preference controls the way by which post-natal experiences leads to the adaptive development of social behaviours.

Filial imprinting gives us a unique opportunity to analyse the initial phases of the behavioural development. Since the pioneering observation by Spalding (1873), imprinting has long been studied in precocial birds like geese (Lorenz, 1937), ducklings (Hess, 1958) and domestic chicks (Horn, 1985, 1998, 2004). It has been shown that these animals would learn to form a social attachment even if the first-seen object was a nonbiological artefact (also see reviews by Sluckin 1964; Bolhuis and Honey 1998, Bolhuis 1999; Matsushima et al., 2003). Under experimental settings, chicks exposed to a moving artefact (e.g., a blue box rotating along its axis; Horn, 2004) learn to follow similar objects based on the memorized colour (blue) and/or the shape (box). Recent studies revealed further that the newly-hatched chicks and ducklings memorise physical, geometrical and conceptual aspects of the object that these animals have been exposed to (colour cues for Maekawa et al. 2006; topological features for Wood & Wood 2015;

3-dimensional geometry for Versace et al. 2016; relational concept for Martinho & Kacelnik 2016; abstract multimodal patterns for Versace et al. 2017b), indicating that the imprinting comprises a complex set of perceptive and cognitive developments.

Despite the arguments of Lorenz (1937), who stressed the irreversible nature of imprinting, the preference does change after imprinting. Even if initially imprinted to an artefact, chicks gradually develop a predisposed preference for a stuffed hen that has natural visual configurations, particularly those of the neck and the head region (Bolhuis et al., 1985; Johnson et al., 1985; Johnson and Horn, 1988; also see Rosa-Salva et al. 2010 for the innate preference for face-like configuration). Chicks may depend on the memorised visual cues as the first step, but they gradually reveal a predisposed preference for more natural objects. It is noticeable that the predisposed preference develops after non-specific experiences such as being placed in a running wheel, if given during a sensitive period within 36 hours post-hatch (Johnson et al., 1989). A similar transient time window has been shown for predisposed preference for moving objects of changing speed (Versace et al. 2019). A series of behavioural, localized brain-lesion and pharmacological experiments clearly showed that the predispositions have neural substrates and behavioural processes distinct from those responsible for the memory formation (Bolhuis and Honey 1998). However, it remains unclear how the memory formation interacts with the predispositions through the course of filial imprinting.

Biological motion (BM) is another factor of the imprinting stimulus for which predisposition has been observed. Johansson (1973) reported that simple animations composed of relatively few light points created a vivid and immediate percept of locomotion for human perceivers, if these points were appropriately placed to represent major parts of the body. In a manner similar to the configurational visual predispositions described above, the BM preference appears in visually inexperienced naïve chicks if they are primed by a certain amount of motor activity (Vallortigara et al., 2005). The predisposed BM preference thus precedes the imprinting memory formation, so are the predisposed preference for a rotating stuffed hen described above (Johnson et al., 1985; Johnson and Horn, 1988). The idea of the BM predisposition gained further evidence from a distinct gravity bias where chicks do not show tendency to align with an upside-down inverted version of the BM animation of a walking chicken

(Vallortigara and Regolin, 2006). Furthermore, the BM preference was significantly strengthened after nonspecific visual experience, where newly-hatched chicks were exposed to different kinds of motion pictures, not necessarily BM animations (Miura & Matsushima, 2012). In addition, BM preference was not induced in 4-day-old chicks if chicks had been raised in darkness, suggesting a transient "time window" like other forms of predisposition described above. If the emergence of the BM predisposition preceded the imprinting memory formation, we would expect these two processes to interact. Accordingly, BM point-light animations proved to be among the most potent imprinting stimuli, and those chicks with a higher BM preference showed a higher imprinting score (Miura & Matsushima, 2016).

The induction of BM preference is also closely linked with activation of a hormone that controls the sensitive period of imprinting (triiodo-thyronine T_3; Yamaguchi et al., 2012; Miura et al., 2018). Imprinting enhances expression of the converting enzyme (*Dio2*) that leads to a higher content of T_3 in the telencephalon, which then primes the memory system of imprinting so that even aged (4 to 8 days old) chicks will become imprintable (Yamaguchi et al., 2012). In other words, imprinting makes subsequent imprinting possible through feedback activation by thyroid hormone. Even if the first-seen moving object was an artefact, the induced BM preference would cooperate with the T_3-based priming of the memory system, making chicks form lasting social attachment selectively to those natural BM objects such as a mother hen (Takemura et al., 2018).

This scenario fits well with idea of the two-mechanism device underlying face processing in primate infants (Morton and Johnson, 1991). Two-to-four-month-old human babies show an innate preference for face-like visual patterns (Mauer and Barrera, 1981; also see Mondloch et al., 1999). Even new-borns are equipped with a visual processing machinery for face-like visual patterns (Sugita, 2008 in rhesus monkeys; Buiatti et al., 2019 in human babies; Reid et al., 2017 in human foetus) in accord with the idea of the *Conspec* process (Morton and Johnson, 1991). The *Conspec* process guides the initial preference, making infants pay more attention to face-like patterns, but it subsequently becomes un-observable in isolation and replaced by the later *Conlern* process, which makes babies learn the visual characteristics of specific human faces. The *Conspec*-*Conlern* dual processes may also function for the perception

of BM because the preference for BM appears very early in 2-day-old babies (Simion et al., 2008). The induction of the BM preference could act as a *Conspec* process based on core knowledge (Spelke, 2000; Spelke and Kinzler, 2007), which lead neonates (human babies and chicks) to form adaptive social attachments. For comprehensive reviews, see Vallortigara (2012), Rosa-Salva et al. (2015) and Di Giorgio et al. (2017).

Regarding the function of BM preference in imprinting, however, this scenario has some caveats because of several reasons. First, in most studies so far, conclusions were drawn from comparisons among groups of chicks trained by different visual stimuli, but each chick was trained by a single stimulus. This may not be appropriate, because hatchlings in nature are exposed to many moving objects, and subsequently learn to follow one, most preferably the mother hen. We must analyse behavioural consequences after training by simultaneously presented multiple visual stimuli. Furthermore, when we compare groups of chicks, each imprinted to one stimulus, different imprinting scores among groups could be due to different level of arousal or attention, rather than different predisposition of learning. On the other hand, as simultaneous display made chicks attend to the two visual stimuli equally, different preference scores would specifically represent a different degree of learning.

Second, chicks were often tested shortly after imprinting. The BM preference quickly arises in the early post-hatch period (Miura and Matsushima, 2012), but it may not last long enough to maintain subsequent imprinting selectively to biological objects. Third, components of the imprinting memory are not yet fully specified. Colour and shape cues have been identified at the level of neuronal representations in responsible telencephalic regions (IMM: Horn, 1998; Horn, 2004; visual Wulst: Maekawa et al. 2006 for the colour), but the motion cue has not been specified as a critical sub-modality component of the memory; the responsible brain regions involved in the motion cues are also not identified.

In the present study, we examined the following two hypotheses. First, we hypothesized that (1) *the BM pattern would enhance the memorised preference for the associated colour.* Alternatively, chicks could form a learned attachment not solely on the colour cue, but on the association of motion and colour. Chicks might have a biased preference for a specific motion-colour association. Second, (2) *the induced BM preference would last long enough to control subsequent imprinting.* A stronger

imprinting to BM animation could thus emerge when chicks had been pre-induced for a BM preference. Alternatively, the induced BM preference rapidly decays and does not control the subsequent imprinting.

Experiments-1 to -4 were designed to examine the hypothesis (1), and experiments-5 and -6 the hypothesis (2). Experimental procedures, predictions, results and conclusions will be separately described in each section.

GENERAL METHODS

Compliance with Ethical Standard

Experiments were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University (approval number 18-0093). The guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals; after partial amendment No. 68, 2005). After the experiments, the chicks were euthanised by carbon dioxide.

Subjects

Domestic white Leghorn chicks (*Gallus gallus domesticus*, egg-laying strain called as “Julia”) were used. Training started at 15–24 h post-hatch, and it was denoted as Day-1. Fertilised eggs supplied from a local hatchery (Iwamura Co., Hokkaido, Japan) were incubated in the laboratory by using type P-008B incubators (Showa Furanki Co., Saitama, Japan) with its temperature controlled at 37.7°C and the humidity at ~80%. The inside of the incubator was kept in complete darkness until hatch. Chicks were individually hatched in small boxes separated by black plastic walls, so that they could interact acoustically but not visually. To avoid post-hatch visual experiences, hatchlings were individually housed in boxes placed in another incubator of the same type, kept in darkness until the experiment. Chicks were sexed just before the experiment.

In each batch of hatchlings, subjects were pseudo-randomly assigned to groups, until the number of accepted subjects reached n=10 in each group. Male and females were pseudo-randomly assigned to groups in a balanced manner (1:1) in experiment-3 to -6, but not in experiment-1 and -2. In one group of experiment-1, the number of chicks was n=9 by our mistake but we did not add another subject. In experiment-5, the number of chicks was n=11 in one group and we added one more subject in the other group. When chicks did not run at all during both two training sessions on Day-1, these

chicks were discarded and not tested. If, on the other hand, the chick run and hit the sensor once or more during the training sessions, it was accepted and tested. A total of 183 chicks were used, and the present paper is based on 171 accepted chicks (79 males and 92 females); see supplementary_data_file_#3 for the sex and the number of subjects in experiments-1 to -6.

Apparatus and General procedures

We used an I-shaped maze (9 cm × 70 cm) equipped with a 50-cm-long treadmill consisting of a rubber belt at the centre and an LCD monitor at each end (Fig. 1). The apparatus was the same as the one we used in a previous study (Miura et al., 2018). During imprinting, an infrared sensor and a transparent Plexiglass partition were placed at a point 10 cm from one monitor, and the other monitor was occluded by an opaque partition. When chicks ran and hit the sensor, the rubber belt of the treadmill moved for a short period of 0.3 s, drawing the chick backward by about 30 cm at a time. The treadmill motions were digitally counted, and the number of approaches was recorded for each trial.

At tests, the partitions were removed, and the treadmill was turned off. The subject chick was enclosed in a start box placed at the centre of the treadmill for about 10 s and was then allowed to freely exit and choose between the two arms. We recorded the total stay time near each monitor for 5 min, starting from the point when the chick exited the starting box. Each test was repeated twice with inter-test intervals of 1 min, after the sides of test animations were switched. The behaviour of the subject chick was monitored through a CCD camera (250 kilo pixels) placed on the ceiling, and the videos were stored for offline analysis in a video recorder (DCR-SR60, Sony, Japan). The apparatus was placed in a sound-proof wooden box, and the inside of the box was illuminated by infrared LED lamps.

Two sessions of training (each for 1 hour) were repeated with a 1-hour break interval, during which chicks were individually housed in boxes in a dark incubator. For testing the colour preference, chicks were presented with full-screen plates (background colour test, *i.e.*, overall screen was in yellow or red) twice for 5 min each. We measured the total period in which the subject chick stayed on either arm of the test maze, indicated by dashed lines in Fig. 1. The stay time on one side was subtracted by that on the other side, and the difference provided the preference score of +600 s (most

preferred) to -600 s (least preferred). Further details of the procedures will be described separately for each experiment.

Point-light animations and video clips displayed on LCD monitors

Animations and video clips are summarized in Supplementary_document_#1. Four types of point-light animations (each composed of 13 identical light points) were used after combining different motions (Walking or Linear) and colours (red or yellow), namely Wp (red), Wp (yellow), Lp (red), and Lp (yellow). Here, W and L abbreviate Walking and Linear motion, respectively. The suffix p indicates that the animation was composed of light points. In experiment-5, in order to test BM preference, we used two animations Wp (white) and Lp (white) that were composed of white light points. In experiment-2 and -3, two animations were simultaneously presented in combinations; Wp (red) and Lp (yellow), Wp (yellow) and Lp (red) in experiment-2; Wp (red) and Wp (yellow), Lp (red) and Lp (yellow) in experiment-3. The walking directions were alternately switched from one scene to the next.

These animations were made from a video clip of a walking chicken as in our previous study (Miura and Matsushima, 2016). In experiment-4, the original video clip (in black and white) was used after changing the colour (in red or yellow), either the whole body or its parts (head or tail).

Animations and video clips were displayed on black background at a speed of 30 frames/s. We made editing by Adobe Premiere (Elements 7) and the colour was set either to red (R: 255, G: 0, B: 0) or yellow (R: 255, G: 255, B: 0). These stimuli were displayed on the LCD monitors (size 10.4”, 800 × 600 pixels, Logitec LCM-T102AS, Japan; flash rate: 56–75 Hz, brightness: 230 cd/m^2, pitch size: 0.264 × 0.264 mm) using free viewer software (A-player, version 6.0) on a Windows PC. The width of the presentation was set at 9 cm on the monitor.

Statistical analysis

In each group, binary choice data were examined for the difference from the chance level by using one-sample *t*-tests without adjusting p-value by Bonferroni correction. Prior to comparisons among groups, we applied Levene’s test to assess the inequality of variances in each experiment. When Levene’s test failed to detect a significant difference in variance among groups, we applied parametric methods: two-sample t-tests, one-way or two-way ANOVA with or without repeated measures. When a

significant difference was detected by ANOVA, if necessary, we made post-hoc multiple comparisons by using Tukey's multiple comparison of means, or Dunnett's test depending on the design of the experiment. When parametric tests were not applicable, non-parametric methods were used instead; Wilcoxon rank sum test (i.e., Mann-Whitney's U-test), Wilcoxon's signed rank test, or Steel–Dwass's multiple comparisons depending on the design.

To examine the correlation between two behavioural scores obtained from a group of chicks, we used Pearson's product-moment correlation coefficient. Because the number of individuals in a group was small (n=9-11 chicks), we did not adopt the method of converting the computed correlation coefficients to z-values to compare. Instead, to compare the degree of correlation between two groups, we constructed generalized linear models (GLMs) and evaluated them in terms of AIC (Akaike Information Criteria).

We used R (version 3.4.1) and EZR on R commander (version 1.36). The significance level was set at $p = 0.05$. When comparisons were repeated in one experiment, the significance was judged after Bonferroni correction of the p-value. Results of these statistical calculations (number of males and females in each group, results of one-sample t-test and Levene's test of variance equality, and constructions of generalized linear models (GLMs) are summarized in Supplementary_document_#2. Behavioural data are shown in Supplementary_data_file_#3.

RESULTS

Experiment-1: Comparisons of imprinting using single point-light animations

Procedures and Predictions

We examined if BM pattern would enhance the learning of associated colour. Four groups of chicks were examined in experiment-1. In training, chicks were exposed to one type of point-light animation composed of different motions (Walking or Linear in light points, abbreviated as Wp or Lp) and colours (red or yellow) displayed on the training screen (left LCD monitor) (Fig. 1). Notice that the Wp possessed the BM feature, whereas the Lp did not. At 0.5 hour after the end of training, the trained chicks received binary choice tests using full-screen presentation of colours (red or yellow) on the two testing screens (left and right LCD monitors), both without any shape or motion

cues. If the hypothesis (1) was correct, chicks would consistently form an attachment to the colour that was associated with Wp but not Lp.

Chicks were further tested for their preference for the familiar point-light animation over the unfamiliar one, which was made of different motion and different colour. For example, if trained by Wp (red), the unfamiliar animation was Lp (yellow). If the alternative of the hypothesis (1) was correct, chicks would consistently form an attachment to the Wp point-light animation over the Lp.

Results and Discussion

As shown in the left column of Fig. 2, the colour test scores (as the yellow-side stay time subtracted by the red-side) were neutral or positive (indicative of yellow preference) in all 4 groups. Results of t-test against chance level (i.e., preference score = 0) are shown by asterisks (or ns) on the box-whisker plots in this and the following figures; the statistical data are summarized in supplementary_document_#2. Two-way ANOVA revealed a significant main effect of motion (Wp or Lp; $F=6.4483$, $df=1$, $p=0.0157$), but no main effect of colour (red or yellow; $F=0.7657$, $df=1$, $p=0.3875$) nor interaction ($F=3.754$, $df=1$, $p=0.0607$) appeared. This result does not support the hypothesis (1), which predicted that the colour test scores (i.e., yellow preference over red) would be negative in Wp (red) but positive in Wp (yellow), and a significant effect should have emerged on the interaction.

The right column of Fig. 2 shows the memory test scores (as the familiar-side stay time subtracted by the unfamiliar-side). Two-way ANOVA revealed a significant main effect of motion (Wp or Lp; $F=17.5842$, $df=1$, $p=0.0001$), but no main effect of colour (red or yellow; $F=2.7794$, $df=1$, $p=0.1044$) nor interaction ($F=1.9304$, $df=1$, $p=0.1734$) appeared. This result supports the alternative to the hypothesis (1), that chicks form learned preference for the BM motion associated with the colour. In the following experiment-2 and -3, we further examined this possibility by simultaneous presentation training.

Experiment-2 and -3: Comparisons of imprinting using simultaneously presented two point-light animations

Procedures and Predictions

We examined if a BM pattern would enhance the learning in an unbiased manner. Two groups of chicks were examined in each experiment. In training, chicks were exposed to two animations simultaneously displayed on the training screen. These animations were composed of different motion-colour associations. Different preference scores in test should represent different levels of learning, rather than different levels of attention or arousal.

In experiment-2, group-1 was trained by Lp (yellow) and Wp (red), and group-2 by Wp (yellow) and Lp (red) (Fig.3). If the hypothesis (1) was correct, chicks would consistently prefer the colour associated with Wp, namely red in group-1 and yellow in group-2 in the colour test. In experiment-3, group-1 was trained by Wp (yellow) and Wp (red), and group-2 was trained by Lp (yellow) and Lp (red) (Fig. 4). If the hypothesis (1) was correct, chicks would not show a preference at test.

Chicks were further tested for their preference between the two animations used in training. Notice that chicks were familiarized to both animations. If the alternative of the hypothesis (1) was correct, chicks would consistently prefer the Wp animation in experiment-2 irrespectively of the associated colour, but no such preference would emerge in experiment-3. On the other hand, if the corollary of the alternative was correct, chicks would show a biased preference for a specific association of motion and colour, consistently in both experiment-2 and -3.

Results and Discussion

Results of experiment-2 are shown in Fig. 3. As in the left column, the colour test scores were positive (indicative of yellow preference) in both groups, and a two-sample *t*-test failed to reveal a significant difference between them ($df=18$, $t=0.375$, $p=0.7120$). Again, the data do not support the hypothesis (1), which predicted that scores would be negative in group-1 and positive in group-2 with a significant difference between them. The right column of Fig. 3 shows the memory test scores (the yellow-side stay time subtracted by the red-side). A two-sample t-test revealed a significant difference between the groups ($t=4.4013$, $df=18$, $p=0.0003$); chicks preferred Wp if associated with red, but not with yellow.

Results of experiment-3 are shown in Fig. 4. The colour test scores (left column) were positive (indicative of yellow preference), and a two-sample t-test failed to reveal a significant difference between them ($df=18$, $t=0.0322$, $p=0.9746$). The hypothesis (1)

was not supported. The memory test scores (right column) indicated red preference in group-1, and a two-sample t-test revealed a significant difference between the groups (df=18, t=2.6286, p=0.0170). Taking experiment-2 and -3 together, chicks formed a preference for Wp (biological motion pattern) if associated with red, despite their consistent yellow preference in the colour tests.

Experiment-4: Comparisons of imprinting using simultaneously presented realistic chicken videos in different colours

Procedures and Predictions

Biased preference for the walking light-points in red (Wp (red)) emerged consistently in experiment-2 and -3. We examined if a similar preference bias could occur when more naturalistic videos of real chicken were used. Four groups of chicks were examined in experiment-4 (Fig. 5). In training, chicks were exposed to a pair of videos simultaneously displayed on the training screen. These pairs were of the same motion type (Walking motion or Linear movement of a still image, denoted as W or L, respectively), but different in colours (red or yellow) as in experiment-3. In 3 pairs of the Walking videos, the whole body, the head or the tail was coloured. In 1 pair of the Linear videos, only the head was coloured.

If the hypothesis (1) was correct, colour preference would not emerge. Alternatively, if a biased preference for a specific motion-colour association occurred, chicks would prefer the W-x (red) video over the W-x (yellow) in all of the 3 groups (x = whole, head, or tail), but no such bias would appear for the L-head (red) video over the L-head (yellow). The coloured portion of the body could also be critical, as suggested by the configurational predisposition to the head-neck regions of the jungle fowl (Johnson and Horn 1988). Furthermore, the head motion had a bigger change in speed than the tail. Considering the predisposed preference for the changing speed (Rosa-Salva et al. 2016), W-head in red could be a stronger imprinting stimulus than W-head in yellow, whereas less or no preference could emerge in the group trained by W-tail.

Results and Discussion

As shown in the left column of Fig. 6, the colour test scores were neutral or positive (indicative of yellow preference) in 4 groups. A one-way ANOVA revealed no significance ($F=0.4976$, $df=3$, $p=0.6863$); the hypothesis (1) was not supported.

The memory test scores (right column; the yellow-side stay time subtracted by the red) revealed differences among groups. As parametric test was not applicable, Steel-Dwass multiple comparisons were adopted for every pair out of the 4 groups. We detected significant difference between group-1 vs each of groups-2, -3, -4; group-1 vs -2 ($t=3.1039$, $p=0.0103$), group-1 vs -3 ($t=3.6339$, $p=0.0015$), group-1 vs -4 ($t=3.6339$, $p=0.0015$). Although the difference was not significant in the other 3 pairwise comparisons (group-2 vs -3: $t=2.1166$, $p=0.1477$; group-2 vs -4: $t=1.8898$, $p=0.2323$; group-3 vs -4 $t=0.1890$, $p=0.9976$), "*red head*" in biological motion (group-2) tended to show higher scores than that in non-biological linear motion (group-3), but "*red tail*" did not (group-4). Chicks thus showed a clear bias toward "*biological motion in red*".

The present result contrasts with those obtained so far by using artefact (coloured toys) as training and tests (Izawa et al. 2001; Yamaguchi et al. 2012, 2018), where comparable scores were obtained irrespectively of whether chicks were trained by a red toy or a yellow alternative. Although further systematic surveys are necessary, it is possible to assume that the red bias appears only when associated with visual stimuli that bears BM. The predisposed colour bias to red, if any, could strongly depend on the way the colour is presented.

Experiment-5: BM preference induced by non-BM visual stimulus

Procedures and Predictions

To methodologically validate the following experiment-6, we examined the time course of the BM preference induction. Two groups of chicks were compared in experiment-5. On Day-1, chicks were trained by a video of a moving toy (group-1) or stationary toy (group-2) both coloured in red (Fig.7). The trained chicks were tested twice, first at 0.5 hour (Day-1) and second at ~24 hour (Day-2) after the end of training. Each test consisted of a BM preference test and a memory test. The BM preference was tested with Wp (white) vs Lp (white), and the imprinting memory was tested with a moving red toy (familiar) vs a moving yellow toy (unfamiliar).

If the hypothesis (2) was correct, group-1 chicks would consistently show a BM preference in both Day-1 and Day-2, whereas group-2 chicks would not show a BM preference. As argued in the Introduction, the BM preference might increase or decrease on Day-2, but we would anyway find a difference between the two groups. In the memory tests, group-1 chicks would show higher scores than group-2 on Day-1, but the difference between the two groups could be smaller on Day-2, if the imprinting memory of the artefact decayed. Furthermore, if the BM preference was linked with the imprinting memory formation at the individual level, we would find a significant correlation between the two scores in group-1 chicks.

Results and Discussion

If induced on Day-1, the BM preference lasted until Day-2 (Fig. 8(a) (b)). A two-way ANOVA with a repeated measure revealed a significant main effect of the group factor ($F=55.3711$, $df=1$, $p<0.0001$), but not of the Day factor ($F=2.2272$, $df=1$, $p=0.1660$) nor the interaction ($F=0.0769$, $df=1$, $p=0.7876$). This result supports the first part of the hypothesis (2) that the induced BM preference lasts for at least ~24 hours with no signs of increase or decrease.

On the other hand, the memory scores decayed on Day-2 when compared with Day-1 (Fig. 8(c) (d)). A two-way ANOVA with a repeated measure revealed a significant main effect of the group factor ($F=59.9201$, $df=1$, $p<0.0001$) and the Day factor ($F=19.5100$, $df=1$, $p=0.0010$) without significant interaction ($F=1.9398$, $df=1$, $p=0.1941$).

Between the memory score and the BM preference, a significant correlation was not detected in any of the 4 plots (Fig. 8 (e) (f)); group (1)/Day-1 ($t=-0.82946$, $df=9$, $p=0.4283$); group (1)/Day-2 ($t=1.0856$, $df=9$, $p=0.3059$); group (2)/Day-1($t=2.0232$, $df=9$, $p=0.07374$); group-2/Day-2 ($t=0.6356$, $df=9$, $p=0.5409$), after Bonferroni correction of p-value ($=0.05/4$, namely $p=0.0125$). At the individual level, the BM preference is not supposed to predict the memory score.

Taken together, the lasting nature of the induced BM preference validated the procedure of the experiment-6. Imprinting memory of the red toy was also not predictable by the individual BM preference.

Experiment 6: Effects of pre-induction of the BM preference on subsequent imprinting

Procedures and Predictions

To know if a pre-induced BM preference makes chicks specifically learn the coloured animation associated with BM, 3 groups of chicks were examined in experiment-6. Considering the lasting BM preference (experiment-5), we tested whether those chicks with pre-induced BM preference on Day-1 could show enhanced scores in imprinting on Day-2 (Fig. 9). For the Day-2 training, we presented chicks with simultaneously displayed Wp (yellow) and Lp (red), which gave rise to no significant preference in group-2 of experiment-2 (memory test in Fig. 3).

On Day-1, group-1 and -2 were trained by a moving toy (red), whereas group-3 was trained by a stationary toy (red). Chicks were tested for BM preference at 0.5 hour after the end of training. On Day-2, group-1 and -3 chicks received imprinting training, but group-2 chicks were not trained but kept in darkness. Chicks were subsequently tested for recent memory and old memory at 0.5 hour after the training.

If the hypothesis (2) was correct, group-1 chicks would show higher scores in the recent memory test (on Day-2) than group-3 chicks. However, since the recent memory test is accomplished by a binary choice between a BM stimulus (Wp (yellow)) and a non-BM stimulus (Lp (red)), the observed preference could represent the pre-induced and lasting BM preference. Group-2 was thus added as a control to group-1. We predicted that group-1 would show higher scores in the recent memory test than group-2. Or the recent memory scores of group-2 would have a high level of correlation with the individual BM scores compared with group-1.

In addition, we compared the old memory scores in Day-2 among the 3 groups. If newly-formed imprinting memory (on Day-2) interfered with the retention of old memory (on Day-1), group-2 could have higher scores than group-1.

Results and Discussion

BM preference scores on Day-1 were compared between group-1 and -2, and independently between group-1 and -3 (Fig. 10(a)). Pairwise multiple comparisons (Dunnett's test) showed a marginal but significant difference between group-1 and -3 ($t=2.357$, $p=0.0476$). On the other hand, no significant difference occurred between

group-1 and -2 (t=0.821, p=0.6285), allowing us to compare the Day-2 data between these two.

Recent memory scores on Day-2 were compared between group-1 and -2, and between group-1 and -3. Pairwise multiple comparisons (Dunnett's test) showed a significant difference between group-1 and -3 (t=3.231, p=0.0061). Group-1 chicks tended to show a higher score than group-2, but the difference was not significant (t=1.692, p=0.1774).

We made a post-hoc comparison between group-1 of experiment-6 and group-2 of experiment-2. A two-sample t-test revealed a significant difference (t=2.892, p=0.0097). Both groups were trained by simultaneous presentation of Wp (yellow) and Lp (red), and the difference was whether BM preference was pre-induced or not. BM preference was therefore strong enough to override the bias, making chicks prefer Wp (yellow) to Lp (red).

Correlation between the BM preference and the recent memory score was calculated for group-1 (c), -2 (d) and -3 (e) after Bonferroni correction of p-value for the repetition (p=0.05/3, namely p=0.0166). Peason's product-moment coefficient was significant in group-2 (t=3.711, df=8, p=0.0059), but not in group-1 (t=0.7233, df=8, p=0.1286) and group-3 (t=0.4030, df=8, p=0.1410).

To obtain reliable estimates of the contributing factors, we constructed 2 sets of generalized linear models (GLMs) for the recent memory scores, one set for the merged data of group-1 and -2, and another set for group-1 and -3. For each set, we constructed 8 models with all possible combinations of two variables including their interaction, namely the BM preference score and the training (group-1 and -2: Day-2 training or not; group-1 and -3: Day-1 training by moving or stationary toy). Details are shown in Supplementary_document_#2.

In the set (group-1 and -2), the best model (AIC=268.76) was composed of BM score (p=0.0408), group (p=0.0311) and the interaction (p=0.1104) as explanatory variables. The second-best model (AIC=270.05) did not include the interaction, and both coefficients of BM score (p=0.1670) and group (p=0.1350) were less significant. The AIC of the null model was 271.71. The Day-2 training thus contributed to the recent memory of group-2 in a manner stronger than that of group-1.

In the set (group-1 and -3), the best model (AIC=276.32) was composed only of group (p=0.0080) as explanatory variable, and the second-best model (AIC=277.97) was composed of BM score (p=0.0096) and group (p=0.030). The AIC of the null model was 282.33. The effect of the Day-1 training was reconfirmed.

The old memory test on Day-2 failed to reveal a significant difference between group-1 and -2 after pairwise multiple comparisons (Dunnett's test; t=0.3930, p=0.8941). The assumed interference of the old memory by the recent memory formation thus gained no experimental support.

GENERAL DISCUSSION

Induced BM preference functions as a Conspec process, which guides the subsequent imprinting toward selective memorisation of the BM stimulus despite the associated colour

Contrary to our initial prediction, the full-screen colour test of experiment-1 to -3 failed to show a consistent learned preference for the colour associated with BM point-light animation. On the other hand, chicks can show a preference for the imprinting stimuli, when tested by the motion pictures in its associated colour, at least for the BM imprinting stimuli. In experiment-1 (Fig.2), group-1 and -2 preferred Wp (BM), whereas group-3 and -4 did not prefer Lp (non-BM). In experiment-2 (Fig.3), when simultaneously trained by Wp (red) and Lp (yellow) (group-1), chicks preferred the former Wp (red). However, when the motion-colour association was swapped (group-2), chicks did not show a preference between Wp (yellow) and Lp (red). Wp is therefore not powerful if shown in yellow. Accordingly, in experiment-3 (Fig.4), group-1 preferred Wp (red) to Wp (yellow), but group-2 did not show a preference between Lp (red) and Lp (yellow). The first hypothesis (1) is thus rejected, and we conclude a corollary of the alternative idea that chicks form a biased preference for the BM in red.

Results of experiment-4 (right column, Fig.6) confirmed this conclusion, as a strong preference bias appeared for the red chicken walking over the yellow (group-1). Furthermore, the red head tended to be more powerful than the yellow head (group-2), but the preference disappeared in the linear motion (group-3) and the tail in colour (group-4). Our interpretation is that chicks have a predisposed preference for a walking chicken with a red crest on the head, or a red face ("*biological motion in red*"). We must

559 also note that the head movement was jerkier than the tail. The preference for the
560 walking head can be accounted for in terms of the predisposed preference for objects of
561 changing speeds as a visual cue of animacy (Rosa-Salva et al. 2016). Conversely, the
562 motionless colour plate test (left column, Fig.6) almost consistently revealed a yellow
563 preference in experiment-1 to -4. We are presently unable to explain why yellow was
564 preferred, but this bias could simply represent an innate preference for a brighter screen.
565 In order to understand the functional role played by the early induction of BM
566 preference, the "*biological motion in red*" bias was confounding because BM stimuli
567 did not necessarily caused a successful imprinting as in the case of group-2 of
568 experiment-2 (Fig. 3, memory test). In experiment-6, we therefore tried to separate the
569 Day-1 process (induction of the BM preference) and the Day-2 process (learning based
570 on the BM preference). The results (Fig. 10) showed that the induced BM preference
571 made chicks override the "*biological motion in red*", and chicks formed a learned
572 preference for Wp (yellow), guiding the subsequent imprinting toward selective
573 memorisation of the BM stimulus despite the associated colour.
574 Instead, the different results of experiment-2 (group-2) and -6 (group-1) might be
575 attributed to the other factors, for example, the different ages of the memory test (post-
576 hatch 15~24 h in experiment-2, and 39~48 h in experiment-6). We might assume that
577 the aged chicks showed an unconditional preference bias to Wp (yellow). However,
578 such a bias did not occur in group-2 of experiment-6 (no training on Day-2; central data
579 of Fig. 10(b)), and the preference bias was predicted by the induced BM preference
580 (Fig. 10(d)).
581 Experiment-5 was designed to methodologically validate experiment-6. As a pre-
582 requisite, we examined if the induced BM preference on Day-1 could survive until Day-
583 2, and it did as we had predicted. On the other hand, a decay in the memory test scores
584 (Fig. 8(c) and (d)) occurred. It cannot be ascribed to the repeated testing on Day-1 and -
585 2, because similarly low scores appeared in the old memory test of experiment-6, in
586 which chicks were tested only once (Fig. 10(f)).
587 The main results are summarised in Fig. 11(a). If the BM preference was not pre-
588 induced, the "*biological motion in red*" predisposition predominates. If pre-induced, this
589 predisposition was overridden and a preference for Wp (yellow) could be developed
590 through exposure to the combined imprinting stimuli in which BM was associated with

yellow. We may argue that a stronger *Conspec* mechanism (*i.e.*, the BM preference) leads to a clearer *Conlern* mechanism (*i.e.*, the preference for BM in yellow). However, at the individual level, we found no significant correlations between the BM preference (*Conspec*) and the recent memory scores (*Conlern*) in experiment-6 (Fig. 10(c)). The link between these two processes might not be direct nor strong.

It remains to be asked if other forms of motion preference could contribute beside the BM preference studied here. It has been reported that the visually naïve chicks show preference for a simple ball in proactive motion (self-propelled causal agency: Mascalzoni et al., 2010, Rosa-Salva et al., 2016; also see Lorenzi et al., 2017 for the involved brain areas). At present it remains unanswered as to if these forms of motion preference could share common neuro-cognitive bases.

As a take-home-message, we can summarise our findings by saying that imprinting is composed of dynamical processes in which predispositions and memory formation interact to develop adaptive social attachments, as opposed to the widely accepted view that imprinting is an irreversible memorization of the first-seen object that the chicks happened to encounter during a brief sensitive period after hatching.

Breakdown of imprinting and possible hormonal mechanisms linking Conspec with Conlern processes in chicks

The present study revealed the somewhat complicated nature of imprinting. The breakdown list of the recognition memory is summarised in Fig. 11(b). As Lorenz (1937) pointed out, “imprinting has a number of features which distinguish it fundamentally from a learning process” (as cited by Sluckin, 1973), and it holds true in many senses. Not only is imprinting free of reinforcement process (as well as being independent of the basal ganglia system for reward-value updating; Izawa et al., 2001) , the recognition memory is formed based on several different sensory sub-modalities such as colours, shapes, motions and configurations.

We must notice that we do not know whether the pre-induction of BM preference could improve the BM perception *per se*, as would be expected as a *Conspec* process. Through a consequent *Conlern* process, chicks would have an improved discrimination of objects by the motion cues. Naïve chicks do not discriminate between the mother hen and a possible predator like polecat (see Johnson and Horn 1988; Vallortigara et al.

2005), but chicks could do so after the BM induction. In human adults, capability to discriminate different motions by point-light animations is enhanced by acquiring the novel motor patterns that correspond to the animation without specific visual experience (Casile and Giese 2006). Similar post-imprinting development through behavioural execution might be assumed in chicks.

Induction of the BM preference is correlatively (Takemura et al., 2018) as well as causally (Miura et al., 2018) linked with increased gene expression of *Dio2*, which codes the type-2 deiodinase, an enzyme responsible for the conversion of thyroid hormone (T_4) to its active form (T_3) in the brain. The influx of T_3 would acutely activate the ongoing imprinting, and chronically prime the memory system so that the chicks remain imprintable for considerable days or a week afterwards (Yamaguchi et al., 2012). Cellular/molecular bases of the acute (activational) and chronic (organisational) effects of T_3 are yet largely unknown (see Aoki et al., 2015; Yamaguchi et al., 2018), but the neuro-hormonal actions would link the predisposed BM preference (*Conspec* mechanism) with the further memorisation of the BM object in colour (*Conlern* mechanism). As important topics in future studies, neuropharmacological manipulations could be designed to disconnect these functional links.

Imprinting gains resilience or developmental stability through functional links between the memory formation and the social predispositions

The present study makes us assume a resilient development of social cognition, as reported in the study of socially deprived rhesus monkeys (Harlow and Suomi, 1971). Even if the first-seen object after hatching was not the mother hen, chicks would soon develop a set of predispositions toward a "*biological motion in red*". This would act together with the prolongation of the sensitive period of learning (memory priming) through T_3. The pure colour preference of the initial imprinting object rapidly fades (even before the completion of the 2-hour training; Takemura et al., 2018) and is replaced by the formation of specific motion-colour memory, leading to adaptive social attachment.

In this context, recent findings from the chick model of autism spectrum disorder (ASD) is worthy of attention (Nishigori et al. 2013; Sgadò et al. 2018; Lorenzi et al.

2019). Embryonic exposure to valproic acid consistently impaired development of various types of social behaviours, whereas imprinting memory formation (using artificial objects or video images) remained intact. As the machineries underlying the social development, several neural nuclei (septum, hypothalamic preoptic area and amygdaloid nucleus) have been suggested (Mayer et al. 2017a, b; Lorenzi et al. 2017). Most of these candidate nuclei receive direct projections from the limbic part of arcopallium, and localized lesion to this part is shown to impair social facilitation of foraging behaviour (Xin et al. 2017). Furthermore, neurons in the arcopallium receive excitatory synaptic inputs from intermediate medial mesopallium (IMM; Csillag 1999), which has been known as the hub for imprinting memory formation (Horn 1998, 2004). The embryonic valproic acid treatment could selectively interfere with the development of the social neural network, while sparing the sensory learning mechanisms in IMM and IMHA (Aoki et al. 2015) intact. The link between the BM preference induction and the attachment formation found in this study could be broken down in these ASD model chicks.

It is surprising that chicks retain these adaptive neurocognitive processes for imprinting even after artificial breeding for thousands of generations under domestication. A recent report clearly shows that the preference is maintained in genetically isolated lines for a considerable period over 18 years (Versace et al., 2017a). Furthermore, chicks are equipped with faculties responsible for numerosity comprehension (Rugani et al., 2009, 2013, 2015), probability inference (Santolin et al., 2016), categorisation based on conceptual relationship (Martinho and Kacelnik, 2016; Versace et al. 2017b; also see Hogue et al. 1996 for the transitivity inference in female hens, and Daisley et al. 2010 for chicks) and spatial geometry (Pecchia and Vallortigara, 2010; Pecchia and Vallortigara, 2012; Lee et al., 2012). Intensive domestication could have weakened or erased these “cognitive” capabilities that are not explicitly beneficial in the animal husbandry.

The idea of modular organizations may assume that these distinct processes could independently evolve to meet different ecological needs. What if, on the contrary, these cognitive processes are mutually linked, as suggested by Versace et al. (2018), particularly during the early *Conspec* process? The physiologically linked relationships are unbreakable and make these apparently distinct processes act altogether, allowing

some of the cognitive phenotype to survive even in the absence of any favourable positive selection. Accordingly, exogenous application of T_3 also facilitated the learning reinforced by delayed rewarded, as it did the secondary imprinting (Yamaguchi et al., 2012), suggesting that the underlying cognitive flexibility is tightly associated with the early processes of imprinting. Further analytical surveys would reveal critical developmental linkages shared by these distinct domains of intelligent behaviours.

Supplementary materials

Supplementary_document_#1; list of point-light animations and videos

Supplementary_document_#2; statistical calculations

Supplementary_data_#3; behavioural data (excel file)

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924 **Figure legends**

925 **Fig. 1** Experiment-1, -2 and -3, apparatus and procedures. In training, the stimulus was
926 presented on an LCD monitor on one side, and the other side was occluded. When the
927 chick approached the monitor, an infra-red sensor detected it and triggered the treadmill
928 to withdraw the chick backward. At tests, the treadmill was turned off and chicks
929 walked between the two monitors that displayed different visual stimuli. The stay time
930 spent in each arm was measured during two sessions (300 sec each) with counter-
931 balanced side of presentation, and difference of the stay time represented the preference
932 score. Colour tests were immediately followed by memory tests. See text and following
933 figures for the abbreviations of visual stimuli such as Wp (red) and Lp (yellow).

Fig. 2 Experiment-1, results. Four groups of chicks (n=9 or 10) were compared. In training, chicks were exposed to one of 4 types of animations with different motion patterns (Walking (W) for BM and Linear (L) for non-BM) and colours (red and yellow). The suffix (p) denotes that the animation was composed of light points on dark background screen. In this and the following figures (Fig. 3, 4, 6), results of the colour test (left column) and the memory test (left column) are shown in box-whisker plots (median with upper/lower quadrants and maximum/minimum range), and dots indicate individuals. Asterisks placed on the boxes indicate the significant difference against the chance level revealed by one-sample t-test; *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), ns (not significant). For the inter-group comparisons, see text and Supplementary_document_#2.

Fig. 3 Experiment-2, results. Two groups of chicks (each n=10) were compared. In training, chicks were exposed simultaneously to two of the 4 types of aminations used in experiment-1; Wp (red) and Lp (yellow) in group-1, Wp (yellow) and Lp (red) in group-2.

Fig. 4 Experiment-3, results. Two groups of chicks (each n=10) were compared. In training, chicks were exposed simultaneously to two of the 4 types of aminations in a manner different from experiment-2; Wp (red) and Wp (yellow) in group-1, Lp (yellow) and Lp (red) in group-2.

Fig. 5 Experiment-4, procedures. Walking (W) represents a BM motion, and Linear (L) a non-BM motion. Suffixes (whole, head and tail) denote that the whole body, the head or the tail was coloured. For example, W-whole (red) indicates a Walking chicken video with its whole body coloured in red. Test procedure is the same as experiment-1 to -3. Note that the point-light animations used in experiment-1 to -3 were composed based on the video clip of walking chicken used in experiment-4.

Fig. 6 Experiment-4, results. Biased imprintability of the red animation over the yellow, when chicks were trained by a walking motion pattern, and when the whole or the head region was coloured. Four groups of chicks (each n = 10) were compared.

Fig. 7 Experiment-5, procedures. Chicks were trained either with Moving toy or Stationary toy (both in red) and tested twice at 0.5 h (Day-1) and at ~24 h (Day-2). The first test was for the BM preference (Wp (while) vs. Lp (white)), and second test for the memory (familiar red Toy vs. unfamiliar yellow Toy).

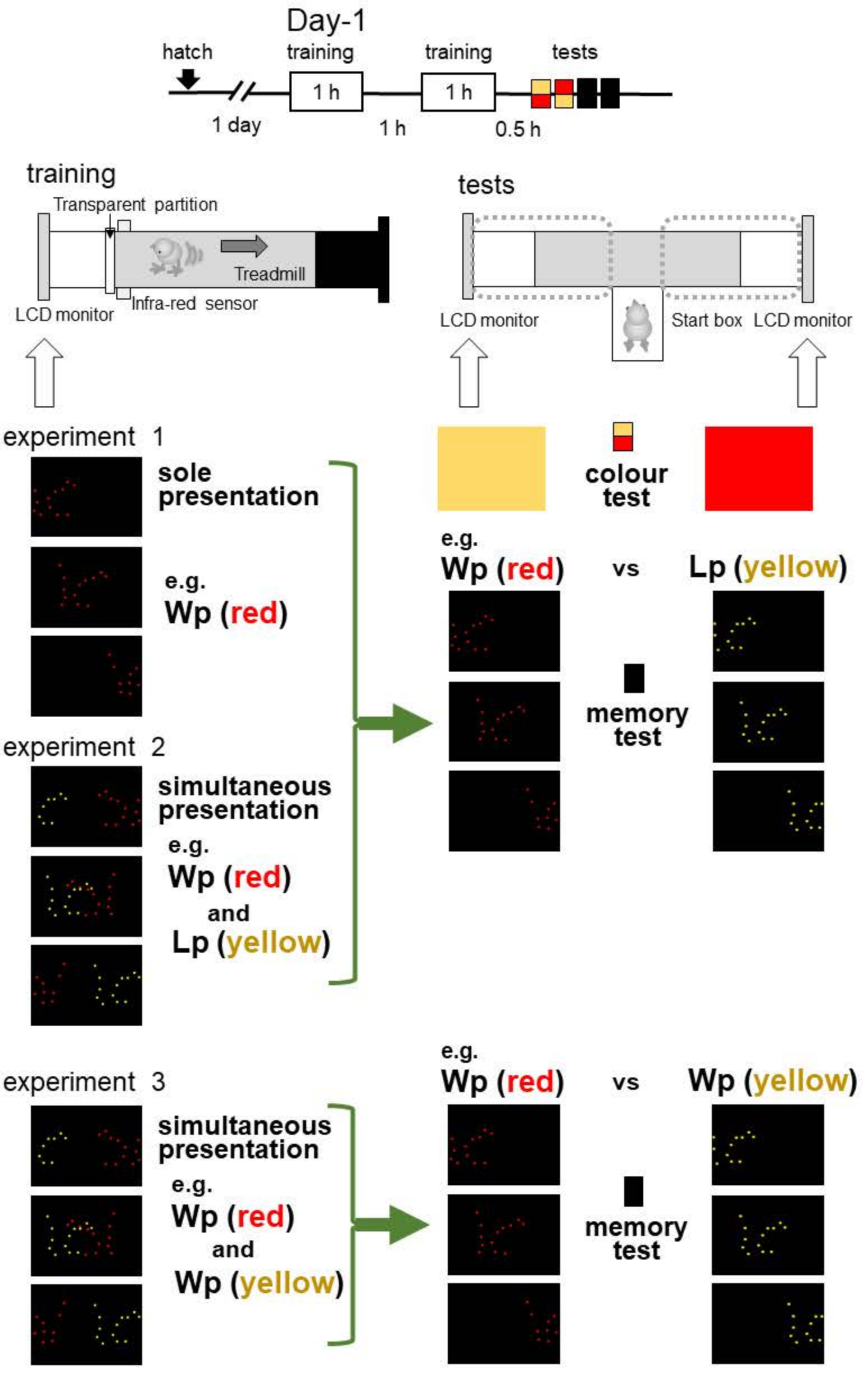
Fig. 8 Experiment-5, results. Data obtained from 2 groups of chicks (group-1 and -2) with different training on Day-1 (Moving toy and Stationary toy, respectively). The Day-1 (open circles) and Day-2 (filled circles) test scores are connected by dashed lines in (a) to (d). In contrast to the stable BM preference (a, b), the memory test revealed decaying scores (c, d) in both groups. Memory scores (y-axis) plotted against individual BM preference scores (x-axis) failed to reveal significant correlations in all 4 sets of data (e, f).

Fig. 9 Experiment-6, procedures. On Day-1, chicks were trained with a moving toy (red) in group-1 and -2, and a stationary toy (red) in group-3. These chicks were tested for the BM preference at 0.5 hour after the end of training. On Day-2, group-1 and -3 chicks were further trained by simultaneous presentation of Lp (red) and Wp (yellow) animations; group-2 chicks were not trained and kept in darkness. The 3 groups of chicks were then tested for the recent memory with point-light animations, and for the old memory with toys.

Fig. 10 Experiment-5, results. Test scores obtained from 3 groups of chicks (group-1, -2, and -3) with different training on Day-1 and Day-2 are shown; (a) BM preference on Day-1, (b) recent memory test and (f) old memory test on Day-2. In (c) to (e), the recent memory test score (as y-axis) was plotted against the individual BM preference score (as x-axis); a significant positive correlation appeared in group-2 (d) but not in -1 (c) and -3 (e) after Bonferroni correction of p-values.

Fig. 11 Graphical summary. (a) Results are compared between experiment-2 and -6. In experiment-2, naïve chicks trained with Wp (yellow) and Lp (red) showed no preference (group-2). In experiment-6, if the BM preference was pre-induced, chicks showed a preference for Wp (yellow) after the same training (group-1). If not pre-induced, no preference emerged (group-3). (b) Possible scenario upon breakdown of the recognition memory of imprinting.

EXPERIMENT 1, 2, 3



EXPERIMENT 1

training **colour test** **memory test**

(a) **group-1**

Wp (red)

(b) **group-2**

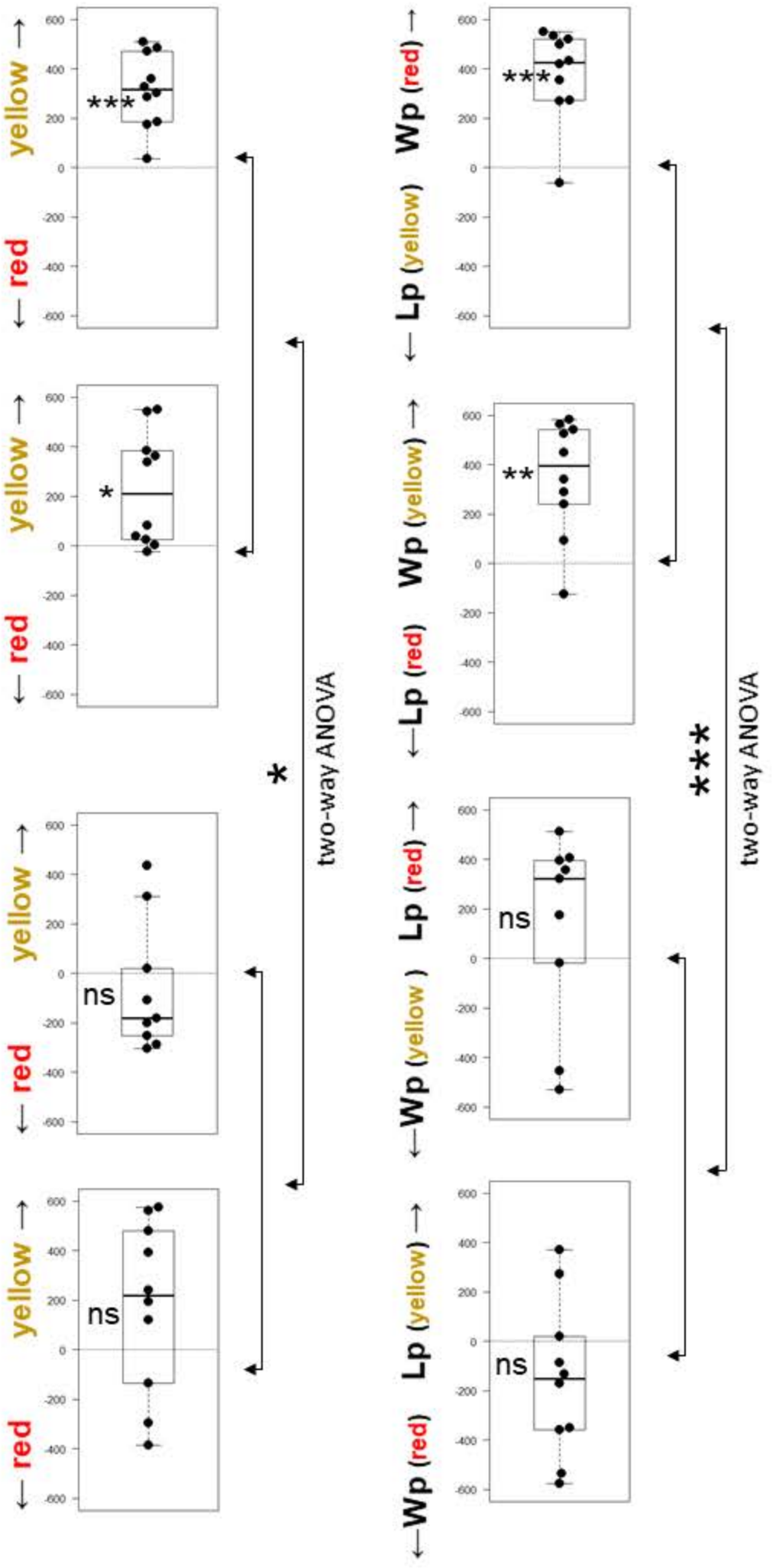
Wp (yellow)

(c) **group-3**

Lp (red)

(d) **group-4**

Lp (yellow)



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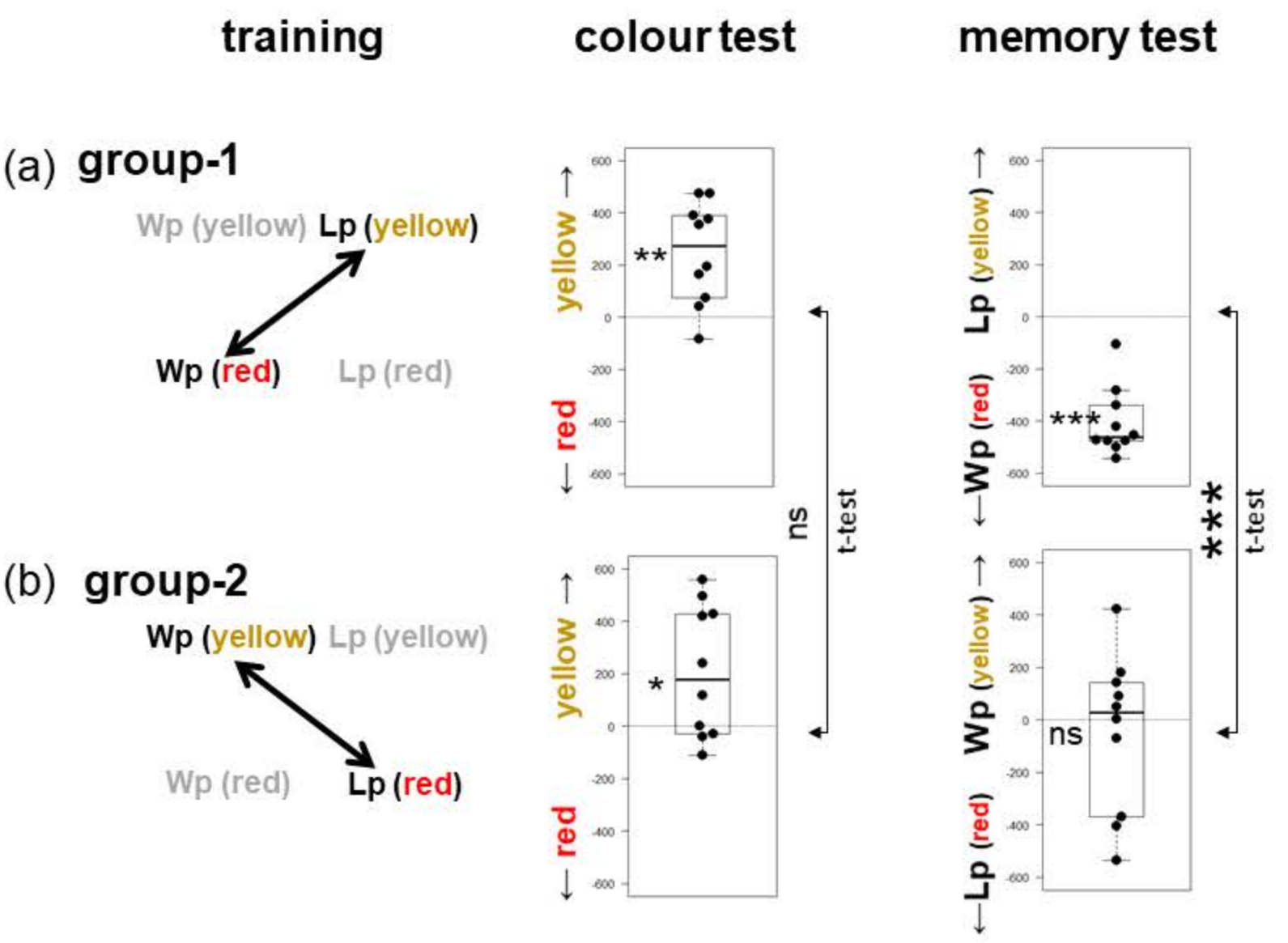

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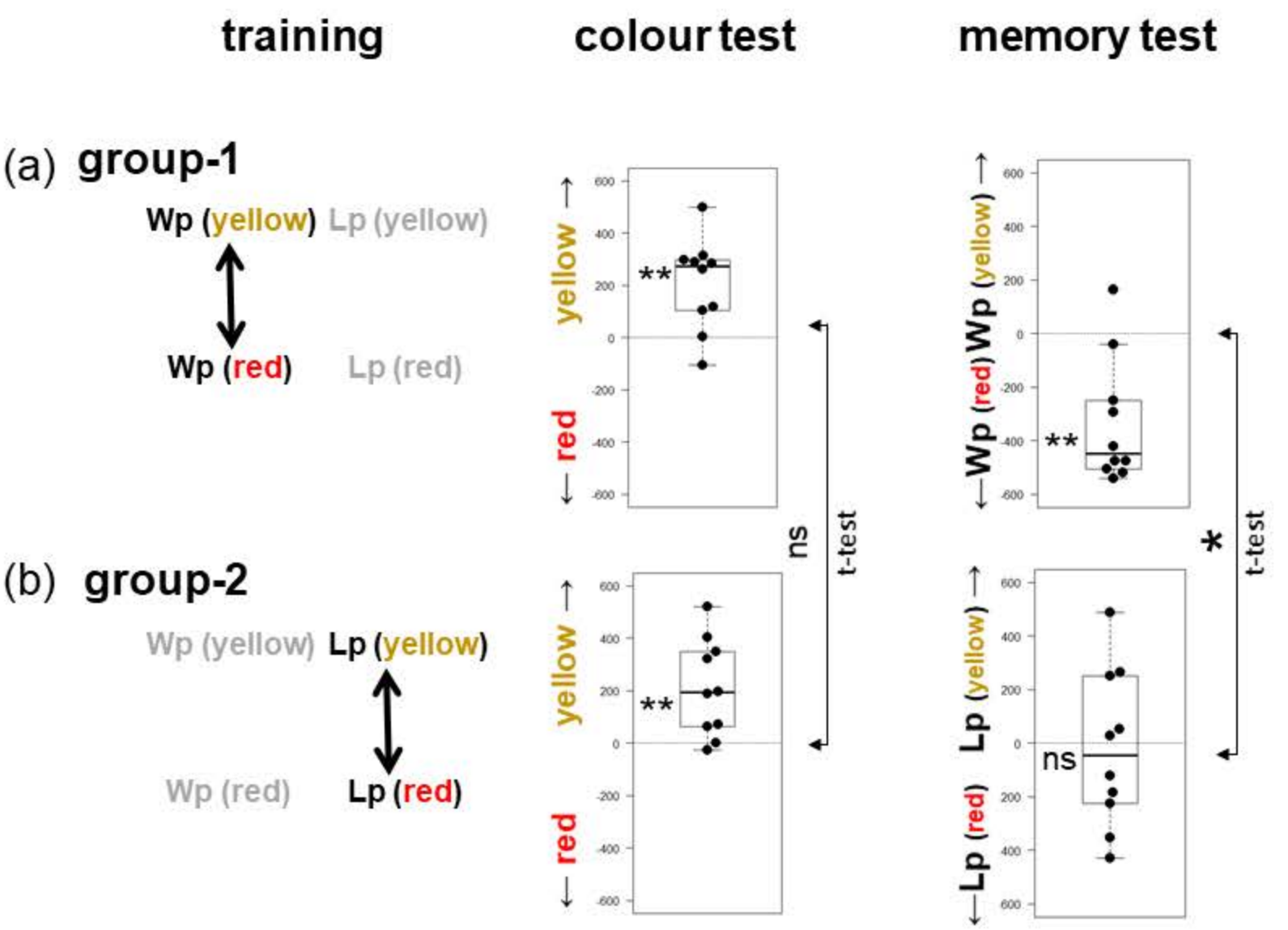

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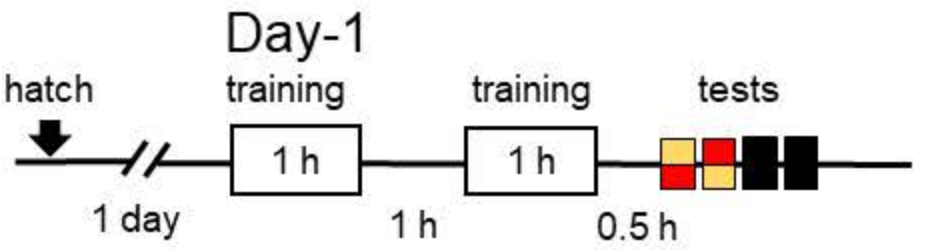

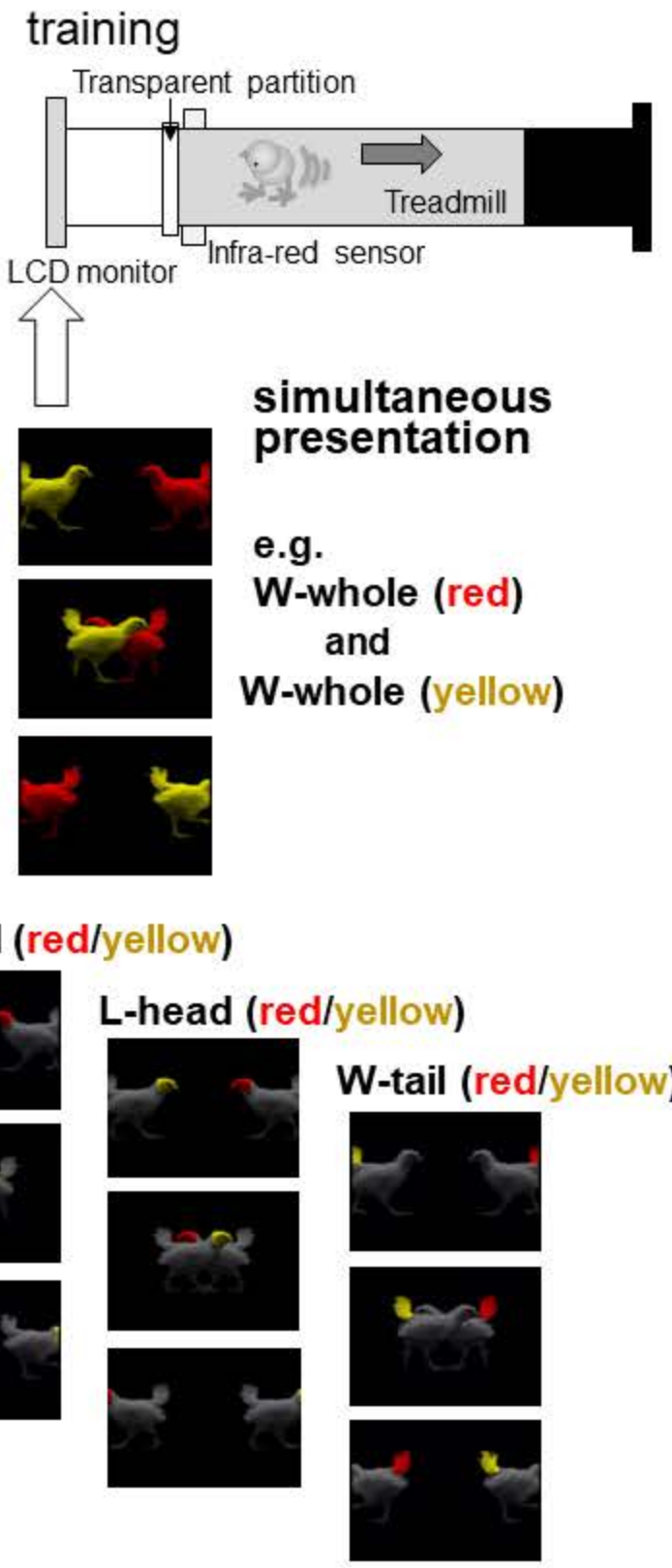

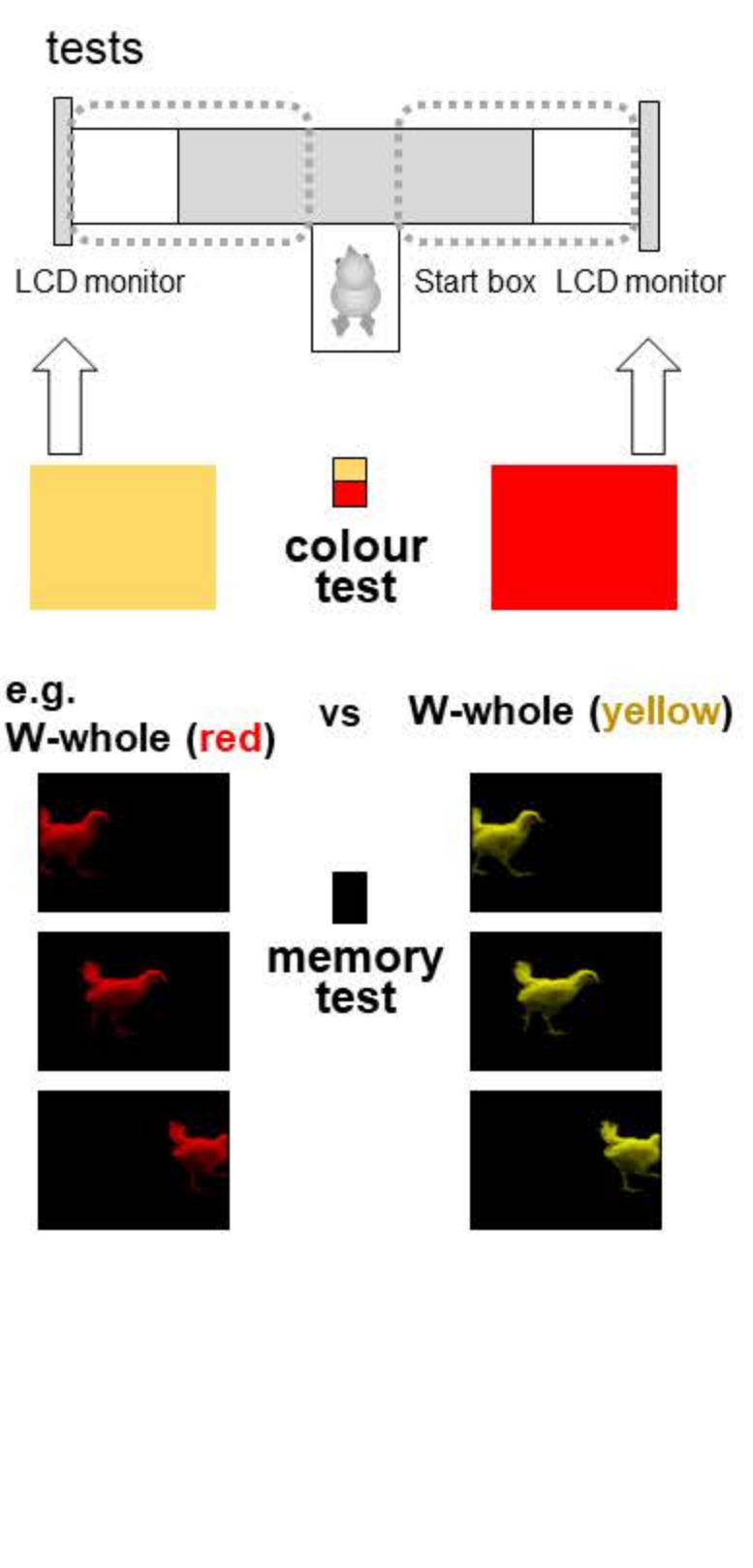

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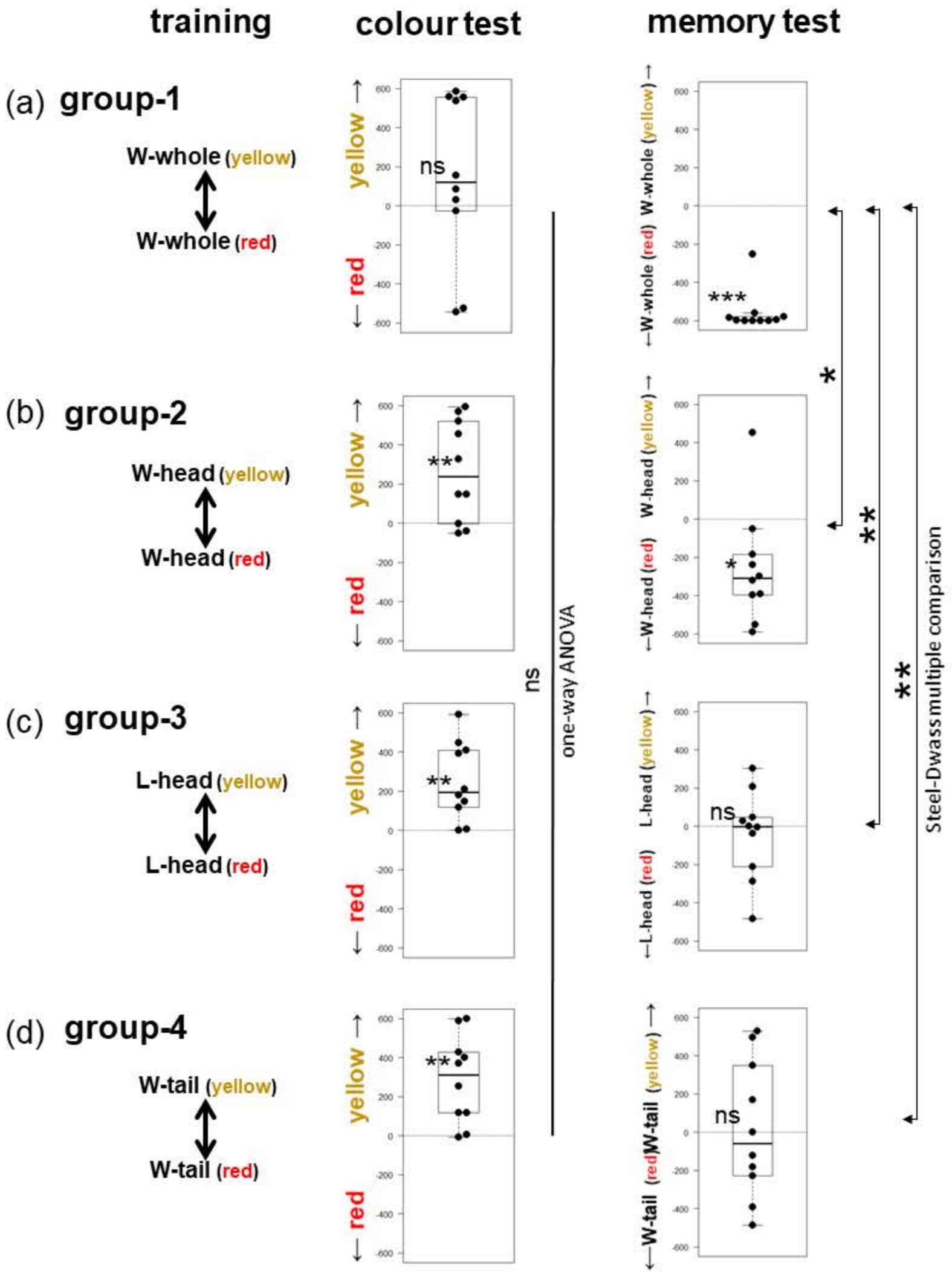
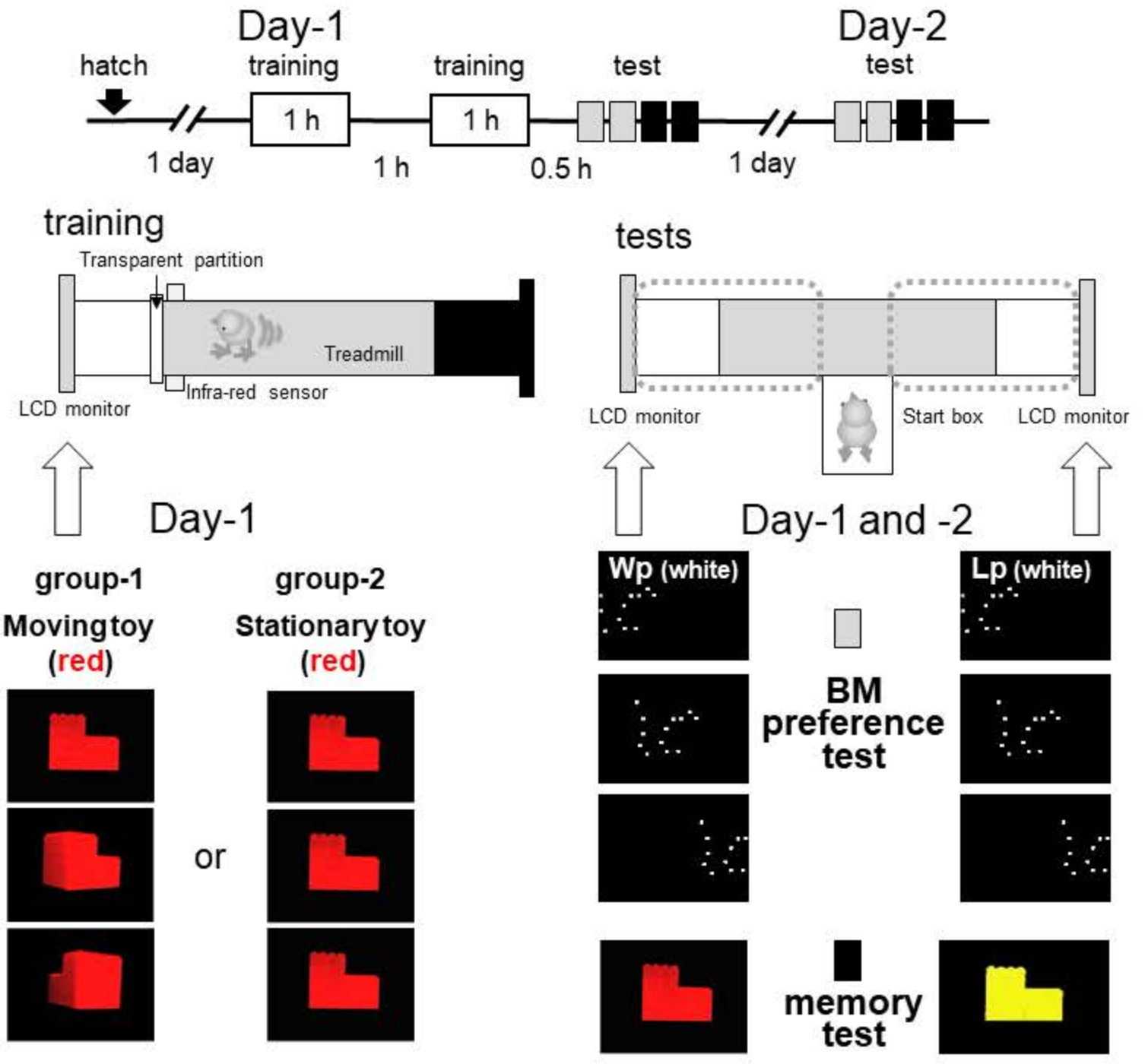
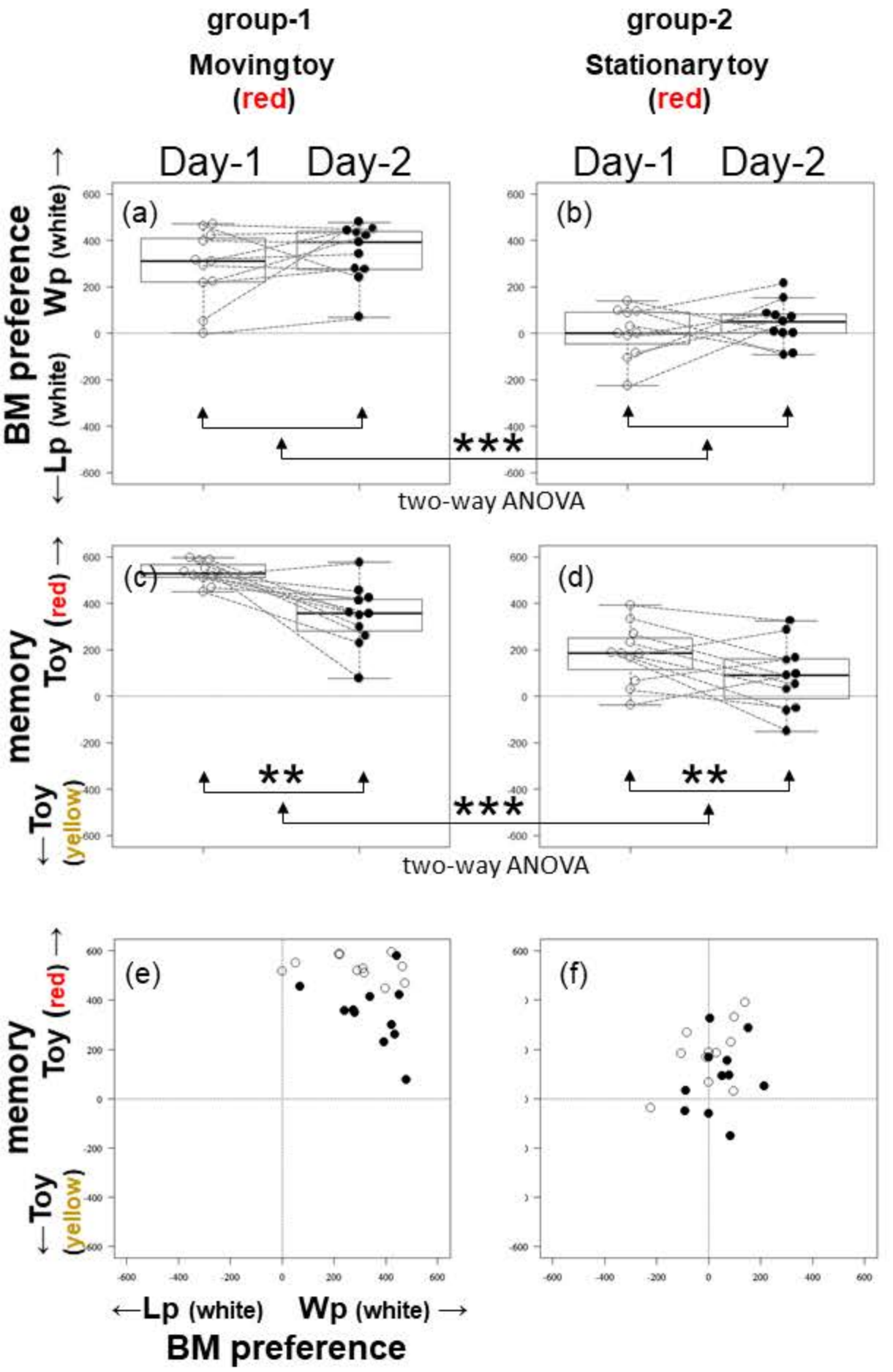


EXPERIMENT 5

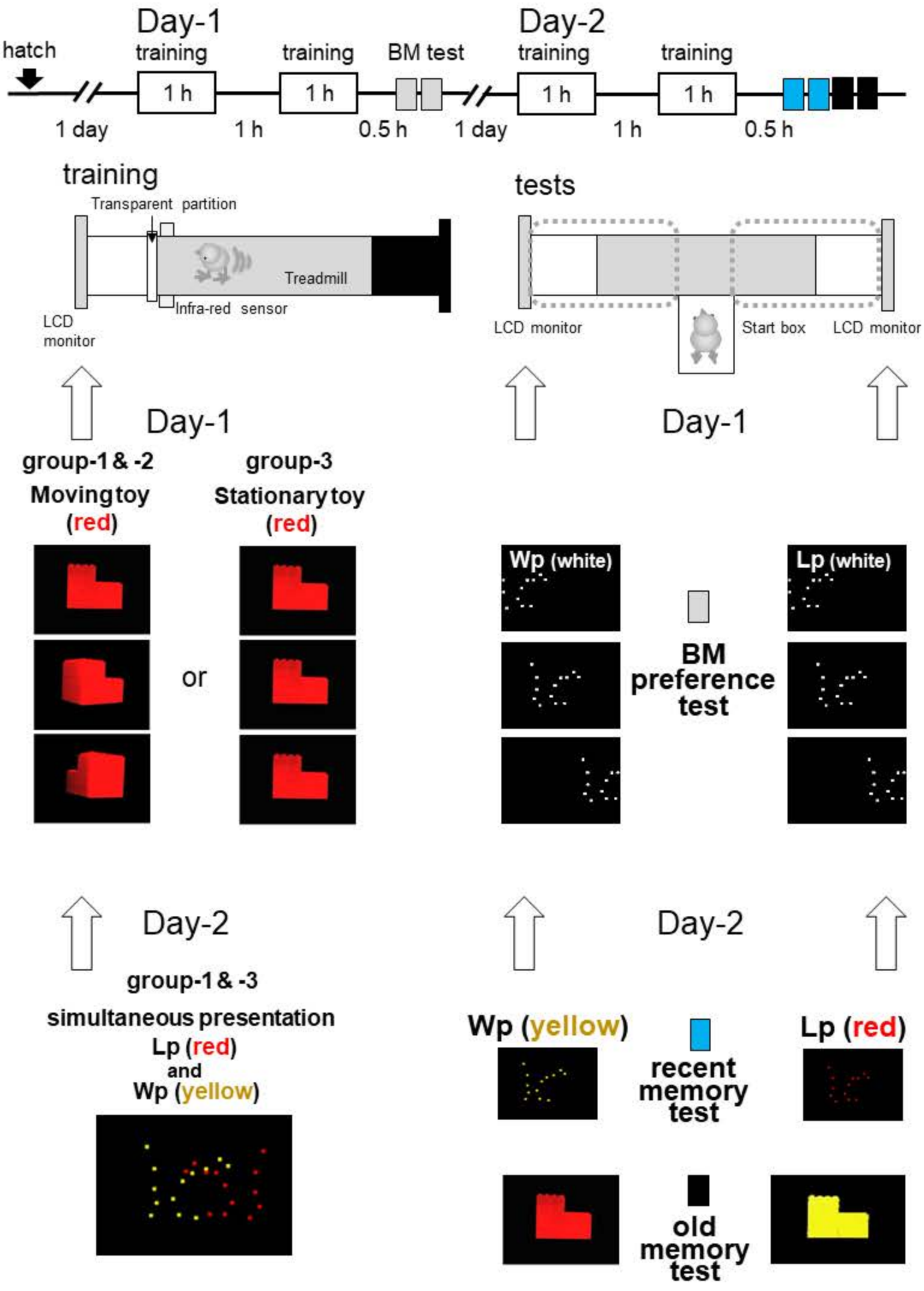


EXPERIMENT 5

ns



EXPERIMENT 6



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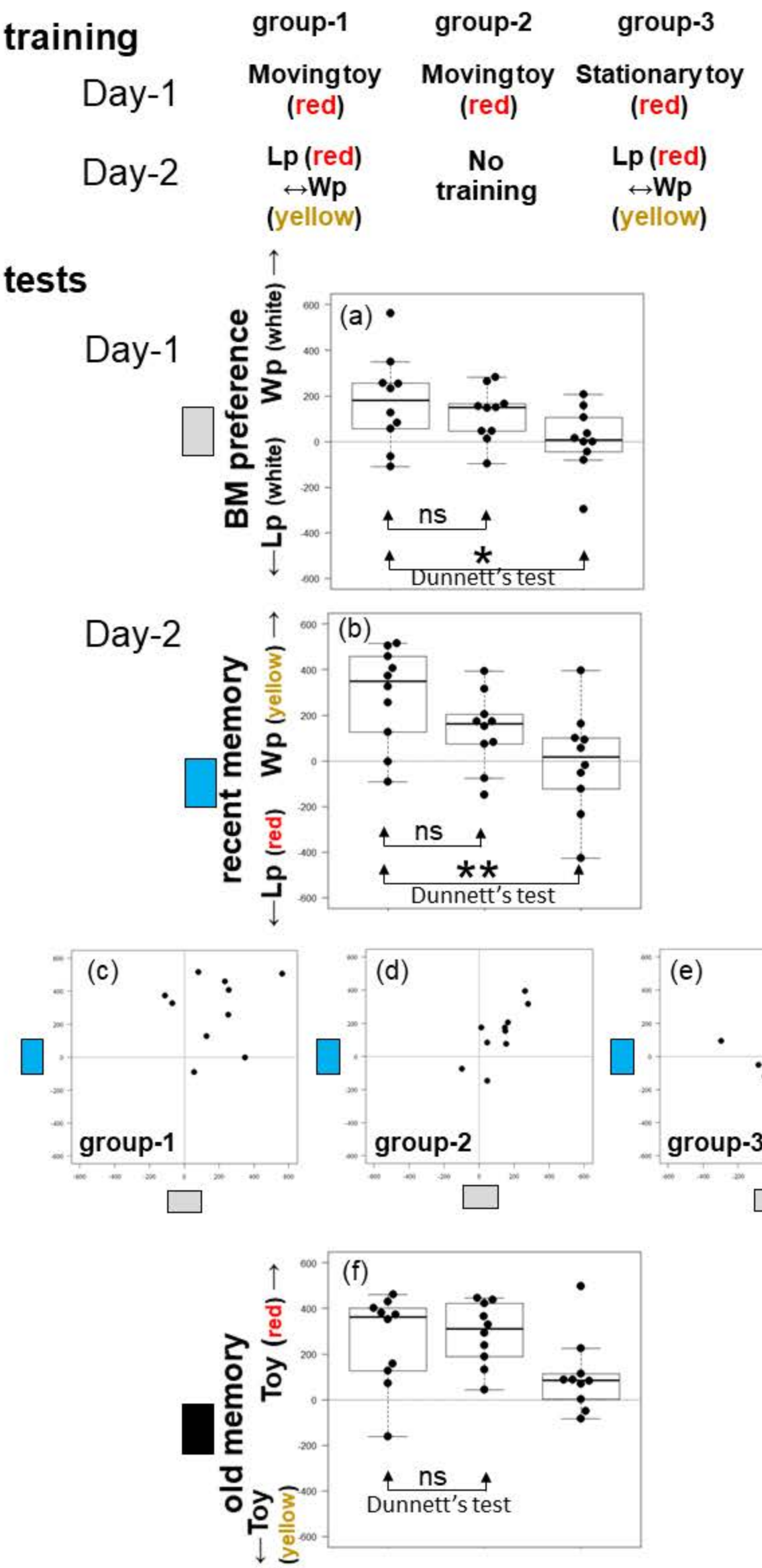
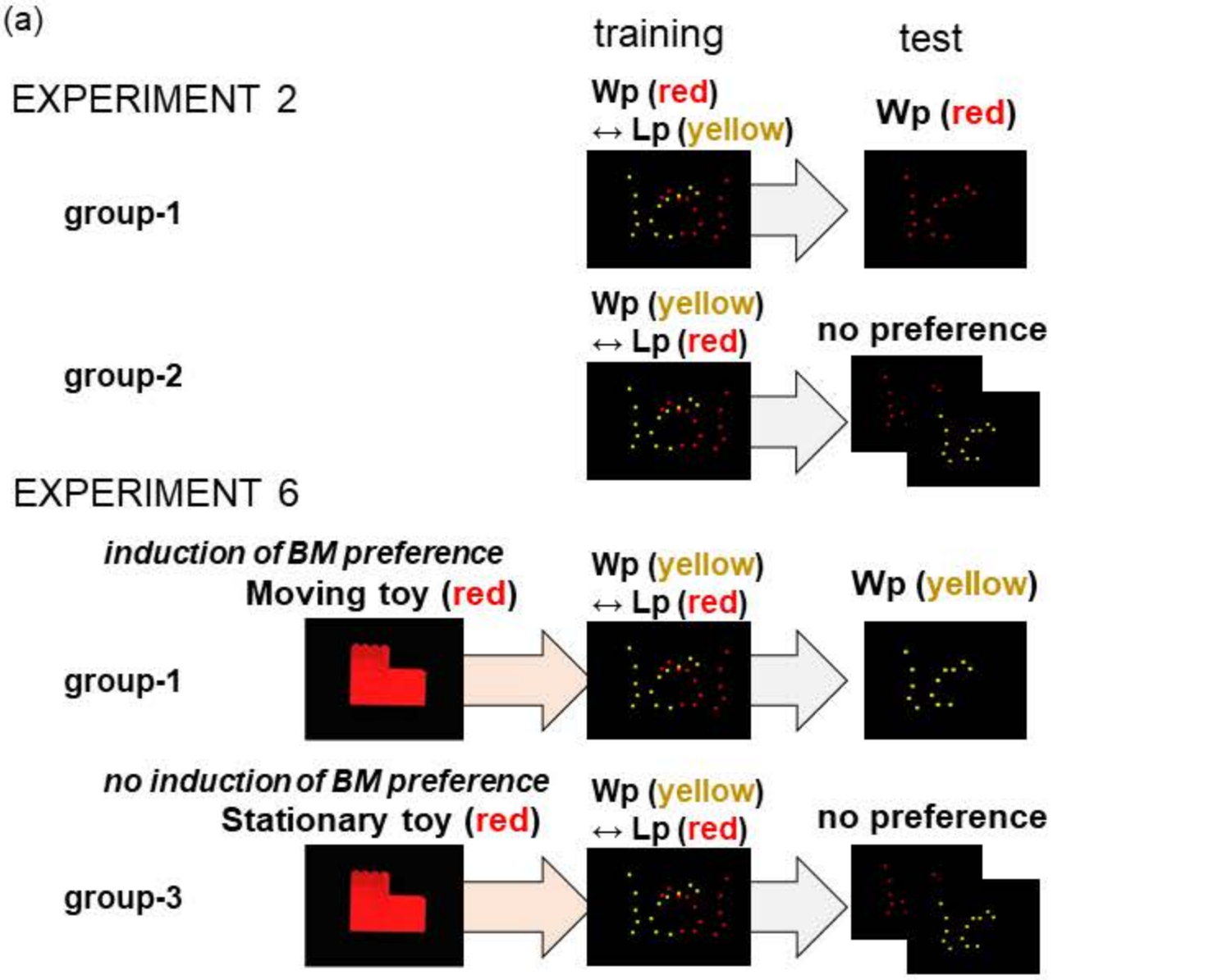
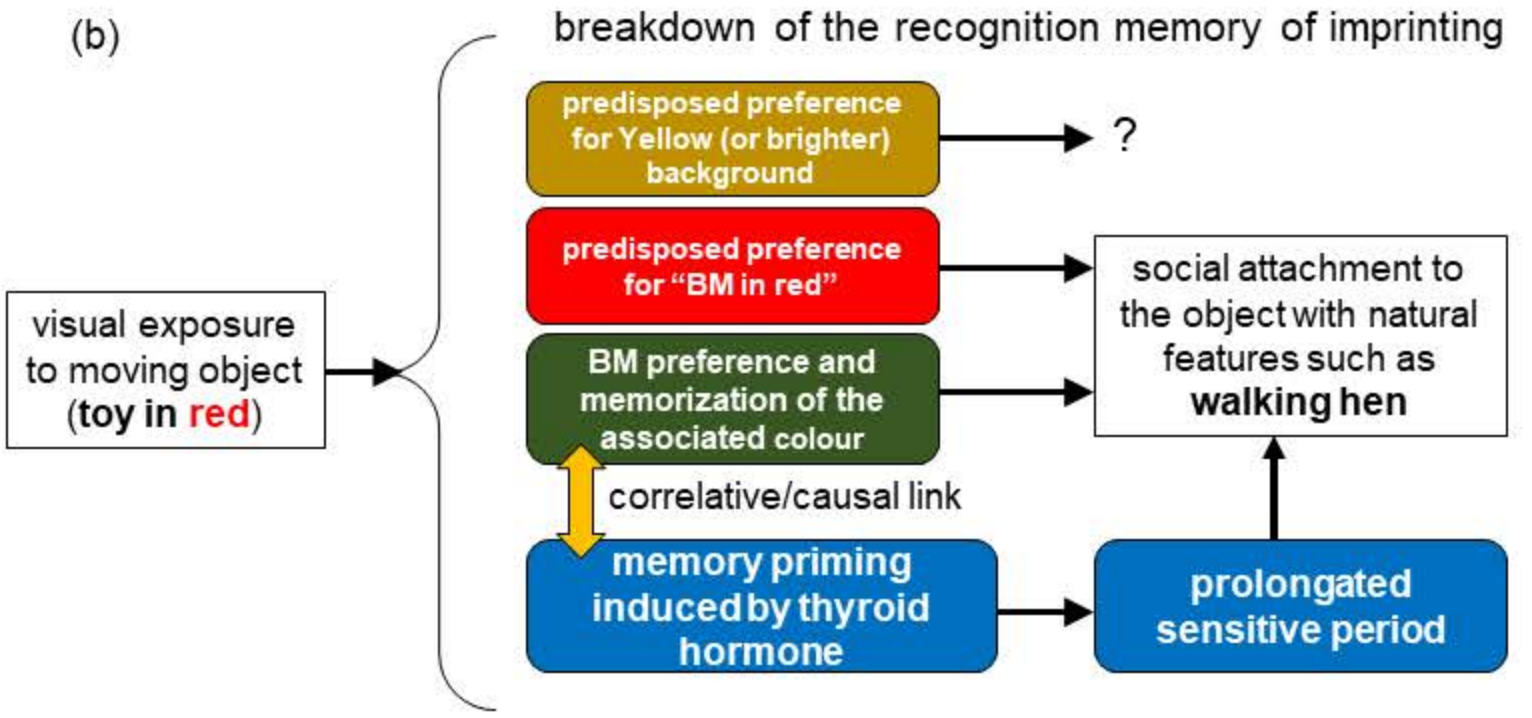


(a)


training
test
EXPERIMENT 2
group-1
Wp (red)
↔ Lp (yellow)
Wp (red)
group-2
Wp (yellow)
↔ Lp (red)
no preference
EXPERIMENT 6
induction of BM preference
Moving toy (red)
group-1
Wp (yellow)
↔ Lp (red)
Wp (yellow)
no induction of BM preference
Stationary toy (red)
group-3
Wp (yellow)
↔ Lp (red)
no preference

(b)


breakdown of the recognition memory of imprinting
visual exposure to moving object (toy in red)
predisposed preference for Yellow (or brighter) background
?
predisposed preference for "BM in red"
BM preference and memorization of the associated colour
social attachment to the object with natural features such as walking hen
correlative/causal link
memory priming induced by thyroid hormone
prolongated sensitive period