RESEARCH ARTICLE

Embodying an artificial hand increases blood flow to the investigated limb [version 1; peer review: 2 not approved]

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Abstract

Background: The autonomic nervous system is the main determinant of the blood flow directed towards a body part, and it is tightly connected to the representation of the body in the brain; would the experimental modulation of the sense of ownership of the limb affect its blood perfusion?
Methods: In healthy participants, we employed the rubber hand illusion paradigm to modulate limb ownership while we monitored the brachial artery blood flow and resistance of the investigated limb.
Results: In all conditions with brush-stroking, we found an initial drop in the blood flow due to tactile stimulation. Subsequently, in the illusion condition where both the rubber and real hand experience synchronous brush-stroking, the blood flow rose significantly faster and reached significantly higher values. Moreover, the increase in blood flow correlated to the embodiment level measured by questionnaires and, negatively, to the change of peripherical vascular resistance.
Conclusions: These findings demonstrate that modulating the representation of a body part impacts its blood perfusion.

Keywords

Autonomic nervous system, upper limb embodiment, blood flow, Rubber Hand Illusion paradigm, peripherical vascular resistance, brachial artery, body representation

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Plain language summary

The autonomic nervous system controls the visceral body and its blood perfusion, by adapting it to our behavior. Its activity is influenced by cognitive and emotional processes, it is bidirectionally connected to the network hosting the body representation, to the creation of which it contributes. By exploiting brachial artery blood flow recording during the rubber hand illusion paradigm, we demonstrated that modulating the belonging of a body part to the body representation increases its perfusion, through a sympathetic-driven downstream vasodilatation. The blood flow increase correlated to the achieved level of fake hand embodiment. This raises intriguing questions on the local specificity of the blood flow enhancement, and on the essence of its causal connection with the alteration of the sense of embodiment of the limbs.

Introduction

The autonomic nervous system (ANS) takes care of the involuntary control of the visceral body. Glands, smooth and cardiac muscles are regulated to maintain the body homeostasis and to adapt the digestion, body temperature, ventilation, cardiac activity and regional blood flow to our behavior.

Despite the ANS being mainly a low-level control system, it is strongly influenced by emotive and cognitive processes. Depending on emotions and feelings, connections between the amygdala and the medial cortices (anterior cingulate, insular, and ventromedial prefrontal cortex) in association with the dorsal pons and hypothalamus, modulate blood pressure, pupil size, heart rate and electrodermal activity[1]. Moreover, ANS homeostatic information related to pain, temperature, pH, carbon dioxide, and oxygen are sent to the insula and interact with somatosensory processing to build the body representation[2].

Pathways and cortical elaboration centers of interoceptive and exteroceptive information often overlap. For example, somatosensori-motor cortices, extra-striate body area and the dorsal precuneus control gastric activity, digestion, cardiac output and heart rate, and they are also involved in mapping bodily space through touch, action and vision. In particular, the primary sensorimotor cortex receives both tactile and visceral afferents combining internal and external bodily information[3–6].

Evidence of the tight connection between the ANS and central body representation derives from complex regional pain syndrome (CRPS)[7], where the alteration of the brain representation of a body part impacts on the autonomic neural pathway subserving that part. In CRPS, an autonomic dysfunction results in changes to the skin blood flow, warmer limbs, change of colour, edema, longer nails and abnormal sudomotor activity[8]. CRPS is usually triggered by a limb-related trauma and a subsequent period of immobilization. The associated pain is related to sympathetic hyperactivity as well, so patients benefit from early sympathetic blockade[9]. The strange association of a 'neglect-like' syndrome[10] with an over-representation of the affected hemispace[11], and of an enlargement of the affected limb motor cortex[12] with a reduction in its primary sensory cortex[13] could imply that the derangement of body representation affects CRPS pathogenesis. Moreover, both pain and autonomic symptoms are relieved with interventions manipulating the representation of the limb, such as mirror therapy[14]; minimizing lens[15]; or prism adaptation[7,11].

Emerging evidence for the existence of a strong relationship between body representation and interoceptive signals are not confined to pathological models, but can be also gathered in the normal context from studies on healthy participants. For example, interoceptive information such as cardiac feedback can modulate the visual body perception[16] and influence one's own body awareness[17,18] or, vice-versa, changes in body-ownership and self-identification can alter the ability to detect internal body signals[19]. Furthermore, interoceptive sensitivity predicts the malleability of participants' body representation[20].

ANS is in charge of blood perfusion; think for instance to the viscera-to-muscle redirection of the flow during the fight or flight response, to the reduction of wound hemorrhages, thermoregulation and thermomimesis[21]. For these responses, the nucleus tractus solitarius integrates signals from the periphery and from higher brain centers, to control vagal and sympathetic outflow[22]. Preoptic hypothalamic and forebrain centers interact with periaqueductal gray and raphe nuclei[23] when the limb flow is modulated by cognitive and emotional processes, the level of attention[24], and anxiety[25–27]. The amygdala, involved in vigilance and arousal, and the habenula, activated by aversive events or missing rewards, control vasoconstriction triggered by salient alerting stimuli[28].

Hitherto, we know that i) the central ANS is tightly connected with circuits subserving the representation of the body, ii) cognitive processes influence central ANS control of the local blood flow, and that iii) a syndrome due to an alteration of the limb representation (i.e. CPRS) presents an autonomic-driven dysfunction of the vascular supply to the affected limb.

Altogether, this suggests that modifying the brain representation of a body part could result in a change of the blood perfusion of that part; however, this has never been demonstrated so far.

A simple way to modulate the body representation is through the rubber hand illusion (RHI), a perceptual illusion caused by the synchronous brush-stroking of the hidden participant's real hand and a visible fake one[29]. Spatio-temporal congruency of visuo-tactile stimuli is mandatory for the illusion to arise and asynchronous stimuli abolish it, because the sense of body ownership depends on Bayesian integration of different information into a pre-existent internal body map[30–32]. Indeed, the illusion is abolished when the visual and somatosensory stimulation are presented asynchronously.

This work assessed whether modulating the belonging of the upper limb to the body representation would impact on its perfusion. In healthy subjects, we recorded the brachial artery flow of the limb involved in three different RHI conditions:

synchronous *(Synch)*, asynchronous brush-stroking (*Asynch*), and the mere sight of the fake hand while the hidden real hand was not stimulated (*VisionOnly*).

Methods

Participants

The participants were selected among a population of students and collaborators of Neurophysiology and Neuroengineering of Human-Technology Interaction (NeXT) Research Unit that volunteers to participate to the study. Inclusion criteria were to be older than 18 years, to be naïve to the RHI protocol, to have normal hand sensation and normal, or corrected to normal, vision. To our best knowledge, we are the first to systematically measure blood flow on the forearm and on the hand while participants experience the rubber hand illusion and, for reason, it was not possible to calculate participants sample size with *a priori* power analysis. Therefore, in this case, the number of participants was chosen equal to previous RHI studies. Participants were enrolled after having signed a written informed consent to the participation and publication of the data, including the permission for their treatment images. The experimental procedures were approved by the Ethics Committee of the Università Campus Bio-Medico di Roma (EMBODY protocol) and carried out according to the Declaration of Helsinki and its future amendments.

Experimental procedure

The study was performed in a dedicated room of the NeXT Research Unit, in a period ranged between September 2018 and June 2019. Participants were placed in front of a custom-made experimental set-up, made of three parallel compartments (L × W × H = 40 × 60 × 20 cm each) covered by a two-way mirror (Figure 1). They could see the content of each compartment only when the experimenter turned the relative internal light on 33,34. Then, participants were invited to place their forearms inside the two lateral compartments while their shoulders were covered by a black cloak. A left rubber hand, matching the participant's gender, was placed in the central compartment of the structure, 15cm apart from the real hidden left hand of the subject. The left hand was tested because it appears to be the side where it is easier to induce the RHI[35].

Three conditions were tested for each participant, administered in a random order:

- *Synchronous* (*Synch*) *condition*: a well-trained experimenter used two identical paintbrushes to stroke both the second digit of the rubber hand and the corresponding digit on the real hidden hand. The tactile stimulation was delivered at a frequency of 1Hz. The brushstroke duration was about 0.6–0.7s, and it was delivered from the proximal to the distal phalanx.
- *Asynchronous* (*Asynch*) *condition*: similarly, the experimenter used the paintbrushes to stroke the second digit of the participant, but a small temporal delay (about 0.5s) was added between the stimulus delivered on the rubber hand and the one delivered on the real hand (Figure 1).
- *Vision only condition*: in this case, no stroking was delivered to either the rubber or the real hidden hand. The participant was instructed to simply look at the rubber hand for the entire duration of the condition. Such condition was performed in order to control for the effect of mere tactile stimulation on the blood

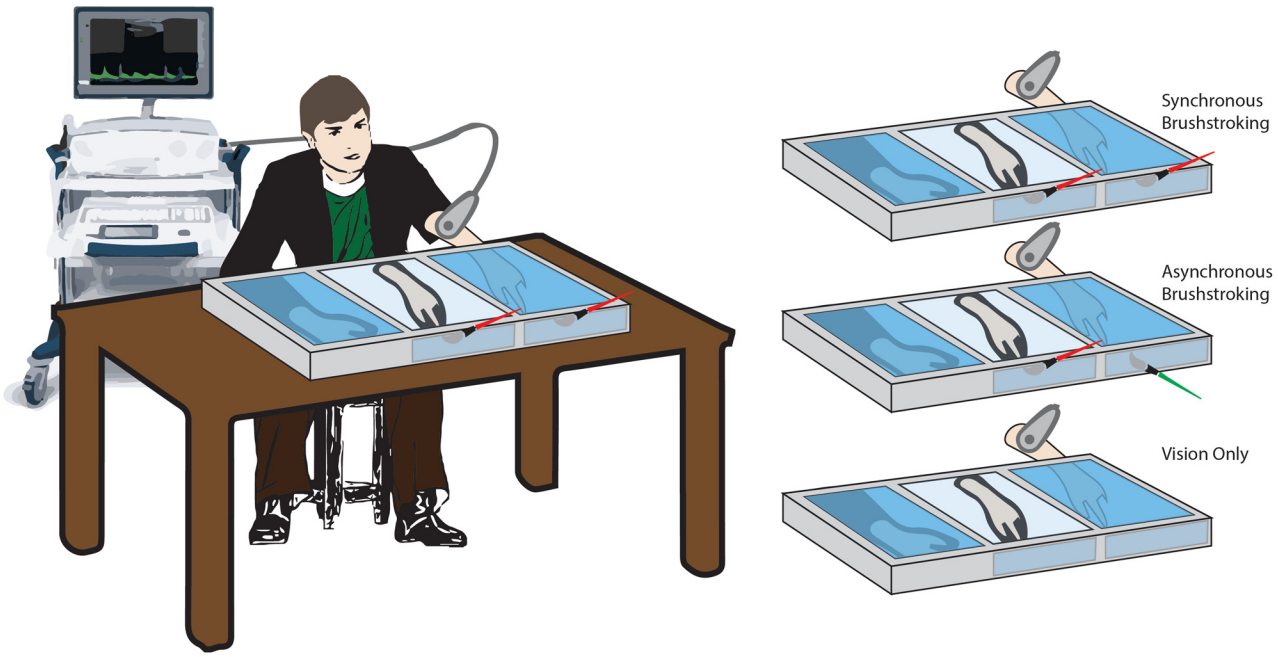

Figure 1. Schematic illustration of the experimental conditions. Setup and rubber hand illusion paradigm conditions.

flow and was considered as an additional condition of no embodiment[36]. In such condition only the blood flow was recorded.

Each condition lasted 100s.

Blood flow was collected at a sampling rate of 100 Hz by using a Multidop-X DWL (Elektronische Systeme GmbH, Germany). The probe of the device was placed at the level of the brachial artery on the medial aspect of the tested (i.e. left) arm. We selected to employ a 4 MHz DWL ultrasound probe, which is suited to monitor the blood flow of the brachial artery considering that it can penetrate roughly 12–30 mm. The probe was kept still by the experimenter during the whole protocol. The brachial artery was selected because it is the major blood vessel located in the upper arm: the main supplier of blood to the arm and hand.

Each 1.3s, the device calculated and saved for further analysis three parameters: the mean blood flow, the peak of systolic flow and the peak of the diastolic blood flow. Once the blood flow was stable, it was recorded for 120s, from 20s before the compartment's lighting was turned on to 100s after it. For each condition, about 92 measures of each parameter were recorded (about 77 if considering the period when compartment's lighting was on).

Embodiment measures

Two measures of embodiment (proprioceptive drift and nine-item self-evaluation questionnaire) were collected to assess the embodiment of the rubber hand induced by brush-stroking in synchronous and asynchronous conditions.

The proprioceptive drift was assessed[29] by asking the participants to verbally report a number on a measuring tape reflected on the two-way mirror that corresponded to the perceived location of their left index finger by maintaining the hands still and relaxed.

For each condition, the perceived location was collected twice, before and after brush-stroking. To guarantee a random offset before every assessment, the measuring tape had the possibility to slide. Positive differences between the hand position estimated post and pre-stimulation indicate a drift of the perceived location of the participants' hand towards the rubber hand.

Then, the experimenter handed to the participant a nine-item questionnaire made up of three questions aimed at investigating the extent of the self-attribution of the rubber hand and six control questions testing participant susceptibility[29] (Table 1). The participants were asked to rate the extent to which these items did or did not apply to them, using a seven-point scale. On this scale, -3 meant ''absolutely certain that it did not apply,'' 0 meant ''uncertain whether it applied or not,'' and +3 meant ''absolutely certain that it applied''. Such questionnaire was provided with two additional items to rate the vividness (0 - 10) of the perceived illusion (i.e. how realistic the illusion was when it was experienced) and the prevalence (0 - 100%), which reflected the percentage of time that the illusion was experienced (i.e. how long with respect to the length of section the perception of the illusion was).

The overall experimental session lasted about 30 minutes for each participant.

Data analysis

The Kolgomorov-Smirnov test ($p > 0.05$) was used to verify that the data relative to the typical RHI outcomes (nine-item

Table 1. List of items of the questionnaire.

Questionnaire	Item	Rating
Item 1	It seemed as if I were feeling the tactile stimulation at the location where I saw the visible hand touched	-3 – +3
Item 2	It seemed as though the stimulation I felt was caused by the touch on the visible hand	
Item 3	I felt as if the visible hand was mine	
Item 4	I felt as if the position of my real hand was drifting towards the visible hand	
Item 5	It seemed as if I had more than two hand or arm	
Item 6	It seemed as if the tactile stimulation I was feeling came from somewhere between my own hand and the visible one	
Item 7	I felt as if my real hand were turning 'rubbery'	
Item 8	It appeared as if the position of the visible hand was drifting towards my real hand	
Item 9	The visible hand began to resemble my own hand, in terms of shape, skin tone, freckles or some other visual features	
Vividness	How realistic and life-like was the illusion that the visible hand was yours when it was experienced?	0 – 10
Prevalence	How long with respect to the length of section the perception of such illusion was?	0 – 100%

questionnaire, vividness, prevalence score and proprioceptive drift) were normally distributed. To verify that the responses to the questionnaires were not due to the participants' suggestibility, the mean score of the three items employed to measure the effective illusion was compared against the mean score of six items served as controls for compliance, suggestibility, and ''placebo effect'', by using a two-tailed paired t-test.

Then, on the basis of questionnaires' responses, a single index was calculated: the RHI index, which expresses the difference between the mean score of the illusion items and the mean score of the other ones[37]. The RHI index was calculated for each condition and considered as the "illusion outcome" in the following analyses.

Questionnaire outcomes and proprioceptive drift were analyzed with paired t-test to highlight differences between the illusion condition (*Synch*) and the asynchronous control condition (*Asynch*). Effect size (d) was also calculated as Cohen's d.

Regarding the blood flow signal analysis, the mean blood flow signal (*f*) was smoothed by using a moving average 5s window to eliminate the high frequency noise. In order to minimize the influence of inter-individual variability and of the circumstance on which the experiment was run (e.g. the room temperature), the extracted measure was expressed as percentage change with respect to a baseline value for each trial (*F(t)*), for simplicity hereafter called mean blood flow and calculated using the following equation:

$$F(t) = 100 * \frac{f(t) - \overline{f}(\Delta t_b)}{\overline{f}(\Delta t_b)} \quad (1)$$

Where *f(t)* is the blood flow value at certain time t, $\overline{f}(\Delta t_b)$ is the value of baseline calculated as blood flow values averaged on the last 5s window of the baseline interval (i.e. Δt_b = [-5s, 0s]). These values were calculated for each condition and participant.

After that, the *F*(t) values were averaged across participants for each condition.

The statistical analysis was performed on three contiguous equally-long time-intervals, altogether lasting the entire duration of the trial: Δt_1 = [0.01s, 33.33s], Δt_2 = [33.34s, 66.66s] and Δt_3 = [66.66s, 100s]. The average value of the blood flow was extracted in the different time-intervals for each condition and participant ($\overline{F}(\Delta t_1)$, $\overline{F}(\Delta t_2)$ and $\overline{F}(\Delta t_3)$). After checking the normality of the data by using Kolmogorov-Smirnov test ($p > 0.05$), a Mauchly test was employed to verify the sphericity of the distribution of the values and a repeated measures ANOVA (rmANOVA) with two factors (i.e. *condition* and *time*) was performed with Greenhouse-Geisser adjustment. Hence, a paired t-test with Tukey-Kramer adjustment was employed as post-hoc analysis. Additionally, the effect size (Cohen's d) was calculated for each comparison.

A drop of the mean blood flow values was identified at around 10s from the beginning of the conditions. Thus, we corrected the blood curves of all the three conditions to make all of them starting from the same value after the drop, the value of the drop was subtracted to the mean blood flow by using the following equation:

$$\Delta F(t) = F(t) - \overline{F}(\Delta t_d) \quad (2)$$

where $\overline{F}(\Delta t_b)$ was calculated as blood flow value averaged on a 10s window centered 10s after the beginning of the trial (i.e. the drop of the signal) (i.e. Δt_d = [5s, 15s]).

The obtained signal in the interval between 10s and 100s were fitted by using an exponential curve:

$$y(t) = a * (1 - e^{(b*(t-10s))}) \quad (3)$$

where *a* and *b* are the coefficients of the curve employed to fit the data.

a is the value to which the curve asymptotically tends (i.e. trend value): the higher the *a*, the higher the trend value. *b* is the rate to reach the trend value: the higher the absolute value of *b*, the faster the curve rate. *a* and *b* coefficients in the different conditions were compared using a Friedman test, and post hoc tests with Tukey-Kramer correction were employed for pairwise comparisons. Effect size (r) was calculated as $z/\sqrt{n}$, where z is test statistic for signed-rank test and n is the number of observations.

The link between blood flow changes and embodiment was investigated by correlating (Spearman's) *a* and *b* coefficients with the illusion outcomes in *Synch* and *Asynch* condition.

The resistance index (*ri*)[38–40] was calculated as:

$$ri(t) = \frac{f_{Syst}(t) - f_{Dias}(t)}{f_{Syst}(t)} \quad (4)$$

where f_{Syst} is the systolic peak blood flow, f_{Dias} is the diastolic blood flow. The signal of the resistance index was smoothed and normalized by using the same strategy of equation (1), the result is hereafter simply named resistance index (*RI(t)*). The average value of the resistance index was extracted in Δt_1, Δt_2 and Δt_3 time-intervals for each condition and participant ($\overline{RI}(\Delta t_1)$ $\overline{RI}(\Delta t_2)$ and $\overline{RI}(\Delta t_3)$). Correlations (Spearman's coefficients) between the resistance index and mean blood flow values in all conditions were calculated for all three time intervals (Δt_1, Δt_2 and Δt_3).

The analysis was performed with Matlab2015a (Mathworks), a freely available alternative software is GNU Octave and JASP for statistical analysis.

Results

Twenty volunteers took part in the experiment (age: 29.55 ± 6.12; 12 M, 8 F; 20 right-handed as by self-report).

For both stroking (*Synch* and *Asynch*) conditions, the mean value of the illusion items of the self-evaluation questionnaire was higher than the mean value of the control items (*Synch*:

d = 1.82; t(19) = 7.95, p <0.001; *Asynch*: d = 0.50; t(19) = 2.19, p = 0.041), thus the group of participants were generally not suggestible (Figure 2)[41].

The illusion items' score in the *Synch* condition was significantly higher than that in the *Asynch* condition for all the embodiment measures employed (RHI index: d = 1.17; t(19) = 5.12, p <0.001; vividness: d = 1.34; t(19) = 5.84, p <0.001; prevalence: d = 1.22; t(19) = 5.31, p <0.001; proprioceptive drift: d = 0.84, t(19) = 3.68, p = 0.002) (Figure 3). This confirms that participants effectively experienced the RHI in the *Synch* condition.

By analyzing the behavior of the mean blood flow (*F(t)*) averaged among the participants, it is possible to note that right after the experiment began there was a drop in the mean blood flow, peaking at 10s. This drop was present in all conditions, but it was more evident for *Synch* and *Asynch* conditions (Figure 4). After this drop, the *F(t)* tends to increase in all conditions. In particular, the mean blood flow behavior for *Synch* and *Asynch* conditions is similar until 50 s; after which, the value for the *Synch* condition has a higher increasing trend. The blood flow value for the *VisionOnly* condition is higher on average than for the other two conditions, probably because of the lower extent of the drop for this condition. For further analysis, the whole duration of each session (100s) has been divided into three time windows equal in length, in line with the neurogenic dynamic of the slow changes of the flow[42].

The rmANOVA run on the mean blood flow of the three temporal intervals of the trial showed the presence of both of the main factors time (F(2, 38) = 6.56, p = 0.006) and condition (F(2, 38) = 8.55, p = 0.004), and of their interaction (F(4, 76) = 2.89, p = 0.047). Post-hoc analysis showed that mean blood flow in the *VisionOnly* condition was significantly higher than in the *Asynch* one (d = 0.80, t(19) = 3.60, p = 0.005) and that mean blood flow value in the Δt_3 temporal interval was higher than the other temporal intervals (Δt_1: d = 0.70, t(19) = 3.15,

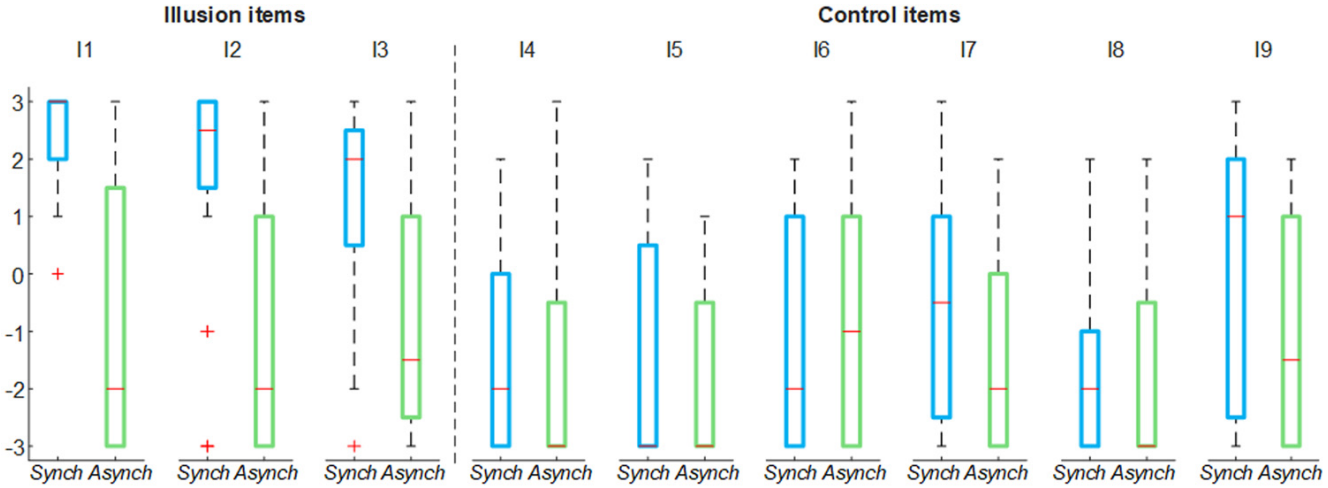

Figure 2. Nine-item questionnaire outcomes. Box and whisker plots of nine-item questionnaire outcomes for *Synch* and *Asynch* conditions: median (red lines), 1st and 3rd quartiles (box), lowest and highest values comprised within 1.5 times the interquartile range from the 1st and 3rd percentiles (whisker).

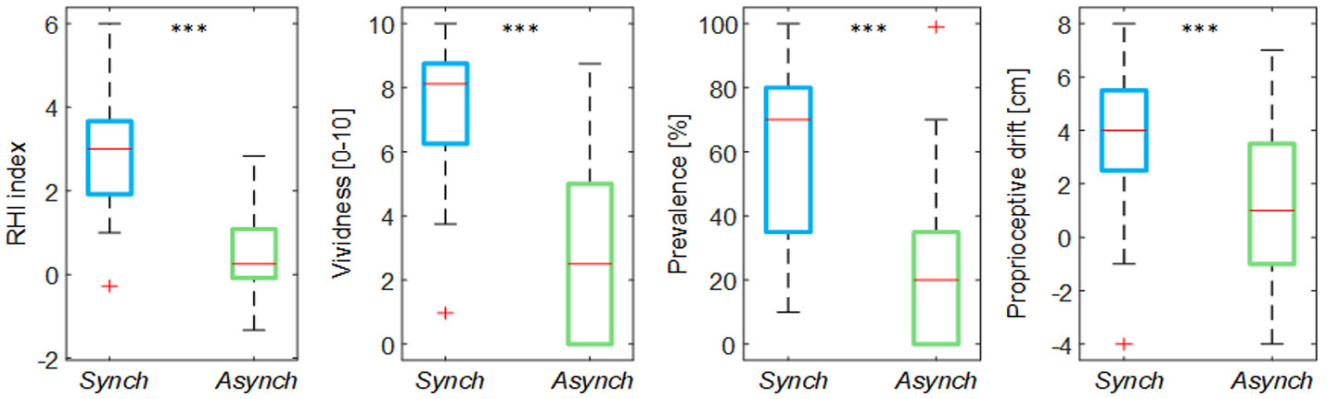

Figure 3. Illusion outcomes. Box and whisker plots of the illusion outcomes (rubber hand illusion [RHI] index, vividness, prevalence rating and proprioceptive drift) for *Synch* and *Asynch* conditions: median (red lines), 1st and 3rd quartiles (box), lowest and highest values comprised within 1.5 times the interquartile range from the 1st and 3rd percentiles (whisker). *** indicates a p-value <0.001.

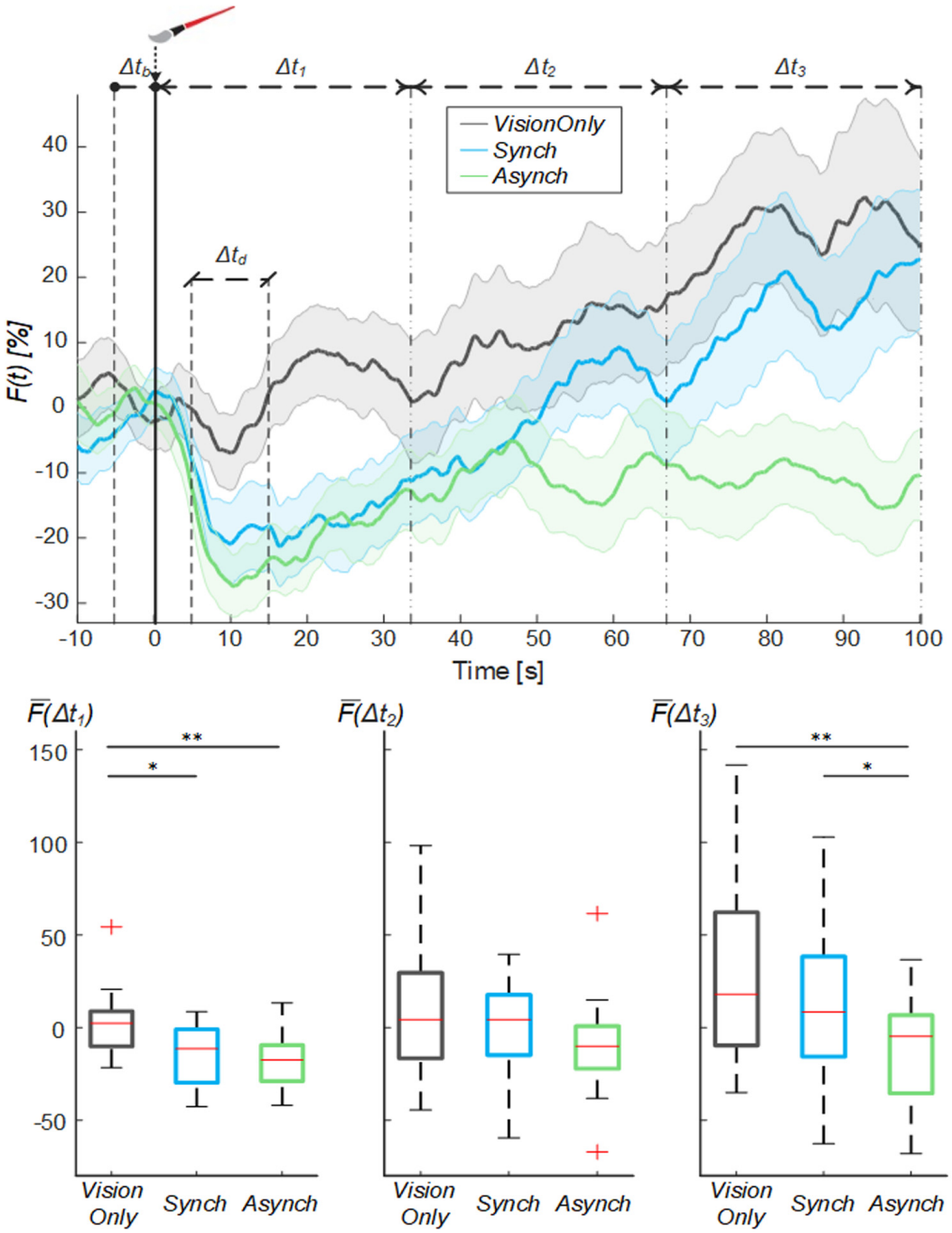

Figure 4. Mean blood flow data. Mean blood flow (F) averaged across participants for each condition, the shade represents the standard error (SEM), dashed lines indicate the time intervals where the mean baseline and drop values were calculated (Δt_b and Δt_d) and the analysis performed (Δt_1, Δt_2 and Δt_3). Time=0 sec is when the trial began. (Upper panel). Box and whisker plots relative to averaged blood flow values calculated in the selected intervals for Synch, *Asynch* and *VisionOnly* conditions (Lower panel): median (red lines), 1st and 3rd quartiles (box), lowest and highest values comprised within 1.5 times the interquartile range from the 1st and 3rd percentiles (whisker). * indicates a p-value <0.05.; ** indicates a p-value <0.01.

p = 0.014; Δt_2: d = 0.59, t(19) = 2.64, p = 0.041). Considering the interaction between the factors and given that our aim was to find a difference among conditions in a single time interval, we made three separate analysis, one for each time-interval: the *VisionOnly* flow in the first interval was significantly higher than both the others (*Asynch*: d = 0.87, t(19) = 3.88, p = 0.003; *Synch*: d = 0.59, t(19) = 2.62, p = 0.042), whereas in the third interval the *Asynch* flow was significantly lower than both the *Synch* and *VisionOnly* ones (*Synch*: d = 0.60, t(19) = 2.67, p = 0.038; *VisionOnly*: d = 0.85, t(19) = 3.80, p = 0.003). No

significant differences were identified in the second interval (Figure 4).

In this analysis, the flow value for each time window was not independent from the value in the previous window, so that higher *VisionOnly* value may have been the effect of its milder drop. To avoid such influence, an exponential curve was employed to fit the behavior of the blood flow from the drop identified at 10s (Figure 5): the *a* fitting coefficient corresponds to the curve trend value; whereas the *b* coefficient corresponds to the

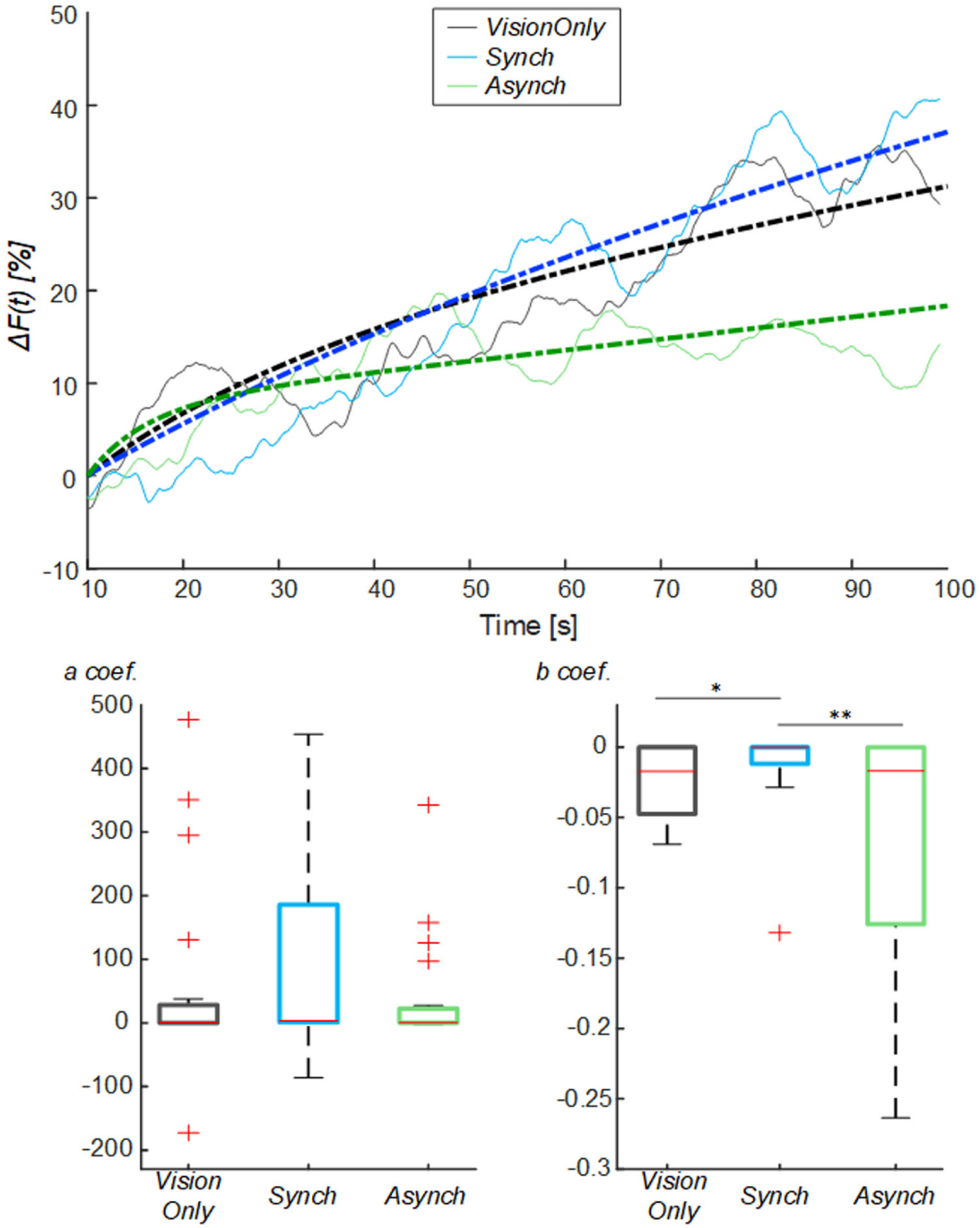

Figure 5. Exponential fitting of the mean blood flow data. Mean blood flow value from the drop (*ΔF*) averaged across participants for each condition, dashed lines indicate the exponential curves that fit the data, averaged across participants (Upper panel). Box and whisker plots relative to *a* and *b* coefficients calculated in the selected intervals for *Synch* and *Asynch* and *VisionOnly* conditions (Lower panel): median (red lines), 1st and 3rd quartiles (box), lowest and highest values comprised within 1.5 times the interquartile range from the 1st and 3rd percentiles (whisker). * indicates a p-value <0.05.; ** indicates a p-value <0.01.

rate to reach the trend value. The statistical analysis showed a difference in the curve fitting *b* coefficients (*a* coefficient: $\chi^2(2, 38) = 3.70$; $p = 0.157$; *b* coefficient: $\chi^2(2, 38) = 11.20$; $p = 0.004$): the *b* values for the *Synch* condition were significantly higher than those of both the *VisionOnly* and *Asynch* conditions ($r = 0.47$, $z = 2.10$, $p = 0.031$; $r = 0.49$, $z = 2.21$, $p = 0.005$; respectively). This means a faster growth rate for the *Synch* condition (Figure 5).

Both *a* and *b* coefficients were correlated to RHI index, vividness and prevalence scores ($\rho > 0.30$, $p < 0.05$) (Table 2). In particular, the *b* coefficient related to the blood flow growth dynamics was more strongly correlated to questionnaire scores ($\rho > 0.40$, $p < 0.05$). There was no correlation with proprioceptive drift.

Moreover, from the comparison of the systolic and diastolic variation of the flow, a resistance index reflecting the resistance caused by microvascular bed distal to the site of measurement[38–40] was calculated (Figure 6).

A significant negative correlation between the averaged blood flow and the resistance index values was highlighted in all time intervals (Δt_1: $\rho = -0.36$, $p = 0.005$; Δt_2.: $\rho = -0.38$, $p = 0.003$; Δt_3: $\rho = -0.62$, $p < 0.001$), demonstrating that the measured increase in the blood flow was tightly related to a decrease in peripheral vessels resistance of the tested limb.

Discussion

This study was designed to investigate possible changes in the blood flow directed towards the hand and forearm induced by the modulation of sense of limb ownership. To modulate limb ownership, we employed the RHI paradigm while the brachial artery blood flow of the homolateral limb was monitored.

Embodiment of a fake hand induced by the synchronous stimulation of the fake visible hand and real hidden hand of the participant (*Synch* condition) was tested against the commonly-adopted control condition where embodiment was inhibited because the rubber hand and the participant's own hand were asynchronously stroked (*Asynch* condition).

Table 2. Correlation analysis fitting coefficients and illusion outcomes.

	Coef. *a*		Coef. *b*	
	ρ	p	ρ	p
RHI index	0.33	0.037	0.43	0.007
Vividness	0.30	0.050	0.40	0.012
Prevalence	0.36	0.024	0.41	0.010
Prop. Drift	0.15	0.372	0.09	0.576

RHI, rubber hand illusion; Prop. Drift, proprioceptive drift.

Since we suspected that brush-stroking itself could have affected the flow independently from the achieved embodiment, a third condition was introduced as further control, where participants were instructed to simply look at the fake hand, without receiving any paintbrush stimulation on the real or on the fake hand (*VisionOnly* condition). In the latter case, tactile stimuli were not present.

The first element to note is that the adopted experimental process induced a consistent modulation of the blood flow, characterized by having different behaviors for different conditions, but small variability across participants (SEM lower than 14, 11 and 16 % for *Synch*, *Asynch* and *VisionOnly* conditions, respectively). This suggest that the designed experiment was suited to assess the targeted phenomenon.

Furthermore, looking at the average blood flow dynamics, in all conditions, we found a common initial drop beginning at the start of the experiment, when the light was turned on in the fake hand compartment and the fake hand began to be stimulated. This drop was always followed by a progressive increase in the blood flow, which reached its maximal value at the end of the stimulation period (Figure 4). Considering that the dynamic of the blood flow oscillation at frequencies of 0.02–0.05 Hz are mainly affected by sympathetic nerve activity[42], we chose to analyze the blood flow signal by splitting the recording session into three time intervals (33s each).

In the first time-interval ($0 < \Delta t_1 \leq 33.33$s), a significant difference in mean blood flow was found between the conditions with brush stimulation (*Synch* and *Asynch*) and the *VisionOnly* condition, while no significant difference was found between *Synch* and *Asynch* conditions.

The drop was a lot less evident in the *VisionOnly* condition, which was the only condition without any brush-stroking applied to the real hand. This suggests that the drop was due to the initial, mostly unexpected, tactile stimulation of the hidden hand caused by the brush, regardless of whether the stroke was synchronous or asynchronous and if an illusion was achieved. A blood flow drop due to tactile stimulus has already been reported[43].

While the flow in the *VisionOnly* condition had the milder drop and remained higher throughout the experiment, in the *Asynch* control condition the flow had a deep drop due to brush-stroking and it remained lower throughout the experiment. The only condition in which the blood flow dramatically increased after experiencing a deep drop was the synchronous brush-stroking condition, which was precisely the one designed to test the effect of the fake hand embodiment.

Indeed, focusing on the third time-interval ($66.66 < \Delta t_3 < 100$s), the blood flow of the asynchronous condition was significantly lower than the blood flow of all others. From the visual inspection of the evolution of the flow, different causes may

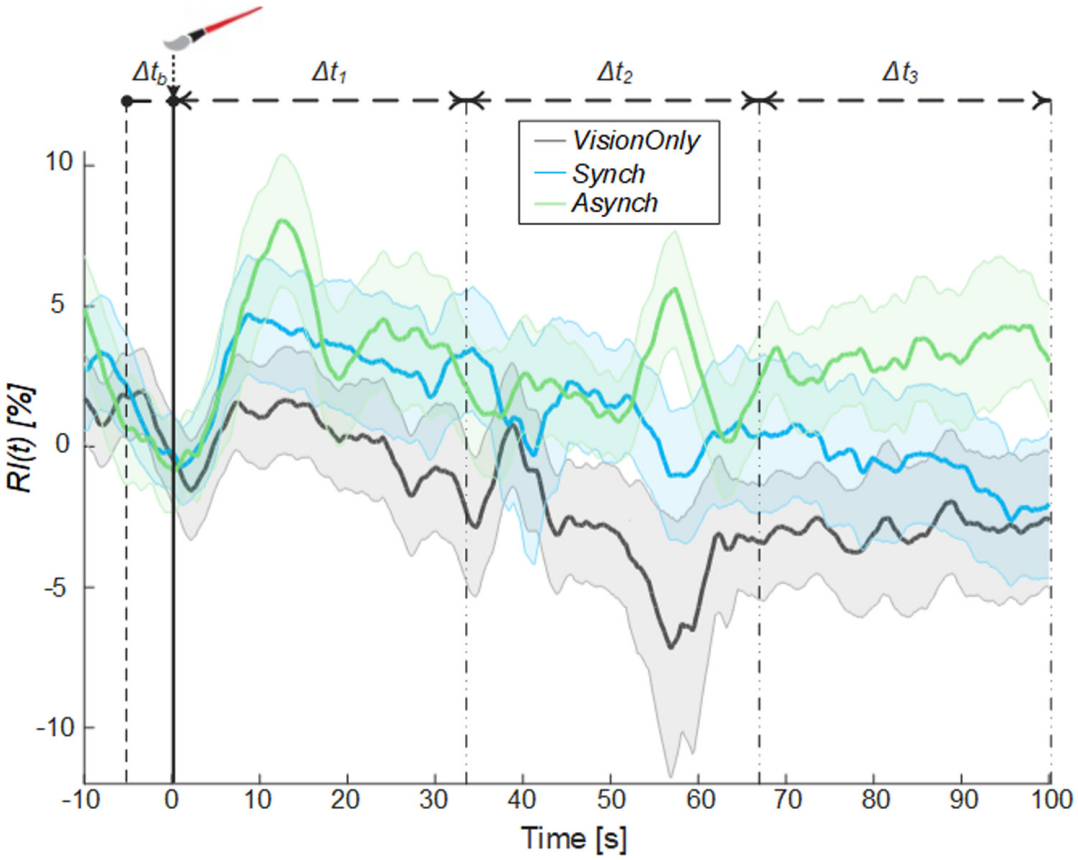

Figure 6. Resistance index. Resistance index (*RI*) averaged across participants for each condition, the shade represents the standard error (SEM), dashed lines indicate the time intervals where the mean baseline value was calculated (Δt_b) and the analysis performed (Δt_1, Δt_2 and Δt_3). Time=0 sec is when the trial began.

be supposed to determine the difference between *Asynch* and *VisionOnly*, and the one between *Asynch* and *Synch*.

Compared to *Asynch*, the higher *VisionOnly* value in the third interval may be the effect of the previous milder *VisionOnly* drop or may be the effect of a slight embodiment induced by *VisionOnly.*

Indeed, despite embodiment illusion being strongly dependent on the integration of coherent multisensory afferences, previous studies hypothesized the mere vision of a fake hand placed in a congruent position as being able to induce some mild degree of embodiment[44,45], while another study did not[46]. In this work, the *VisionOnly* condition was introduced to control for the cause of the initial drop of the flow and collecting the embodiment measures during this condition was outside the original scope of the investigation. There are both technical and scientific reasons that may suggest not recording embodiment measures in *VisionOnly*: the most significant Botvinick and Cohen questions focus on being touched by the brush, and they lose meaning if the hand is not touched. Furthermore, the collection of the proprioceptive drift without relying on self-assessment measures could be misleading, considering that such measures are related to different aspects of the embodiment process[36]. However, the lack of embodiment evaluation in the *VisionOnly* condition, especially of the proprioceptive drift, should be considered a limitation of the study and it is envisaged for future studies investigating the topic.

On the contrary, *Asynch* and *Synch* conditions did not experience different magnitudes of drop. Their significantly different value can only be due to their different effect on the body representation of the hand. This finding confirms our hypothesis: the embodiment illusion is able to significantly modulate the blood flow directed towards the tested forearm.

The main aim of the experiment was to investigate the modulation of the limb blood flow due to the embodiment of a fake hand and not the modulation due to tactile stimulation. To better highlight the effect of embodiment, we further corrected the blood flow changes for the initial drop by subtracting the value of the drop. Thus, we corrected the blood flow curves of all three conditions to have all of them start from the same value after the drop and modelled the following flow increase with

an exponential curve. The comparison of the growth rate between conditions, employing the *b* fitting parameter, showed that the synchronous illusion condition had a significantly faster growth rate than the other conditions.

Moreover, the fitting coefficients positively correlate with most of the widely-validated measures of the illusion (i.e. RHI index, vividness and prevalence scores), confirming that the variation in the blood flow dynamics is linked to the change of embodiment level during the trials.

Generally, an increase in blood flow can be due to vasodilatation and /or an increase in cardiac output, both of which are mainly driven by the ANS.

To our knowledge, we are the first to demonstrate that embodying an artificial limb enhances the blood flow directed to the tested limb.

However, this is not the first finding involving an overactivation of the sympathetic nervous system in correlation with the illusion. Indeed, the skin conductance response, which is known to be mainly driven by the sympathetic branch of the ANS, is modulated by the illusion as well; a threat to the fake hand induces a stronger event-related skin conductance response when the hand is embodied[30,47–49]. More recently, studies have demonstrated that the embodiment induced by the synchronous RHI brush-stroking, by itself without the need of any threat, enhances the spontaneous fluctuations of the skin conductance[50,51].

Two tightly interconnected questions remain to be addressed: i) Is the hyperactivation of the ANS a local or a systemic response? And ii) Is the hyperactivation of the ANS just due to an alert after the perceived abnormalities linked with the experimental manipulation or, more intriguingly, is it due to a mismatch between the sensory, the motor and autonomic representations of the limb in the brain?

Limb vasoconstriction/dilatation is mainly affected by the ANS, specifically by the sympathetic and, to a lesser extent, by the parasympathetic activity[52,53]. Most systemic blood vessels, particularly those of the abdominal viscera and skin of the limbs, are constricted by the sympathetic stimulation. Contrarily, parasympathetic stimulation has almost no effect on most blood vessels, except for vasodilatation in certain restricted areas, such as in the blush area of the face[52].

Theoretically, the activation of the sympathetic branch of the ANS is a systemic response that recruits the whole body. However, in favor of the local response hypothesis, previous works found a selective cooling of the investigated hand compared to the contralateral one, when ownership over the rubber hand was induced[54–57]. For the sake of completeness, few other studies called into question the consistency of this phenomena[58,59].

In the attempt to test the local specificity of our hypothesis, in a preliminary experiment run before the study, we tried to record the blood flow from both arms at the same time, but unfortunately, we realized that our experimental setup was not robust enough for that. Nonetheless, an indirect cue on the local specificity of the autonomic response can be gathered from the resistance index we extracted. Indeed, the resistance index value is determined by the arterial compliance (as opposite to the vessel's stiffness) and vascular resistance, mainly due to the diameter of the vessels, that results in the normal loss of pulsatility as flow progresses from the arteries to the capillaries[38–40].

If the ultrasonographic probe remains in the same spot, a decrease in the index is a sign of vasodilatation. The significant negative correlation between blood flow and resistance index percentage suggests that the change in the blood flow that we highlighted was tightly linked with the peripheral vessel resistance change. This, together with previous works not reporting significant heart rate variability differences between RHI illusion and control conditions[50], indirectly suggest the local specificity of the described phenomena.

Previously, it has been shown that the synchronous brush-stroking of the RHI procedure limited the increase in peripheral perfusion of the pierced skin of the hand induced by acupuncture[60]. The reduction of a further evoked increase in skin perfusion coexists well with an increase in the general flow, them being competitive causes for a limited possible increase in the flow.

We reported an enhancement in limb blood flow with fake hand embodiment. Would this fit with reduced hand skin blood perfusion and with the previous reported cooling of the RHI tested hand, considering that blood perfusion is the main parameter affecting hand temperature[61]? Skin perfusion may well not be representative of the whole flow directed towards the limb.

Indeed, the brachial artery blood flow we recorded is a cumulative measure of the flow through all the vessels placed distally with respect to the position of the probe (in our case the vessels of forearm and hand). The main part of this flow goes to the muscles (59% of the total flow), then to the bones and fat, which are relatively avascular under normal conditions (28%), and the remaining part to the skin (13%)[62]. Blood flow recorded on the brachial artery is, hence, predominantly a measurement of the flow to the forearm and hand muscles and may not be correlated with what happens in the cutaneous bed where thermoregulation is performed.

In regard to the second question, whether the embodiment induced sympathetic hyperactivity is an unspecific alert response or the effect of the mismatched image of the body, there are conflicting hypotheses.

On one side, there is evidence towards the unspecific response: a state of anxiety has been reported to raise the blood flow in the forearm at rest[25–27]. Indeed, an increase in the sympathetic response can enhance the heart rate and decrease the resistance of peripheric vessels in the limb, increasing its blood flow. Therefore, sympathetic-induced skin vasoconstriction and

muscle blood vessel dilatation may be explained as an unspecific alerting state to the defense "fight or flight" reaction: a preparatory adjustment for the muscular activation inseparable from these activities[25–27].

On the other side, previous studies interpreted selective cooling of the tested hand and the increase in histamine reactivity after the RHI as an illusionary disownership and as a sign of rejection of the real hand in favor of the artificial limb[54,63]. A similar interpretation was provided for the downregulation of the somatosensory[64,65] and motor[66–68] cortices when the fake hand is embodied, resulting in a reduction of the amplitude of the recorded somatosensory and motor evoked potentials. In line with our finding, mounting an immune response towards a disowned limb would likely go through an increase in the blood flow towards the targeted limb. Also, this hypothesis fits with the presence of a correlation between the reduction of the skin conductance response to the threatening of a fake hand and the loss of its self-attribution[69]. In regard to the time course of the measured effect, the difference between illusion and control conditions was demonstrated in the 66–100s time window after the beginning of the trial, whereas previous research demonstrated a sympathetic-induced increase in the variability of the non-specific skin conductance response in the 10–55s range[50]. This temporal mismatch between the effect seen for the skin conductance response and the one seen for blood flow could be either due to the time that the flow needed to bounce back after the initial drop, or to the different sudomotor and vasomotor dynamics induced by the sympathetic activation. Indeed, a temporal dissociation between responses to sympathetic activity in the skin and muscle tissue was unveiled while monitoring sympathetic neural activity during handgrip. The former abruptly raised at the onset of the task and the latter increased slowly after a 60s latency[70]. Despite having a cumulative faster growth rate, the synchronous illusory condition had slower initial (<30 s) dynamics. Interestingly, this behavior could be explained by the temporal dissociation of the ANS effect on skin and muscles. The more marked skin vasoconstriction elicited by a higher sympathetic activity in the *Synch* condition could slow down the rise of the blood flow in the first phase of the trial. However, in the following phase, when the increment of the vasodilation in skeletal muscles supersedes skin vasoconstriction, the blood flow level in the *Synch* condition rapidly grows and overcomes the others.

Previous work highlighted that it is possible to induce an increase in arousal just approaching a rubber hand placed in a congruent way with respect to the real hidden hand[71] and this effect could contribute to our outcomes. In order to assess the effect of the visual stroking per se, future studies could measure the blood flow when the stroking is delivered only on the rubber hand.

The RHI paradigm is an easy test to perform to evaluate embodiment. For its simplicity, low requirements and costs, it has been extremely widespread in research related to the representation of the body. It is not free of possible weaknesses[72–74]; one of them is that it lacks objective measures to evaluate its outcome. As previously suggested for the fluctuation in the non-specific skin conductance response[50], the blood flow may be a good candidate to evaluate the achieved embodiment as well. Indeed, the increase in the blood flow significantly correlated with all the other employed measures designed to rate the strength of the illusion (RHI index, vividness and prevalence scores), except for proprioceptive drift, which is often a dissociated measure weighting different aspects of the embodiment process[36].

In conclusion, we demonstrated that the modulation of the sense of limb ownership impacts on the blood flow directed to that limb. It is likely that the fake hand embodiment induced a sympathetic driven vasodilatation of the muscular territories downstream of the brachial artery.

This is a further proof that there is a bidirectional influence between the ANS and body ownership. Interoception, led by the afferent branch of the ANS, contributes to shape the sense of body ownership and, in turn, this modulation changes the autonomic outflow and becomes manifested through changes of the sudomotor[50] and vasomotor activity. Another interesting manifestation of such bidirectional influence is that embodiment of a fake hand seems to alter real hand temperature and, in turn, the propensity to perceive the embodiment illusion seems to be influenced by the hand temperature itself[50].

An important overlap of the brain circuits in charge of the representation of the body with those processing interoception and controlling body temperature, heart and vessel function has been recently confirmed by several experimental, meta-analytic and theoretical works[75–78], which highlighted the main role played by premotor, parietal-temporal, cingulate cortex, the amygdala and the insula.

This is the first study demonstrating that the update of the perceptual status leading to a change of a limb presence in the body representation is paralleled by an enhancement in the perfusion of the tested limb. It also opens the intriguing question of whether the reported changes are unspecific effects of an alert response regarding the whole body or, on the contrary, are specifically causally and topographically related to the limb, the representation of which was modulated. We speculated on this topic providing cues in favor of the latter. This, however, remains an extremely interesting question, a matter still open for future research.

Data availability

Underlying data

Mendeley Data: Embodying an artificial hand increases blood flow to the investigated limb. http://dx.doi.org/10.17632/pcbtb8xfg6.1[41].

This project contains the following underlying data:

- Dataset.mat (matrices of data, Matlab dataset)
- Table_MeanBF.csv (mean blood flow [20 participants X 12000 samples (from -20s to 100s at 100Hz) X 3 conditions (order: VisionOnly, Synch, Asynch)])

- Table_PD.csv (proprioceptive drift [20 participants X 2 conditions (order: Synch, Asynch)])
- Table_RHIi.csv (RHI index [20 participants X 2 conditions (order: Synch, Asynch)])
- Table_RI.csv (resistance index [20 participants X 12000 samples (from -20s to 100s at 100Hz) X 3 conditions (order: VisionOnly, Synch, Asynch)])
- Table_T.csv (prevalence score [20 participants X 2 conditions (order: Synch, Asynch)])
- Table_V.csv (vividness score [20 participants X 2 conditions (order: Synch, Asynch)])

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Reviewer Report 01 September 2021

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✗ **Henrik Ehrsson**
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1. Summary

The study is the first to examine the relation between arm blood flow and body ownership in a multisensory bodily illusion, a digital version of the rubber hand illusion. Although the authors ask an interesting and novel question about the link between body ownership and autonomic nervous system activity, the study has some major methodological problems. These issues could potentially be resolved by conducting a second experiment, providing conclusive evidence.

2. Is the work clearly and accurately presented and does it cite the current literature?

The authors provide a background of their study that is sufficient to understand its rationale. However, the literature on interoception, the autonomic system, and body ownership is complex and results are mixed, and this is not always conveyed to the reader. For example, the fifth paragraph of the introduction "...strong relationship between body representation and interoceptive signals" and "interoceptive sensitivity predicts the malleability of participants' body representation ref 20". Actually, there are several previous studies that have failed to replicate relationships between rubber hand illusion and interoception (Horváth *et al* 2020; Crucianelli *et al*. 2018; Critchely *et al*, 2021) making the relationship between body ownership RHI and interoception somewhat unclear. These, or some of these, studies should probably be cited and a more balanced view presented.

The work is accurately presented overall; however, some parts need clarification (see sections below).

3. Is the study design appropriate and does the work have academic merit?

The study has several major problems. The biggest is that the results only provide partial support for the main conclusion and that the results are explorative in my opinion and it is unclear if they

can be replicated. The changes in blood flow occur with a very large delay (about 50 s) compared to the likely onset of the hand ownership illusion (about 10s), and we do not understand why. Of course, it could be a slow physiological response that takes time to get going, but it could also be a random fluctuation in the signal that will not be replicated. The statistical analysis is problematic in this case because the authors arbitrarily divide the data into three periods and then find significant differences only at the last, most delayed period. Noteworthy, in the whole period from 15 to 45 seconds, there is no difference between the synchronous and the asynchronous conditions although the illusion is for sure vividly experienced during this period (Gentile et al 2013; Ehrsson *et al* 2004; Lloyd 2007), which can be seen as an observation that goes against the main conclusions of the study. In my opinion, this first experiment corresponds to an explorative, descriptive approach that is OK to use in a first experiment, but now the authors need to conduct a second experiment to test the specific hypothesis generated from the first experiment of a delayed blood flow change induced by the illusion. As it is now, I would not feel comfortable citing this study; I do not know if the finding is real or merely a statistical false positive or an artifact.

Additional serious problems are that the visual-only condition is not matched for tactile stimulation, and thus difficult to compare with the illusion condition. Moreover, this control condition shows higher blood flow than the synchronous illusion condition in the critical time period of 50 to 100 seconds, which is an observation that speaks against the authors' main conclusion of illusion-induced increases in blood flow. Also, the fitted exponential models for the VisionOnly and the Sync conditions look very similar.

Related to this, the authors do not collect questionnaire data from the VisionOnly condition, so we cannot know for sure the illusion was significantly weaker in this condition (even if it is likely). The authors motivate their decision of not including the questionnaire in the VisionOnly condition because "the most significant Botvinick and Cohen questions focus on being touched by the brush, and they lose meaning if the hand is not touched". But this does not make sense to me because they could remove the referral of touch statements and just analyze the ownership statement; many previous studies have done so.

A further limitation that the authors already discuss is that it would be good to record the blood flow from both arms at the same time to confirm that the observed effect is specific to the limb exposed to the illusion. However, I do not understand what the authors mean by saying that "our experimental setup was not robust enough for that"? Please edit for clarity.

A further methodological point is how it was ensured that the recording device's probe was kept still during the experiments, given that it was held by the experimenter manually throughout the procedure. Could this way of recording have produced any artifacts that potentially influenced the outcome?

4. Are sufficient details of methods and analysis provided to allow replication by others?

The Methods and Results sections provide a sufficient amount of details regarding the procedure and the statistical analyses. However, I do not think the arbitrary division of the data into three time periods is well motivated.

The sample size was not justified well. The authors write that "the number of participants was chosen equal to previous RHI studies". However, they do not cite any studies. Moreover, basic

demographics information of the participants should be reported in the Methods section.

5. Are all the source data underlying the results available to ensure full reproducibility?

Yes.

6. Are the conclusions drawn adequately supported by the results?

Not really. I think you need to run a second experiment to demonstrate that the main finding of a delayed illusion-related increase in blood flow is real. The experiment you have so far is hypothesis-generating. Now you need a hypothesis testing experiment.

Additional control conditions would also be good to have to further strengthen the conclusion that the effect is related to changes in embodiment and not to visuotactile synchrony or asynchrony *per se*. This could be, for example, control conditions with synchronous visuotactile stimulation, but the hand presented in an anatomically implausible position, or the strokes are delivered in different directions (spatial incongruence manipulations) (e.g. Gentile *et al* 2013).

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and does the work have academic merit?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cognitive neuroscience, body representation research, and bodily illusions.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Oct 2021

Marco D'Alonzo, Campus Bio-Medico University of Rome, via Alvaro del Portillo, 5, Rome, Italy

The revised version of the manuscript entitled “Embodying an artificial hand increases blood flow to the investigated limb” is updated. We wish to thank the Reviewers for the time spent on our manuscript. We are really pleased with the interest toward our work. We carefully considered the Reviewers’ observations and suggestions that helped us to refine the quality of the draft. Point-by-point replies to the Reviewers’ comments (in *italics*) are provided below, and changes to the text are highlighted in red here and in the manuscript. We look forward to your response. Sincerely, on behalf of all co-authors Marco D’Alonzo and Giovanni Di Pino.

The study is the first to examine the relation between arm blood flow and body ownership in a multisensory bodily illusion, a digital version of the rubber hand illusion. Although the authors ask an interesting and novel question about the link between body ownership and autonomic nervous system activity, the study has some major methodological problems. These issues could potentially be resolved by conducting a second experiment, providing conclusive evidence.

Authors’ Response: Dear Prof. Ehrsson, we are really honored that one of the worldwide most expert in this topic devoted his precious time to read our paper and suggest ways to improve it. We took into account all your inputs and where we could we have tried to implement them as much as possible. We modified the text in line with your suggestions. Unfortunately, at the present, we are not in the possibility to replicate the experiment for the reasons explained below. However, we have re-analyzed our data from the scratch, deleting the previous arbitrary division so much criticized and we have also added the analysis of the VisionOnly proprioceptive drift, suggesting no embodiment in this condition, which we previously decided not to include in the manuscript. This work costs big effort and we deeply trust on the reliability of the results we found. We hope that in its present version it will be worth of your trust and approval.

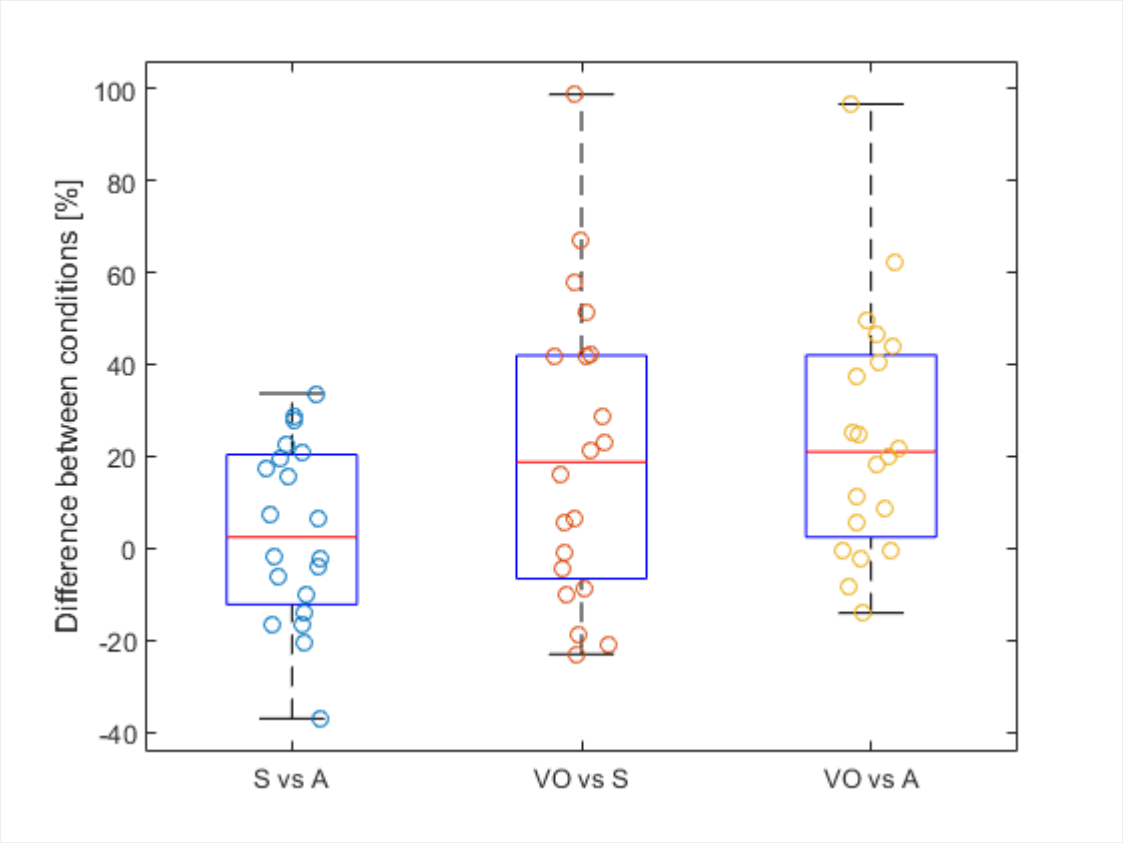
The authors provide a background of their study that is sufficient to understand its rationale. However, the literature on interoception, the autonomic system, and body ownership is complex and results are mixed, and this is not always conveyed to the reader. For example, the fifth paragraph of the introduction "...strong relationship between body representation and interoceptive signals" and "interoceptive sensitivity predicts the malleability of participants' body representation ref 20". Actually, there are several previous studies that have failed to replicate relationships between rubber hand illusion and interoception (Horváth et al 2020; Crucianelli et al. 2018; Critchely et al, 2021) making the relationship between body ownership RHI and interoception somewhat unclear. These, or some of these, studies should probably be cited and a more balanced view presented.

Authors' Response: We trust on the impact of ANS on body representation, thus we may have been biased in the presenting the introduction in favor of this hypothesis. Thank you for having make us realize this. Accordingly with your suggestion, the introduction has been rewritten to present a more balanced view:
"Moreover, ANS homeostatic information related to pain, temperature, pH, carbon dioxide, and oxygen are sent to the insula and interact with somatosensory processing. This has been suggested to have a role in the construction of the body representation [2]." "Evidence of the tight connection between the ANS and central body representation may be derived from complex regional pain syndrome (CRPS) [7]" "Beside pathological models, in healthy subject the meaning and strength of the relationship between body representation and interoceptive signals is still matter of debate. For example, emerging evidence suggests interoceptive information such as cardiac feedback to modulate the visual body perception [16] and influence one's own body awareness [17, 18] or, vice-versa, changes in body-ownership and self-identification to alter the ability to detect internal body signals [19]. Furthermore, interoceptive sensitivity seems to predict the malleability of participants' body representation [20]." Moreover, an additional paragraph was inserted in the introduction: "On the other hand, the relationship between ownership generated by the RHI paradigm and these interoceptive measures was not always confirmed [80] and other interoceptive indexes were found not correlated to the strength of the illusion during the RHI paradigm: e.g. the capability of participants in heartbeat counting tasks [81, 82]."

The changes in blood flow occur with a very large delay (about 50 s) compared to the likely onset of the hand ownership illusion (about 10s), and we do not understand why. Of course, it could be a slow physiological response that takes time to get going, but it could also be a random fluctuation in the signal that will not be replicated.

Authors' Response: "it could also be a random fluctuation in the signal that will not be replicated" Considering that the reviewer asked about the replicability of the results, we performed an additional analysis in order to check how much our results are robust and replicable among the different participants (i.e. whether the obtained results could be due to peculiar behavior of few outlier participants). The difference in mean blood flow between conditions in each participant was calculated in the intervals of interest, and the distribution of these data was plotted (attached figure 2R: Δt_1 = [5 - 31s] data in the left side and Δt_2 = [69 - 100s] data in the right side). All the resulting distributions were found normal by using Kolmogorov Smirnoff test, demonstrating that the distribution of the differences was not

skewed. Focusing on the most interesting distribution of data (i.e. the difference in blood flow value between Synch and Asynch conditions in the second interval of interest, in the ellipse in figure 2R), a higher value of Synch condition with respect to Asynch one was highlighted for main part of participants (15 out of 20 had difference between Synch and Asynch higher than 0, mean and median values of the distribution were also higher than 0), additionally, no outliers were present. This demonstrate a consistent behavior (homogeneity of the sample) among the participants, showing a tendency to have higher Synch value with respect to the Asynch one, and demonstrating the replicability of our results.



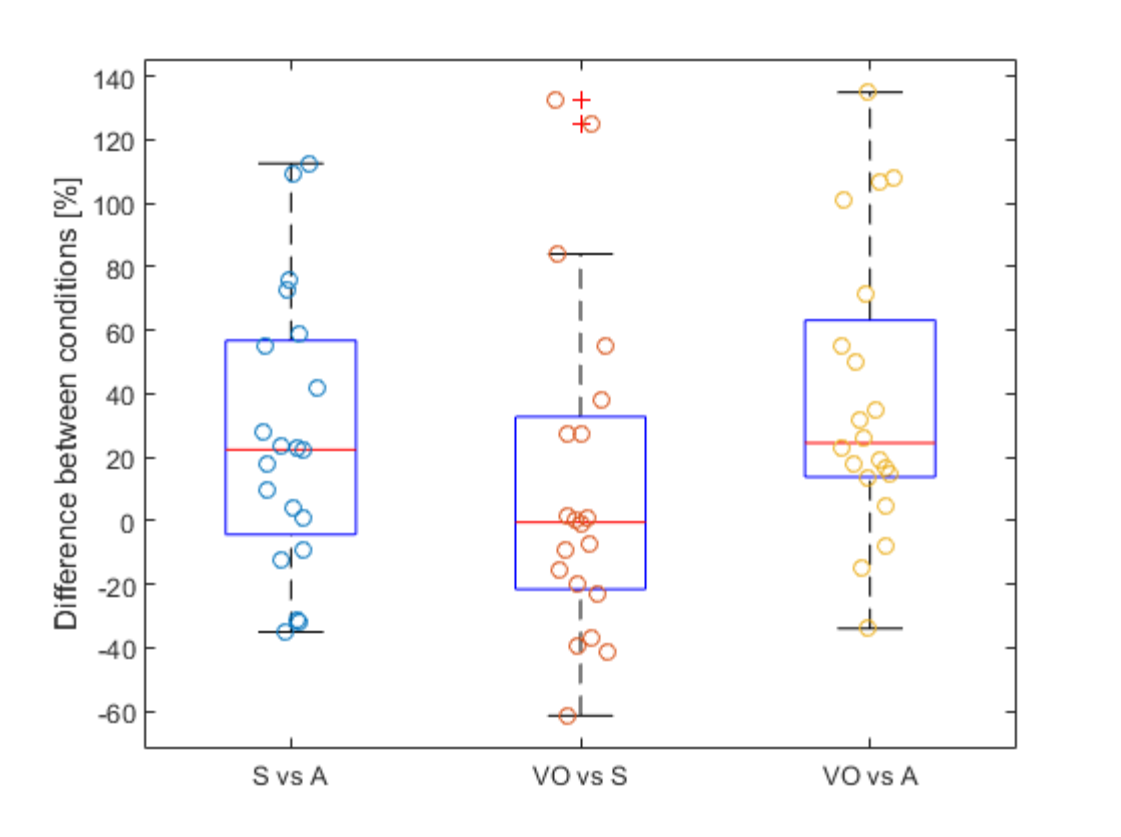


Figure 2R: Difference in blood flow value between conditions in the time-intervals of interest (Δt1, left; Δt2, right; S, Synch; A, Asynch; VO, VisionOnly). Box and whisker plots: median (red lines), 1st and 3rd quartiles (box), lowest and highest values comprised within 1.5 times the interquartile range from the 1st and 3rd percentiles (whisker).

The statistical analysis is problematic in this case because the authors arbitrarily divide the data into three periods and then find significant differences only at the last, most delayed period. Noteworthy, in the whole period from 15 to 45 seconds, there is no difference between the synchronous and the asynchronous conditions although the illusion is for sure vividly experienced during this period (Gentile et al 2013; Ehrsson et al 2004; Lloyd 2007), which can be seen as an observation that goes against the main conclusions of the study.

Authors' Response: In the new version, the statistical analysis has been re-run without the criticized a priori division of time periods (please see the answers below). We agree with the reviewer that from 15 to 45 seconds the illusion is already present. We suppose that after the initial drop, the flow needs 70 seconds to reach significantly different average flow value because of the slow time course of the signal. However, please consider that the growth rate of the Synch signal becomes different from the Asynch already at 25s and it becomes different from VisioOnly at about 50s (Figure 5).

This was highlighted in Results section:
"After this drop, the *F(t)* tends to increase in all conditions. In particular, the mean blood

flow behavior for *Synch* condition starts to have a higher growth than *Asynch* already after 25 s, and higher than *VisionOnly* after about 48 s." To overcame the arbitrary division in time windows, we decided to make a new analysis to identify the intervals where perform the statistics. We compute a point-by-point ANOVA using multiple datasets and a permutation testing to find the cluster of significant difference among the three conditions. In particular, we employed 250 permutations in *clustersize-based permutation testing* and *percentile of mean cluster sum* as method to define the threshold distinguishing between "significant" and "non-significant" clusters. We found two different "significant" clusters one in the interval between 5 and 31 s and the other between 69 and 100 s. In this way, we focused our analysis only on these two intervals. "In order to identify the time intervals where perform the statistics, we computed a point-by-point ANOVA using multiple datasets and a permutation testing to find the cluster of significant difference among the three conditions. In particular, we employed 250 permutations in clustersize-based permutation testing and percentile of mean cluster sum as method to define the threshold distinguishing between "significant" and "non-significant" clusters. We found two different "significant" clusters: one in the interval between 5 and 31 s (Δt_1) and the other between 69 and 100 s (Δt_2). We focused our analysis only on these two intervals."

In my opinion, this first experiment corresponds to an explorative, descriptive approach that is OK to use in a first experiment, but now the authors need to conduct a second experiment to test the specific hypothesis generated from the first experiment of a delayed blood flow change induced by the illusion.

Authors' Response: As regard as the request of a second experiment, please see the last reply (below).

Additional serious problems are that the visual-only condition is not matched for tactile stimulation, and thus difficult to compare with the illusion condition.

Authors' Response: In principle, we wanted to test just synchronous vs asynchronous condition. When we performed preliminary recordings to test the setup, we noted that the touch of the brush, independently if synchronous or asynchronous, produced an initial drop of the blood flow. When multiple factors determine an effect, to control for the contribution of one of those factors either a control condition with only that factor or a control condition without that factor can be used. Since our experimental question was not linked to the change of flow induced by the touch, to be able to isolate the searched effect, theoretically we had two possibilities: either i) introducing a condition with only brush-stroking, which however would have left several open questions (related to how managing visual feedback of the real and rubber hand), or, ii) on the contrary, introducing a condition not affected by touch. Considering that in the RHI literature a control condition without brush-stroking was previously and largely employed, we decided to proceed with the latter option and use the VisionOnly condition. In few words, having a condition without touch was done purposely to isolate the effect of our enquire from the unwanted mere effect of touch.

This is clearly stated in the text. "Since we suspected that brush-stroking itself could have affected the flow independently from the achieved embodiment, a third condition was introduced as further control, where participants were instructed to simply look at the fake

hand, without receiving any paintbrush stimulation on the real or on the fake hand (*VisionOnly* condition). In the latter case, tactile stimuli were not present." And "Such condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an additional condition of no embodiment [36]."

Moreover, this control condition shows higher blood flow than the synchronous illusion condition in the critical time period of 50 to 100 seconds, which is an observation that speaks against the authors' main conclusion of illusion-induced increases in blood flow.

Authors' Response: This is true only before correcting for the drop (new Figure 4), because VisionOnly was not affected (or only marginally), by the initial drop. Once we corrected for the initial drop (Figure 5) from around 35 sec to the end VisionOnly (black line) is below the Synch condition (blue line). Thus, we do not see this observation speaking against our main conclusion.

Also, the fitted exponential models for the VisionOnly and the Sync conditions look very similar.

Authors' Response: When we corrected the blood flow for the initial drop and modelled it with an exponential curve we tested two parameters, a connected to the average value and b, which was the slope of the curve, i.e. the growth rate. Actually, the growth rate (the b values) of the Synch was significantly higher than VisionOnly (as well as higher than Asynch), while there was no significant difference between VisionOnly and Asynch, which means that there was a similar growth dynamics for these two conditions. Please see Figure 5. However, it is true that the blood flow curves between VisionOnly and Synch condition are closer in terms of mean values, even when the drop was subtracted. We tried to speculate on the reason of it, suggesting two possible hypotheses: i) the higher VisionOnly value in the second interval may be the effect of the previous milder VisionOnly drop; ii) it may be the effect of the concomitant absence of stroking of the real hidden hand. This speculation has been added to the text It is likely that, in case of VisionOnly condition, the absence of stroking on the hidden hand during the session could have affected the blood flow value and caused a higher increase in the blood flow with respect to the case of the asynchronous condition. This was reported in the main text: "Compared to *Asynch*, the higher *VisionOnly* value in the second interval may be the effect of the previous milder *VisionOnly* drop or due to the absence of stroking on the hidden hand during the session."

Related to this, the authors do not collect questionnaire data from the VisionOnly condition, so we cannot know for sure the illusion was significantly weaker in this condition (even if it is likely). The authors motivate their decision of not including the questionnaire in the VisionOnly condition because "the most significant Botvinick and Cohen questions focus on being touched by the brush, and they lose meaning if the hand is not touched". But this does not make sense to me because they could remove the referral of touch statements and just analyze the ownership statement; many previous studies have done so.

Authors' Response: The issue of original Botvinick and Cohen questions losing meaning if the hand was not touched has already been raised by Rhode *et al* (2011), the first authors to test this condition (in Rhode *et al* (2011), the ownership questionnaire was not recorded for such condition).

As the Reviewer suggests, we could change the list of the questionnaire, by deleting the questions relative to the touch. However, either we should have deleted those items for all conditions (losing an important part of the illusion outcome also for Synch and Asynch), or we should have tested a different number of items in different comparisons. We thought the latter solution would have messed the experimental design. We collected the proprioceptive drift, however not having the questionnaire, for homogeneity and for maintaining simpler the study design, we originally decided not to analyze and include it in the manuscript. These data were included now: "Such condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an additional condition of no embodiment [36] . As in previous studies [36], questionnaire outcomes were not recorded in this condition." A posteriori, we agree with the Reviewer that not having collected the questionnaire in VisionOnly was a bad choice. Considering Reviewer's suggestion, we have now analyzed VisionOnly proprioceptive drift and we found it not significantly different from Asynch and significantly lower than Synch (new Figure 3), supporting the claim of no embodiment for VisionOnly, as much as concerning this measure. We hope that this may help to ease Reviewer's concern about the absence of cue on VisionOnly embodiment. Nevertheless, we cannot hide that the absence of the questionnaire in this condition is a limitation of our design. Indeed, this is clearly acknowledged in the discussion section of the manuscript (see below). RHI is a model with several constraints, of which we are aware and which we accept each time we gather any insight from it. Considering that the main control condition was the Asynch, and that VisionOnly was introduced only to test the effect of the absence of touch on the initial drop, while testing VisionOnly embodiment was not part of the scope of the paper, we strongly think that, once acknowledged, this limitation is not enough to preclude the publication of our results. . "Indeed, despite embodiment illusion being strongly dependent on the integration of coherent multisensory afferences, previous studies hypothesized the mere vision of a fake hand placed in a congruent position as being able to induce some mild degree of embodiment [44, 45], while another study did not [46]. Being the proprioceptive drift significantly lower in *VisionOnly* than in *Synch,* and similar to *Asynch*, *VisionOnly* induced illusion should be very low. Unfortunately, we cannot take a conclusive position on this possibility, because we did not collect questionnaire in *VisionOnly* for two reasons: i) this condition was introduced to control for the cause of the initial flow drop while testing its embodiment was not its original scope; ii) as previously raised [36], several very important items of Botvinick and Cohen questionnaire focus on being touched by the brush, and they lose meaning if the hand is not touched. We collected proprioceptive drift, but considering that this measure is related to different embodiment aspects than the questionnaire [36], the absence of questionnaires in the *VisionOnly* condition should be considered a limitation of the study and it is envisaged for future works investigating the topic."

A further limitation that the authors already discuss is that it would be good to record the blood flow from both arms at the same time to confirm that the observed effect is specific to the limb exposed to the illusion. However, I do not understand what the authors mean by saying that "our experimental setup was not robust enough for that"? Please edit for clarity.

Authors' Response: We did not record the blood flow in the non-tested limb. The flow should have been recorded at the same time in the two limbs. We had already tried to

record at the same time in the two limbs, but results were not trustable with the experimental setup we implemented, because for a single experimenter was not feasible to hold still two probes and accurately monitor the blood flow on the two arms. Blood flow recording should be done by an ultrasonography expert, being it very operator-dependent, and the only involved author with this skill was Dr Altamura (this skill is confirmed by her publication record).

This was better explained in the text: “we realized that our experimental setup was not robust enough for that, because for a single experimenter it was not feasible to hold still two probes and accurately monitor the blood flow on the two arms.”

A further methodological point is how it was ensured that the recording device's probe was kept still during the experiments, given that it was held by the experimenter manually throughout the procedure. Could this way of recording have produced any artifacts that potentially influenced the outcome?

Authors’ Response: Up today, most of neurophysiology experiments still relays on stimulation, recording and perturbation performed manually by experimenters. For instance, let’s think to all TMS experiments where the coil is manually held upon the designed hotspot by the experimenter. For such reason, especially when the task is really operator-dependent, the only caution we can have is to rely on experimenter expertise.

In our case, the experimenter devoted to maintaining still the probe was a Neurologist expert in ultrasonography, thus in measuring the blood flow using the doppler device who perform on average twenty clinical exams per day, this was better specified in Methods section: “During the whole protocol, the probe was kept still by a second experimenter (CA) expert in ultrasonography.” This was a further reason which did not allowed to record two arms simultaneously. Thus, it is unlikely that blood flow recording was affected by artifact; however, even if any artifact was produced, this should be present in each condition, considering that the way how the experimenter maintaining the probe did not change among conditions. Additionally, the experimenter maintaining the probe was not confident with the RHI protocol and she was not in the position to see the real hand brushing performed by the other experimenter. Thus, she was blind about the stimulation condition, unable to bias blood flow recording. This was added to the Methods. “The second experimenter maintaining the probe was not confident with the RHI protocol and was not in the position to see the real hand brushing performed by the other experimenter.”

The Methods and Results sections provide a sufficient amount of details regarding the procedure and the statistical analyses. However, I do not think the arbitrary division of the data into three time periods is well motivated.

Authors’ Response: We had a reason to divide the three periods, however we are sorry if we were not able to explain it, thus making our choice to look arbitrary. In order to avoid any doubt, we decided to re-run a brand new analysis to identify blindly the intervals of interest where statistics was then performed. For such reason, we compute a point-by-point ANOVA using multiple datasets and a permutation testing to find the cluster of significant difference among the three conditions. In particular, we employed 250 permutations in

clustersize-based permutation testing and *percentile of mean cluster sum* as method to define the threshold distinguishing between "significant" and "non-significant" clusters. We found two different "significant" clusters one in the interval between 5 and 31 s and the other between 69 and 100 s. Then, we focused our analysis only on these two intervals, where we found the same results previously found with the three time periods a priori chosen.

The sample size was not justified well. The authors write that "the number of participants was chosen equal to previous RHI studies". However, they do not cite any studies. Moreover, basic demographics information of the participants should be reported in the Methods section.

Authors' Response: We are really sorry for the missing information. The references relative to the sample size were added in the sentence: "the number of participants was chosen equal to previous RHI studies [30, 32, 34, 36, 84, 85]."

References Armel KC, Ramachandran VS. Projecting sensations to external objects: evidence from skin conductance response. Proc Biol Sci. 2003;270(1523):1499-506. Abdulkarim Z, Ehrsson HH. No causal link between changes in hand position sense and feeling of limb ownership in the rubber hand illusion. Attention, Perception, & Psychophysics. 2016;78(2):707-20. D'Alonzo M, Mioli A, Formica D, Vollero L, Di Pino G. Different level of virtualization of sight and touch produces the uncanny valley of avatar's hand embodiment. Scientific reports. 2019;9(1):1-11. Rohde M, Di Luca M, Ernst MO. The rubber hand illusion: feeling of ownership and proprioceptive drift do not go hand in hand. PloS one. 2011;6(6):e21659. Kalckert A, Ehrsson HH. The moving rubber hand illusion revisited: Comparing movements and visuotactile stimulation to induce illusory ownership. Consciousness and cognition. 2014;26:117-32. Peviani V, Magnani F, Ciricugno A, Vecchi T, Bottini G. Rubber hand illusion survives ventral premotor area inhibition: a rTMS study. Neuropsychologia. 2018;120:18-24.

As regard the suggestion to include basic demographic information in Methods, we have to say that, originally, such info "Twenty volunteers took part in the experiment (age: 29.55 ± 6.12; 12 M, 8 F; 20 right-handed as by self-report)" was indeed placed on the Methods section. Then, the information was placed in Results following the suggestions of the editorial team of Open Research Europe. However, the number of participants is also reported now in Methods section: "Twenty participants were selected among a population.."

I think you need to run a second experiment to demonstrate that the main finding of a delayed illusion-related increase in blood flow is real. The experiment you have so far is hypothesis-generating. Now you need a hypothesis testing experiment.

Additional control conditions would also be good to have to further strengthen the conclusion that the effect is related to changes in embodiment and not to visuotactile synchrony or asynchrony per se. This could be, for example, control conditions with synchronous visuotactile stimulation, but the hand presented in an anatomically implausible position, or the strokes are delivered in different directions (spatial incongruence manipulations) (e.g. Gentile et al 2013).

Authors' Response: We agree with the Reviewer that additional control conditions would have enriched the study and the strength of our claim. We would like very much to comply

with reviewer's suggestion and conduct a new experiment. Unfortunately, this is not in our possibilities because the Doppler ultrasonographic machine we employed for the data collection was part of the University Hospital equipment, and the operator (CA) was a neurologist part of the staff. Since the Covid-19 restrictive measures started, it is no longer possible for us to conduct experiments on healthy subjects within the Hospital, neither we are allowed to move the machine elsewhere. The machine needed for this experiment is one in a hundred because 99% of Doppler machines does not allow continuous reporting and export of the flow, but they measure just discrete intervals. We are well-aware that our work has limitations, which maybe would have been improved by further experiments.

However, please allow me a more general consideration. Once resolved the issue of an arbitrary division of time windows with a brand new blind analysis, and once partly resolved the absence of cue on embodiment of the supplementary control condition (VisionOnly) and having it acknowledged as limitation we can affirm that our study design is sound. Moreover, study limitations are clearly stated in the manuscript (Discussion):

"the absence of questionnaires in the *VisionOnly* condition should be considered a limitation of the study and it is envisaged for future works investigating the topic." "This is the first study demonstrating that the update of the perceptual status leading to a change of a limb presence in the body representation is paralleled by an enhancement in the perfusion of the tested limb. It also opens the intriguing question of whether the reported changes are unspecific effects of an alert response regarding the whole body or, on the contrary, are specifically causally and topographically related to the limb, the representation of which was modulated. We speculated on this topic providing cues in favor of the latter. This, however, remains an extremely interesting question, a matter still open for future research."
Our sample size is suited to our conclusions. More importantly, we did not just find significantly different flow between test and two controls, but also the correlation of such differences with embodiment measures. I'm pretty sure the Reviewer is aware of how much this is rare when working with heterogeneous healthy participants and mixing physiological and behavioral outcomes. Considering all those points, we respectfully think that our work is worth to be disseminated. As typically in science, if the design of a study and its statistics are correct, confirming its results and expanding them, e.g. through further control conditions, will be matter for future studies, which we hope will consider worth to investigate this topic more deeply. Publishing our pioneering results is worth also to allow others to be involved in the matter.

The aspects highlighted by Prof. Ehrsson were reported in the discussion: "Additionally, to assess the repeatability of our findings, further studies could be performed to assess the difference of synchronous brush-stroking with additional control conditions, such as with the fake hand placed in an incongruent position. In such way, it will be possible to confirm whether the found effect is related to changes in embodiment and not to manipulation of visuotactile stimulation synchrony."

Competing Interests: No competing interests were disclosed.

Reviewer Report 09 August 2021

https://doi.org/10.21956/openreseurope.14712.r27284

Valeria Peviani
Department of Neuroscience, Max Planck Institute for Empirical Aesthetics, Frankfurt, Germany

This work investigates changes in blood flow of the arm during the Rubber Hand Illusion, a well-known procedure that induces the sense of ownership towards a fake hand, by triggering a multimodal mismatch. Participants' blood flow was measured in two conditions of visuo-tactile stimulation (fake hand and unseen participant's hand were stroked simultaneously [synchronous condition], fake hand and unseen participant's hand were stroked non simultaneously [asynchronous condition], and one control condition (same set-up, but no tactile stimulation).

The manuscript is well-written and clear (but see some questions below); results are clearly reported and described by figures. I think the authors raise a very relevant question, and this work could potentially contribute to our knowledge on physiological correlates of transitory abnormal experiences in the context of body ownership.

However, I have major concerns on the experimental design and data analysis, detailed below. In my view, these issues, which undermine the reliability of results and their interpretation, need to be carefully addressed before approval.

I hope my comments and suggestions will be useful to the authors to improve the quality of their work.

Major

1. In the introduction, the authors well describe evidence on the role of interoceptive signals in body perception and awareness. I think it would be also relevant to briefly describe studies that investigated physiological changes possibly related to ANS activity (e.g., skin conductance, temperature) during the RHI and other multisensory illusions.

   Relatedly, I think this paper may be very relevant when introducing the work and discussing results: Teaford, M., Fitzpatrick, J., & James Smart Jr, L. (2021). The impact of experimentally induced limb ischemia on the rubber hand illusion. Perception, 50(1), 88-96.

2. *"A drop of the mean blood flow values was identified at around 10s from the beginning of the conditions. Thus, we corrected the blood curves of all the three conditions to make all of them starting from the same value after the drop, the value of the drop was subtracted to the mean blood flow by using the following equation: …"*

   If I correctly understood, the authors re-aligned the signals for the curve-fitting and coefficient analysis. What was the precise extent of the re-alignment (in time) for each

condition?

3. *"This suggests that the drop was due to the initial, mostly unexpected, tactile stimulation of the hidden hand caused by the brush, regardless of whether the stroke was synchronous or asynchronous and if an illusion was achieved"*

   "[the vision-only] condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an additional condition of no embodiment"

   From these sentences, it reads that the signal drop results from tactile stimulation, and that the vision-only condition was included to control for the effect of tactile stimulation. However, no tactile stimulation was delivered in the vision-only condition, which makes it not suitable to account for signal changes due to mere touch, such as signal changes due to non-repeated tactile stimulation.

   Relatedly, as the authors acknowledged in the discussion, the signal increase in the vision-only condition may be due to increased sense of body ownership. However, in this paradigm, the vision-only condition is not an effective no-embodiment condition, especially since no subjective reports on the feeling of ownership towards the fake hand were collected.

   In this regard, authors state: *"There are both technical and scientific reasons that may suggest not recording embodiment measures in VisionOnly: the most significant Botvinick and Cohen questions focus on being touched by the brush, and they lose meaning if the hand is not touched."*

   In my view, existent ownership questionnaires could be adapted to the experimental set-up, as it has been done in other studies (e.g., for virtual set-ups: Tieri *et al.*, 2017; for mirror-box: Medina *et al.*, 2015).

4. Analyses and discussion seem to rely on the assumption that the signal drop described in the signal in the vision-only condition is due to tactile stimulation, despite no tactile stimulation was delivered in this condition. In my view, this is a critical issue that needs to be carefully addressed.

   How do the authors justify the presence of a drop in the vision-only condition, and which is the criterion used to establish the presence of this drop? How did the authors establish that the drop in the vision-only condition does not reflect a physiological oscillation of the signal? Was this drop systematically present in the sample?

   I would ask the authors to comment on this, revise their analyses and discussion accordingly, and consider collecting further evidence.

   In light of observations reported in comments 3 and 4, I think that this work would substantially improve by including more suitable control condition(s) in a follow-up experiment. The present design poses important limits to the interpretation of results. The vision-only condition does not effectively control for neither touch, since no tactile stimulus was delivered, nor no-embodiment, since no ownership measures were collected.

5. The present investigation does not allow to establish the specificity of the effect, i.e., whether the effect is specific to the arm involved in the RHI, thus related to the feeling of ownership towards it, or represent a more diffuse physiological change associated to the multisensory illusion.

   While the authors put forward indirect evidence for the local specificity of the observed effect in the discussion, this could be clarified by including blood flow measures for the homologous arm or even another body part. This would significantly improve the impact of this work.

6. Order effects are relevant in the context of RHI experiments. Why was the order of conditions randomized and not counterbalanced?

7. *"Considering that the dynamic of the blood flow oscillation at frequencies of 0.02–0.05 Hz are mainly affected by sympathetic nerve activity, we chose to analyze the blood flow signal by splitting the recording session into three time intervals (33s each)."*

   This sentence is not very clear to me. Can the authors further elaborate on the choice of splitting the signal into three intervals?

8. As the authors stated, blood flow dynamics are relatively slow, in the range of 2-to-5 cycles per 100 seconds. In light of this, can the authors justify the choice of a 5-seconds baseline?

Minor

1. Introduction: "*Emerging evidence for the existence of a strong relationship between body representation and interoceptive signals are not confined to pathological models*". I think grammar needs to be adjusted.

2. Method: *"Therefore, in this case, the number of participants was chosen equal to previous RHI studies."* Can the authors cite the studies on which their sample size estimation rely on?

3. Method: I suggest the authors to report the sample size in the participants section as well.

4. Method, Experimental Procedure: I would suggest the authors to clarify how references 33 and 34 relate to the sentence: *"They could see the content of each compartment only when the experimenter turned the relative internal light on"*

5. Method, Results (pg. 8): *"Considering the interaction between … were identified in the second interval"* Were these contrasts corrected for multiple comparisons?

6. It is not clear which *alfa* and *beta* coefficients were included in the correlation analysis whose results are reported in Table 2. I guess those belonging of the synchronous condition, but I would suggest the authors make it explicit.

7. Figure 2: axis label and units are missing in top panel.

References
1. Medina J, Khurana P, Coslett HB: The influence of embodiment on multisensory integration using the mirror box illusion.*Conscious Cogn*. 2015; **37**: 71-82 PubMed Abstract | Publisher Full Text
2. Teaford M, Fitzpatrick J, James Smart L: The Impact of Experimentally Induced Limb Ischemia on the Rubber Hand Illusion.*Perception*. 2021; **50** (1): 88-96 PubMed Abstract | Publisher Full Text
3. Tieri G, Gioia A, Scandola M, Pavone EF, et al.: Visual appearance of a virtual upper limb modulates the temperature of the real hand: a thermal imaging study in Immersive Virtual Reality. *Eur J Neurosci*. **45** (9): 1141-1151 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Body perception and representation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Oct 2021

Marco D'Alonzo, Campus Bio-Medico University of Rome, via Alvaro del Portillo, 5, Rome, Italy

The revised version of the manuscript entitled “Embodying an artificial hand increases blood flow to the investigated limb” is updated. We wish to thank the Reviewers for the time spent on our manuscript. We are really pleased with the interest toward our work. We carefully considered the Reviewers’ observations and suggestions that helped us to refine the quality of the draft. Point-by-point replies to the Reviewers’ comments (in *italics*) are provided below, and changes to the text are highlighted in red here and in the manuscript. We look forward to your response. Sincerely, on behalf of all co-authors Marco D’Alonzo and

Giovanni Di Pino

This work investigates changes in blood flow of the arm during the Rubber Hand Illusion, a well-known procedure that induces the sense of ownership towards a fake hand, by triggering a multimodal mismatch. Participants' blood flow was measured in two conditions of visuo-tactile stimulation (fake hand and unseen participant's hand were stroked simultaneously [synchronous condition], fake hand and unseen participant's hand were stroked non simultaneously [asynchronous condition], and one control condition (same set-up, but no tactile stimulation).

The manuscript is well-written and clear (but see some questions below); results are clearly reported and described by figures. I think the authors raise a very relevant question, and this work could potentially contribute to our knowledge on physiological correlates of transitory abnormal experiences in the context of body ownership.

However, I have major concerns on the experimental design and data analysis, detailed below. In my view, these issues, which undermine the reliability of results and their interpretation, need to be carefully addressed before approval.

I hope my comments and suggestions will be useful to the authors to improve the quality of their work.

Authors' Response: We really thank Prof Peviani for her extremely useful remarks, which allowed us to strength the manuscript. We deeply trust that in its new form it reached the needed level of clearness to deserve her approval.

In the introduction, the authors well describe evidence on the role of interoceptive signals in body perception and awareness. I think it would be also relevant to briefly describe studies that investigated physiological changes possibly related to ANS activity (e.g., skin conductance, temperature) during the RHI and other multisensory illusions.

Relatedly, I think this paper may be very relevant when introducing the work and discussing results: Teaford, M., Fitzpatrick, J., & James Smart Jr, L. (2021). The impact of experimentally induced limb ischemia on the rubber hand illusion. Perception, 50(1), 88-96.

Authors' Response: Thanks for your suggestion. We followed it integrating the suggested reference and now the introduction seems a lot better balanced. The introduction was edited following the suggestion of the Reviewer:
"A link between autonomic mechanisms and cognitive processes behind body representation has been previously demonstrated using RHI paradigm, such as the altered temperature regulation while inducing body ownership over the fake hand [20 79]. The occurrence of the RHI results in a disownership and a decrease of the skin temperature of the real hand [54], but the consistency of such finding is still under debate [58, 59]. Furthermore, increased fluctuations in the skin conductance have been proven to correlate with the onset and the strength of the illusion during the RHI [50]. On the other hand, the relationship between ownership generated by the RHI paradigm and these interoceptive measures was not always confirmed [80] and other interoceptive indexes were found not correlated to the strength of the illusion during the RHI paradigm: e.g. the capability of participants in

heartbeat counting tasks [81, 82]. Interestingly, artificially-induced peripheral ischemia modulated the proprioceptive drift during the RHI paradigm [83]."

"A drop of the mean blood flow values was identified at around 10s from the beginning of the conditions. Thus, we corrected the blood curves of all the three conditions to make all of them starting from the same value after the drop, the value of the drop was subtracted to the mean blood flow by using the following equation: …" *If I correctly understood, the authors re-aligned the signals for the curve-fitting and coefficient analysis. What was the precise extent of the re-alignment (in time) for each condition?*

Authors' Response: We did not realign the signals in time (along the x-axis), but only in amplitude of the flow (dependent variable). All the data was treated with the same procedure of analysis to focus on the flow behaviour beyond the initial drop and to make the results comparable among the three conditions. To make all the curves starting from a common ground and eliminate the offset, we subtract the relative drop (the mean amplitude of the blood flow on the interval between 5 and 15 s after the beginning of the condition) to each condition. We are sorry if this was not clear enough. In the new version Methods have been modified to make this clear:

"we corrected the blood curves (relative shift along the y-axis) of all the three conditions to make all of them starting from the same flow value after the drop. This was done by subtracting the value of the drop to the mean blood flow as in the following equation:

$\Delta Ft=Ft-F\Delta td$ $\Delta F(t) = F(t) - \bar{F}(\Delta t_d)$ (2) where $\bar{F}(\Delta t_d)$ (i.e. the drop of the signal) was calculated as blood flow value averaged on a 10s window centered 10s after the beginning of the trial (i.e. Δt_d = [5s, 15s])... The obtained signal in the interval between 10s and 100s were fitted by using an exponential curve"

"This suggests that the drop was due to the initial, mostly unexpected, tactile stimulation of the hidden hand caused by the brush, regardless of whether the stroke was synchronous or asynchronous and if an illusion was achieved"

"[the vision-only] condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an additional condition of no embodiment" *From these sentences, it reads that the signal drop results from tactile stimulation, and that the vision-only condition was included to control for the effect of tactile stimulation. However, no tactile stimulation was delivered in the vision-only condition, which makes it not suitable to account for signal changes due to mere touch, such as signal changes due to non-repeated tactile stimulation.*

Authors' Response: In principle, we wanted to test just synchronous vs asynchronous condition. When we performed preliminary recordings to test the setup, we noted that the touch of the brush, independently if synchronous or asynchronous, produced an initial drop of the blood flow. When multiple factors determine an effect, to control for the contribution

of one of those factors either a control condition with only that factor or a control condition without that factor can be used. Since our experimental question was not linked to the change of flow induced by the touch, to be able to isolate the searched effect, theoretically we had two possibilities: either i) introducing a condition with only brush-stroking, which however would have left several open questions (related to how managing visual feedback of the real and rubber hand), or, ii) on the contrary, introducing a condition not affected by touch. Considering that in the RHI literature a control condition without brush-stroking was previously employed, we decided to proceed with the latter option and use the VisionOnly condition. In few words, having a condition without touch was done purposely to isolate the effect of our enquire from the unwanted mere effect of touch. Regarding the suitability to use VisioOnly to control for the impact of touch on the drop, please see also our reply to your comment n 6 (below). We hope that this explication makes things clearer.

This is clearly stated in the text: "Since we suspected that brush-stroking itself could have affected the flow independently from the achieved embodiment, a third condition was introduced as further control, where participants were instructed to simply look at the fake hand, without receiving any paintbrush stimulation on the real or on the fake hand (*VisionOnly* condition). In the latter case, tactile stimuli were not present." and "Such condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an additional condition of no embodiment [36]."

Relatedly, as the authors acknowledged in the discussion, the signal increase in the vision-only condition may be due to increased sense of body ownership. However, in this paradigm, the vision-only condition is not an effective no-embodiment condition, especially since no subjective reports on the feeling of ownership towards the fake hand were collected. In this regard, authors state: ***"There are both technical and scientific reasons that may suggest not recording embodiment measures in VisionOnly: the most significant Botvinick and Cohen questions focus on being touched by the brush, and they lose meaning if the hand is not touched."***

In my view, existent ownership questionnaires could be adapted to the experimental set-up, as it has been done in other studies (e.g., for virtual set-ups: Tieri et al., 2017; for mirror-box: Medina et al., 2015).

Authors' Response: The issue of original Botvinick and Cohen questions losing meaning if the hand was not touched has already been raised by Rhode *et al* (2011), the first authors to test this condition (in Rhode *et al* (2011), the ownership questionnaire was not recorded for such condition).

As the Reviewer suggest, we could change the list of the questionnaire, by deleting the questions relative to the touch. However, either we should have deleted those items for all conditions (losing an important part of the illusion outcome also for Synch and Asynch), or we should have tested a different number of items in different comparisons. We thought the latter solution would have messed the experimental design. We collected the proprioceptive drift, however not having the questionnaire, for homogeneity and for maintaining simpler the study design, we originally decided not to analyze and include it in the manuscript. Now these data were included: "Such condition was performed in order to control for the effect of mere tactile stimulation on the blood flow and was considered as an

additional condition of no embodiment [36] . As in previous studies [36], questionnaire outcomes were not recorded in this condition."

A posteriori, we agree with the Reviewer that not having collected the questionnaire in VisionOnly was a bad choice. Considering Reviewer's suggestion, we have now analyzed VisionOnly proprioceptive drift and we found it not significantly different from Asynch and significantly lower than Synch (new Figure 3), supporting the claim of no embodiment for VisionOnly, as much as concerning this measure. We hope that this may help to ease Reviewer's concern about the absence of cue on VisionOnly embodiment. Nevertheless, we cannot hide that the absence of the questionnaire in this condition is a limitation of our design. Indeed, this is clearly acknowledged in the discussion section of the manuscript (see below). RHI is a model with several constraints, of which we are aware and which we accept each time we gather any insight from it. Considering that the main control condition was the Asynch, and that VisionOnly was introduced only to test the effect of the absence of touch on the initial drop, while testing VisionOnly embodiment was not part of the scope of the paper, we strongly think that, once acknowledged, this limitation is not enough to preclude the publication of our results. "Indeed, despite embodiment illusion being strongly dependent on the integration of coherent multisensory afferences, previous studies hypothesized the mere vision of a fake hand placed in a congruent position as being able to induce some mild degree of embodiment [44, 45], while another study did not [46]. Being the proprioceptive drift significantly lower in *VisionOnly* than in *Synch,* and similar to *Asynch*, *VisionOnly* induced illusion should be very low. Unfortunately, we cannot take a conclusive position on this possibility, because we did not collect questionnaire in *VisionOnly* for two reasons: i) this condition was introduced to control for the cause of the initial flow drop while testing its embodiment was not its original scope; ii) as previously raised [36], several very important items of Botvinick and Cohen questionnaire focus on being touched by the brush, and they lose meaning if the hand is not touched. We collected proprioceptive drift, but considering that this measure is related to different embodiment aspects than the questionnaire [36], the absence of questionnaires in the *VisionOnly* condition should be considered a limitation of the study and it is envisaged for future works investigating the topic."

Analyses and discussion seem to rely on the assumption that the signal drop described in the signal in the vision-only condition is due to tactile stimulation, despite no tactile stimulation was delivered in this condition. In my view, this is a critical issue that needs to be carefully addressed.

How do the authors justify the presence of a drop in the vision-only condition, and which is the criterion used to establish the presence of this drop? How did the authors establish that the drop in the vision-only condition does not reflect a physiological oscillation of the signal? Was this drop systematically present in the sample?

I would ask the authors to comment on this, revise their analyses and discussion accordingly, and consider collecting further evidence.

In light of observations reported in comments 3 and 4, I think that this work would substantially improve by including more suitable control condition(s) in a follow-up experiment. The present design poses important limits to the interpretation of results. The vision-only condition does not

effectively control for neither touch, since no tactile stimulus was delivered, nor no-embodiment, since no ownership measures were collected.

Authors' Response: On this point, respectfully, it seems to us that the Reviewer misunderstood the message of our work (or we were not enough clear).
VisionOnly had no tactile stimulation, neither it had drop (or really negligible compared to Synch and Asynch). This was the reason why we attributed the initial drop to tactile stimulation. The drop (and its absence) can be easily seen by visually inspecting the signals, which clearly show a consistent decrease for all but the VisionOnly condition after about 10 s from the beginning of the stimulation (new Figure 4).

Which is the criterion used to establish the presence of this drop?

Authors' Response: We firstly defined what we meant with drop: "where $\bar{F}(\Delta t_d)$ (i.e. the drop of the signal) was calculated as blood flow value averaged on a 10s window centered 10s after the beginning of the trial (i.e. Δt_d = [5s, 15s])." Reviewer: "How did the authors establish that the drop in the vision-only condition does not reflect a physiological oscillation of the signal?" We do think that the drop in VisionOnly was mainly the physiological oscillation of the signal. Indeed, the values of the drop were (mean ± st. dev.): -18.4 ± 18.3% and -24.8 ± 16.4% for, Synch and Asynch condition respectively, while just -3.4 ± 16.9%, for VisionOnly.

Was this drop systematically present in the sample?

Authors' Response: How much the drop was systematically present in the participant sample can be gathered from the standard error, which was also graphically represented by the blur of the signal in the new Figure 4 (see zoom in Figure 1R). Then, we run a statistical analysis on the drop values: the distribution of drop values was tested against 0 value (i.e. baseline) by using one sample t-test. Only drop values in Synch and Asynch conditions were statistically lower than the baseline, supporting our hypothesis that only in those condition we have a significant drop, thus linking it to the presence of touch in the Synch and Asynch conditions. These edits were reported in Methods section: "For all condition, the signal drop value was analyzed to assess whether it was significantly lower than the baseline (i.e. 0 value), by using a one-sample t-test." And in Results section: "In particular, focusing on the drop values calculated as blood flow value averaged on Δt_d interval, we found that only drop values in *Synch* and *Asynch* conditions were statistically lower than the baseline (*Synch*: $t(19) = -4.52$, $p < 0.001$; *Asynch*: $t(19) = -6.78$, $p < 0.001$). The values of the drop were (mean ± st. dev.): -18.4 ± 18.3% and -24.8 ± 16.4% for, *Synch* and *Asynch* condition respectively, while just -3.4 ± 16.9%, for *VisionOnly*.." However, even if a lot less represented, the Reviewer is right when she says that a subtle decrease of the signal was also present in VisionOnly, peaking at about the same time (10 sec), which is however not different from the normal fluctuation of the blood signal (black line of new Figure 4). We discussed this mild decrease suggesting that, beyond the touch-dependent strong decrease of the flow seen in Synch and Asynch, also other factors other than the touch of the hidden hand, such as the sudden lighting of the RHI platform compartment, likely have produced the flow decrease.

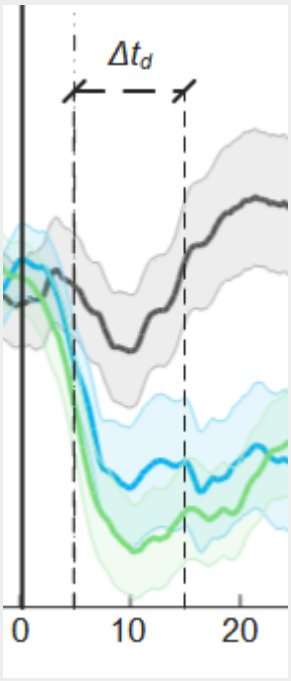


Figure 1R: zoom of Figure 4: Mean blood flow (F) averaged across participants for each condition, the shade represents the standard error (SEM), dashed lines indicate the time intervals where the drop values was calculated (Δt_d).

The present investigation does not allow to establish the specificity of the effect, i.e., whether the effect is specific to the arm involved in the RHI, thus related to the feeling of ownership towards it, or represent a more diffuse physiological change associated to the multisensory illusion.

While the authors put forward indirect evidence for the local specificity of the observed effect in the discussion, this could be clarified by including blood flow measures for the homologous arm or even another body part. This would significantly improve the impact of this work.

Authors' Response: We did not record the blood flow in the non-tested limb. The flow should have been recorded at the same time in the two limbs. We had already tried to record at the same time in the two limbs, but results were not trustable with the experimental setup we implemented, because it was impossible for a single experimenter to hold two probes and accurately monitor the blood flow on the two arms at the same time. Blood flow recording should be done by an ultrasonography expert, being it very operator-dependent, and the only involved author with this skill was Dr Altamura (this skill is confirmed by her publication record).

We are well-aware of this limitation of our study, which we clearly discussed on the manuscript: "In the attempt to test the local specificity of our hypothesis, in a preliminary experiment run before the study, we tried to record the blood flow from both arms at the same time, but unfortunately we realized that our experimental setup was not robust enough for that, because for a single experimenter it was not feasible to hold still two probes and accurately monitor the blood flow on the two arms."

Once established that we were not able to record the flow of the two upper limbs simultaneously, we could have recorded them in different sessions. We agree on that, but unfortunately, we have no the possibility to refine this work with new acquisition. Indeed, the Doppler ultrasonography machine we employed for the data collection was part of the University Hospital equipment, and the operator (CA) is a neurologist part of the staff. Since the Covid-19 restrictive measures started, it is no longer possible for us to conduct experiments on healthy subjects within the Hospital, neither are we allowed to move the machine elsewhere. The machine needed for this experiment is peculiar because 99% of Doppler machines do not allow continuous reporting and export of the flow, but they measure just discrete intervals. To reduce the impact of this limitation, we calculated the resistance index, from which an indirect cue on the local specificity of the autonomic response can be gathered. We found a decrease of the index, sign of vasodilatation, and a negative correlation with flow, suggesting a peripheral vessel origin of the change in the blood flow.

We are well-aware that our work has limitations, which maybe would have been improved by further experiments, as already highlighted in Discussion section: “It also opens the intriguing question of whether the reported changes are unspecific effects of an alert response regarding the whole body or, on the contrary, are specifically causally and topographically related to the limb, the representation of which was modulated. We speculated on this topic providing cues in favor of the latter. This, however, remains an extremely interesting question, a matter still open for future research.” However, our study design is sound, its limitations are clearly stated, our sample size is suited to our conclusions, and we did not just find significantly different flow between test and two controls, but also the correlation of such differences with embodiment measures. Considering all those points, we respectfully think that our work is worth to be disseminated. As typically in science, confirming the results and expanding them, e.g. through further control conditions, will be matter for future studies, which we hope will consider worth to investigate this topic more deeply. Publishing our pioneering results is worth also to allow others to be involved in the matter. We hope that being not in the possibility to acquire new data for a period lasting unpredictably, once the limitations have been clearly acknowledged and the other raised points cleared, Prof Peviani will be in favor to disseminate the results we have gathered.

Order effects are relevant in the context of RHI experiments. Why was the order of conditions randomized and not counterbalanced?

Authors’ Response: Thank you for having raised this point which allowed us to be more detailed in the Methods section. The conditions cannot be completely counterbalanced with 20 participants (in order to have complete counterbalanced order among the conditions, the number of participants to be involved had to be a multiple of six); however, we try to have similar number of conditions for the different ordinal position in our pool of participants (first position in the sequence of performed conditions: 7 VisionOnly, 7 Synch and 6 Asynch; second position: 6 VisionOnly, 7 Synch and 7 Asynch; third position: 7 VisionOnly, 6 Synch and 7 Asynch). This has been now reported in the main test: “Similar number of conditions for the different ordinal position in experimental sequence

was obtained in our pool of participants (first position in the sequence of performed conditions: 7 *VisionOnly*, 7 *Synch* and 6 *Asynch*; second position: 6 *VisionOnly*, 7 *Synch* and 7 *Asynch*; third position: 7 *VisionOnly*, 6 *Synch* and 7 *Asynch*)."

"Considering that the dynamic of the blood flow oscillation at frequencies of 0.02–0.05 Hz are mainly affected by sympathetic nerve activity, we chose to analyze the blood flow signal by splitting the recording session into three time intervals (33s each)."
This sentence is not very clear to me. Can the authors further elaborate on the choice of splitting the signal into three intervals?

Authors' Response: We had a reason to divide the three periods, however we are sorry if we were not able to explain it, thus making our choice to look arbitrary. In order to avoid any doubt, we decided to re-run a brand new analysis to identify blindly the intervals of interest where statistics was then performed. For such reason, we compute a point-by-point ANOVA using multiple datasets and a permutation testing to find the cluster of significant difference among the three conditions. In particular, we employed 250 permutations in *clustersize-based permutation testing* and *percentile of mean cluster sum* as method to define the threshold distinguishing between "significant" and "non-significant" clusters. We found two different "significant" clusters one in the interval between 5 and 31 s and the other between 69 and 100 s. Then, we focused our further analysis only on these two intervals, where we found the same results previously found with the three time periods a priori chosen.

We changed the text accordingly: "In order to identify the time intervals where perform the statistics, we computed a point-by-point ANOVA using multiple datasets and a permutation testing to find the cluster of significant difference among the three conditions. In particular, we employed 250 permutations in clustersize-based permutation testing and percentile of mean cluster sum as method to define the threshold distinguishing between "significant" and "non-significant" clusters. We found two different "significant" clusters: one in the interval between 5 and 31 s (Δt_1) and the other between 69 and 100 s (Δt_2). We focused our analysis only on these two intervals."

As the authors stated, blood flow dynamics are relatively slow, in the range of 2-to-5 cycles per 100 seconds. In light of this, can the authors justify the choice of a 5-seconds baseline?

Authors' Response: We started to acquire blood flow 20 seconds before the light was turned on. Before starting to consider the signal suited for baseline, we wanted to be sure that the signal had time to be stable. From visual inspection, we estimated that 5 out of 20 seconds was the best compromise between stabilization after starting the acquisition and stability of the signal before starting the experiment.

Introduction: "***Emerging evidence for the existence of a strong relationship between body representation and interoceptive signals are not confined to pathological models***". *I think grammar needs to be adjusted.*

Authors' Response: Thank you for noting it, the sentence, after the revision, was re-written: "Beside pathological models, in healthy subject the meaning and strength of the

relationship between body representation and interoceptive signals is still matter of debate. For example, emerging evidence suggests interoceptive information such as cardiac feedback to modulate the visual body perception [16] and influence one's own body awareness [17, 18] or, vice-versa, changes in body-ownership and self-identification to alter the ability to detect internal body signals [19]. Furthermore, interoceptive sensitivity seems to predict the malleability of participants' body representation [20]."

Method: ***"Therefore, in this case, the number of participants was chosen equal to previous RHI studies."*** *Can the authors cite the studies on which their sample size estimation rely on?*

Authors' Response: Sorry for the missing citations. The references were added in the sentence: "the number of participants was chosen equal to previous RHI studies [30, 32, 34, 36, 84, 85]."

References Armel KC, Ramachandran VS. Projecting sensations to external objects: evidence from skin conductance response. Proc Biol Sci. 2003;270(1523):1499-506. Abdulkarim Z, Ehrsson HH. No causal link between changes in hand position sense and feeling of limb ownership in the rubber hand illusion. Attention, Perception, & Psychophysics. 2016;78(2):707-20. D'Alonzo M, Mioli A, Formica D, Vollero L, Di Pino G. Different level of virtualization of sight and touch produces the uncanny valley of avatar's hand embodiment. Scientific reports. 2019;9(1):1-11. Rohde M, Di Luca M, Ernst MO. The rubber hand illusion: feeling of ownership and proprioceptive drift do not go hand in hand. PloS one. 2011;6(6):e21659. Kalckert A, Ehrsson HH. The moving rubber hand illusion revisited: Comparing movements and visuotactile stimulation to induce illusory ownership. Consciousness and cognition. 2014;26:117-32. Peviani V, Magnani F, Ciricugno A, Vecchi T, Bottini G. Rubber hand illusion survives ventral premotor area inhibition: a rTMS study. Neuropsychologia. 2018;120:18-24.

Method: I suggest the authors to report the sample size in the participants section as well.

Authors' Response: The sample size was reported also in Participants section. "Twenty participants were selected among a population.."

Method, Experimental Procedure: I would suggest the authors to clarify how references 33 and 34 relate to the sentence: ***"They could see the content of each compartment only when the experimenter turned the relative internal light on"***

Authors' Response: Sorry, this was a typo, the references were shifted in the previous paragraph, considering that the cited references refer to the experimental setup employed here.
"Participants were placed in front of a custom-made experimental set-up, made of three parallel compartments (L x W x H = 40 x 60 x 20 cm each) covered by a two-way mirror (Figure 1) [33, 34]."

Method, Results (pg. 8): ***"Considering the interaction between ... were identified in the second interval"*** *Were these contrasts corrected for multiple comparisons?*

Authors' Response: Yes, the contrast for the two previously identified time windows between conditions were corrected for multiple comparison. In particular the Tukey-Kramer adjustment was employed, as was stated in the Methods:
"Hence, a paired t-test with Tukey-Kramer adjustment was employed as post-hoc analysis."
".. Considering the interaction between the factors and given that our aim was to find a difference among conditions in the single time interval, we made two separate post-hoc analysis using a Tukey-Kramer adjustment, one for each time-interval."

It is not clear which alfa and beta coefficients were included in the correlation analysis whose results are reported in Table 2. I guess those belonging of the synchronous condition, but I would suggest the authors make it explicit.

Authors' Response: In order to calculate the correlation between the fitting coefficients and illusion outcomes both the Synch and Asynch conditions, data were employed, pooling them together. This was reported in Methods section, and now better explicated:
"The link between blood flow changes and embodiment was investigated by correlating (Spearman's) *a* and *b* coefficients with the illusion outcomes in *Synch* and *Asynch* condition pooled together." "Table 2: Correlation values between fitting coefficients and illusion outcomes calculated pooling together *Synch* and *Asynch* conditions."

Figure 2: axis label and units are missing in top panel.

Authors' Response: Thank you for noting it, the figure was corrected.

Competing Interests: No competing interests were disclosed.