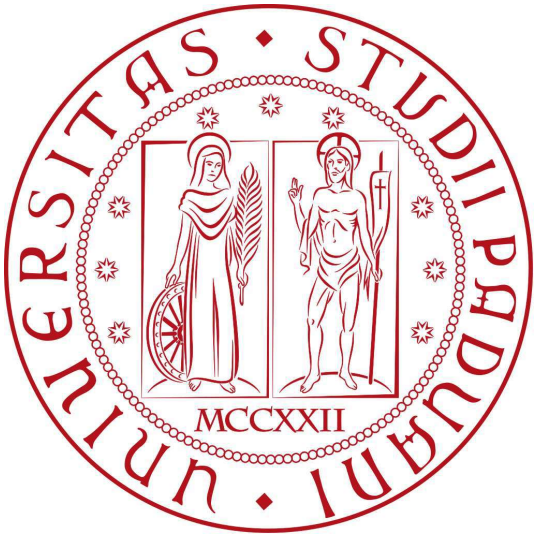

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Final dissertation:

Neurobiological bases for quantity discrimination at birth: A study on newly-hatched domestic chicks

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To my mother, whose sacrifices made it possible for me to be here.

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ABSTRACT

Numerical estimation abilities are extremely important in animals, as they allow for more efficient decision-making in ecological contexts regarding, for example, feeding, conflicts, or reproduction. Furthermore, in humans these abilities are fundamental as they constitute the foundation for subsequent formal mathematics learning during the school period.

These abilities, which do not require a cultural symbolic system such as that provided by human language, appear to be supported by two different cognitive systems: the OTS (Object Tracking System), which is precise and operates with small quantities (approximately 3-4 units), and the ANS (Approximate Number System), which can also be used for larger quantities but is less precise, as it follows Weber's law.

The present study aims to adapt a procedure previously validated in zebrafish (*Danio rerio*) to newly hatched domestic chicks (*Gallus gallus*), thus investigating the neurobiological bases of the ANS in a different phylogenetic group.

The procedure combining the behavioral paradigm of habituation-dishabituation with molecular biology techniques (RT-qPCR). Separate groups of chicks were presented with sets of 3 or 9 dots during the first day of life exploiting the filial imprinting phenomenon for the habituation. The chicks were then tested for dishabituation with a change in number (from 3 to 9 or vice versa, keeping the area or perimeter constant based on the condition) or with a change in size (threefold increase or decrease in total area) while keeping numerosity unchanged. In the control group all conditions remained unchanged. The expression of Immediate Early Genes, as markers for neuronal activation, was quantified in the nucleus rotundus, entopallium, and Wulst, visual areas that we hypothesized to be involved in visual numerosity discrimination.

Preliminary results showed significant main effects of brain area and dishabituation test, without significant interactions. However, no significant differences emerged in subsequent analyses between the different areas or between conditions. Nevertheless, a marginally significant difference between the two experimental conditions (balanced area and balanced perimeter) is noteworthy despite the halved sample size of these two groups compared to the others. Therefore, it will be necessary to better investigate this possible difference in future studies.

1. INTRODUCTION

1.1. Animals and quantity

1.1.1. Different quantities and different species

In the literature, various empirical evidence of numerical abilities in animals can be found, showing that it is not exclusively a human ability. For example, rhesus monkeys (*Macaca mulata*) can choose the greater quantity of food pieces between two sets of different numerosity (Hauser et al., 2000), or dogs (*Canis familiaris*) choose whether to engage in combat based on the numerical ratio between the number of members in their group and that of the opposing group (Bonanni et al., 2011).

Similar evidence has been collected in other tetrapod groups, namely amphibians (*Bombina orientalis*; Stancher et al., 2015), reptiles (*Podarcis sicula*; Miletto Petrazzini et al., 2018), and birds (*Gallus gallus*; Rugani et al., 2009); showing that these abilities are not limited to the class of mammals.

Considering these data as a whole, together with further evidence in fish (*Gambusia holbrooki*; Agrillo et al., 2009), there seems to be a cognitive mechanism common to vertebrates. However, more and more evidence has also been collected in various species of invertebrates, such as spiders (*Portia africana*; Nelson & Jackson, 2012) bees (*Apis mellifera*; (Dacke & Srinivasan, 2008).

This could be due to convergence (Güntürkün, 2012) or alternatively to a common phylogenetic origin that is probably more ancestral than the origin of vertebrates.

1.1.2 Evolutionary purpose

Being able to discriminate different quantities is an aspect that can lead to various evolutionary advantages. In "Numeri senza linguaggio" by Agrillo (2012, p. 17), a review of the main advantages found in an ecological context is made, reported below (see Table 1.1).

One aspect where distinguishing numerosity can prove useful is in social strategies, such as being able to distinguish the numerosity of one's own group and that of the rival group to decide whether or not to engage in combat, which happens in the chimpanzees (*Pan troglodytes*; Wilson et al., 2002) or, as we have seen, in dogs (Bonanni et al., 2011).

Utility is also present in antipredatory defense; in fact, it can be useful, for example, to approach the larger group of conspecifics to have less probability that the single individual will be preyed upon, which seems to actually happen in fish (*Pimephales promelas*; Hager & Helfman, 1991). Similarly, distinguishing numerosity is also useful in hunting strategies, since targeting the larger group of prey increases the probability of success, or more generally it is useful in food choices, to maximize the amount of food taken by choosing the more numerous quantity among several options (Hauser et al., 2000).

A further advantage is present in the search for a sexual partner (Shifferman, 2011); for example, a male might find it advantageous to approach the larger group of females to maximize the probability of mating.

Finally, an advantage can be found in controlling brood parasitism in birds; in fact, by recognizing the numerosity of one's own brood, it is possible to expel foreign eggs from the nest (*Fulica americana*; Lyon, 2003).

Evolutionary advantages of numerical elaboration:
Social strategies
Anti-predator defence
Hunting strutegies
Foraging choices
Mate-seeking behavior
Control of brood parasitism

Table 1.1. Summary of main evolutionary advantages associated with numerical elaboration in animals (adapted by Agrillo, 2012).

1.2. Differences with humans

Since the 1980s, several cognitive models of number related to humans have been developed (Myers & Szücs, 2015).

One of the first was the *abstract modular model* (McCloskey et al., 1985), developed based on dissociations in number processing and calculation found in acalculic patients. This model consists of a central abstract representation, which connects 3 numerical systems: comprehension, calculation, and production (see Figure 1.1).

A subsequent model, the *encoding complex model* (Campbell & Clark, 1988), was developed by analyzing the reading error data of Arabic numerals by McCloskey and colleagues and seemed to adapt to empirical observations better than the previous one. This model proposes, instead of a central abstract representation of the number, that there is a network of specific numerical representations, dependent on notation. Each representation is able to assist in the comprehension, production, and calculation of numbers.

A particularly influential model still today, with aspects that are a combination of the two models seen above, is Dehaene's *triple code model* (1992), developed by integrating knowledge about numerical representations observed in animals, infants, neurotypical adults and those with high abilities, as well as in patients with brain injuries. The model assumes that numbers are mentally manipulated through three distinct representational systems of numerical type: Analog magnitude code, Arabic code and Verbal code.

The analogic code refers to the Mental number line (which would seem to explain the SNARC effect observed in humans and more recently observed also in chicks by Rugani et al. (2015)) and allows the resolution of simple tasks such as: quantity estimation, subitizing (see Section 1.4), numerical comparison, and approximate calculation. The Arabic code allows reading/writing of Arabic numbers, performing parity judgments, and multi-digit operations. The verbal code instead allows reading/writing of number-words, listening/speaking, counting, and arithmetic facts (i.e., simple one-digit addition or multiplication operations that are learned by heart, such as multiplication tables) (see Figure 1.2).

While the analogic representation constitutes a form of non-symbolic representation, the other two modalities described in the model are symbolic in nature and, therefore, exclusively human. The latter in fact require the use of language, acquired through the cultural context, which allows "translating" each analogic representation of quantities into a symbolic representation and also vice versa (Vallortigara & Panciera, 2014, p. 119).

Non-human animals, in fact, not possessing a language like the human one, do not know how to count in the strict sense of the term, that is, to make a one-to-one correspondence between the elements of a set and a symbolic representation; unless the animals are subjected to rigorous and long training, which has been successful with two chimpanzees (Boysen & Berntson, 1989; Matsuzawa, 1985) and with the parrot Alex (Pepperberg, 1994).

However, this inability does not prevent them from performing simple numerical tasks, even in the absence of specific training, that is, those tasks referred to the analog magnitude representation of Dehaene's model and which can therefore be performed even in the absence of a language like the human one. For example, rhesus macaques in a spontaneous choice know how to choose between 2 plates of food the one containing more pieces (Hauser et al., 2000).

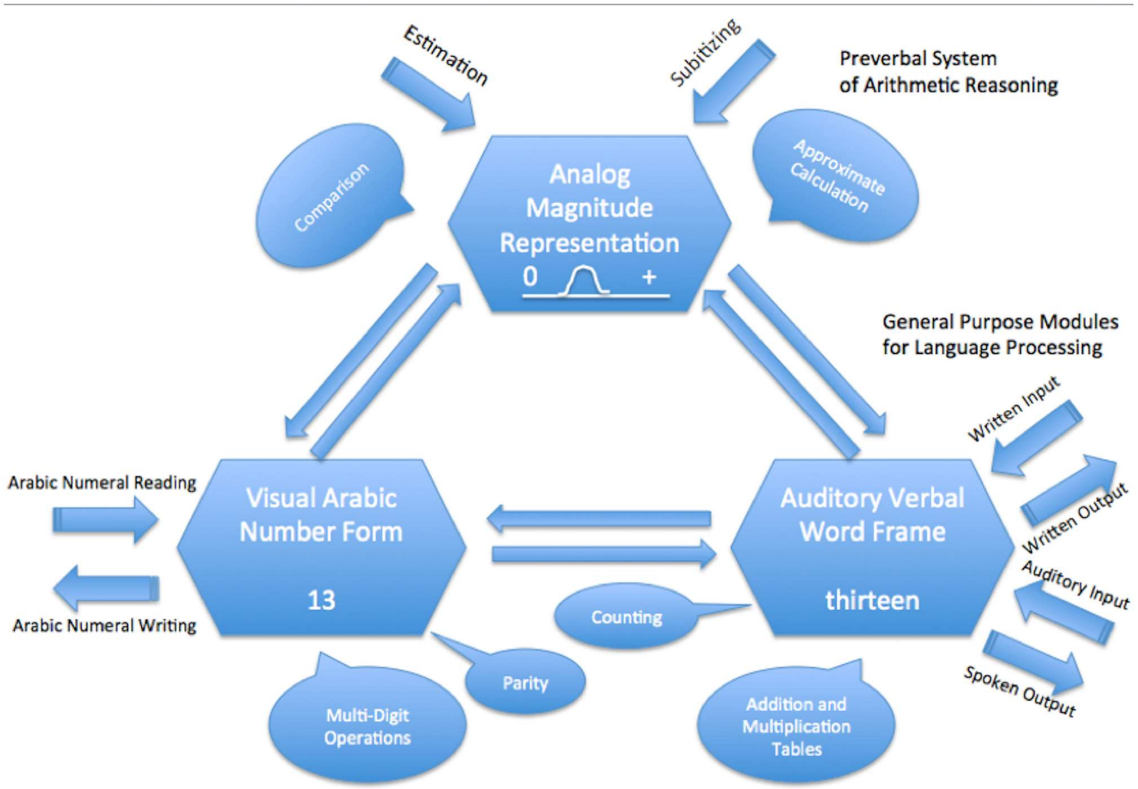

Figure 1.1. Schematic summary rapresentation of Triple code model (Dehaene, 1992; adapted from Myers and Szucs, 2015)

1.3. Do they really use numbers?

1.3.1. Difference between continuous and discrete quantity

Regarding these simple tasks we have talked about, one might wonder if they are solved by non-human animals by actually processing numerosity or through a different strategy. Indeed, at this point, it is necessary to make a clarification: A physical quantity, that is, a property of a system or object that can be measured, can be discrete or continuous (Vallortigara et al., 2022). A discrete quantity is characterized by a plurality of elements or units that, ultimately, cannot be further divided, while continuous quantity is extended and unitary and can be infinitely subdivided into smaller continua. The number of leaves on the crown of a tree is an example of a discrete quantity, while the length of a branch is an example of a continuous quantity.

In nature, these two types of quantities tend to co-vary; in fact, returning to the previous example, longer branches will tend to have a greater number of leaves than shorter branches. This means that if I wanted to know between two trees which contains the greater number of leaves, it would not be necessary to count them all one by one, but it would be enough to observe a continuous quantity that co-varies with the number of leaves, such as the length of the branches or the surface of the shadow projected on the ground by the crown. In this way, I would have reasonably good probability of answering the question correctly even without the need to know the exact number of leaves.

1.3.2. Isolation of discrete variables

All this raises a fundamental methodological question: how to isolate the discrete variable (numerosity) when one wants to determine if an animal is able to solve numerical tasks without resorting to continuous variables?

This problem is particularly complex because numerous continuous variables are inversely related to each other (Agrillo, 2012, p. 40). When creating two sets of stimuli with different numerosity, these inevitably differ also in other perceptual parameters. For example, the numerically larger stimulus will typically occupy a larger total surface area. On the other hand, if the size of the elements in the more numerous set is reduced to equalize the total area between the two groups, the animal might identify the more numerous stimulus simply by recognizing the set with smaller objects, rather than perceiving the numerosity itself.

There are different strategies to address this problem. A first approach consists of sequential control of different continuous variables across the various trials, so that numerosity remains the only consistently informative parameter for the correct resolution of the task. Additionally, it is possible to use heterogeneous rather than homogeneous stimuli (for example those used by Brannon & Terrace, 1998; see Figure 1.2). In fact, using different and irregular shapes discourages the use of global continuous cues by the subjects. In parallel, the use of random geometric configurations among the elements avoids that certain numerical patterns are associated with specific spatial arrangements, such as an error could be to always arrange the numerosity "4" to form a square during the various trials. Furthermore, the adoption of static elements in experimental protocols

allows eliminating density of movement as a potential continuous cue (Agrillo, 2012, p. 42). Finally, a further methodological precaution consists in matching the overall clarity of the stimuli.

A different approach is represented by the sequential presentation of stimuli, by inserting elements one by one into containers. The subject observes the insertion of the stimuli without however being able to observe inside the containers. This methodology eliminates the recourse to continuous visual variables, however, continuous variables of a temporal nature remain, such as the total insertion time or the insertion frequency in case the overall insertion duration is matched. This criticality can be overcome through the insertion of irrelevant elements, such as stones can be for example if in a spontaneous choice task, the animal tries to choose the container in which more pieces of food have been inserted. In fact, the concomitant insertion of non-food objects allows presenting different numerosities of food pieces but balancing the temporal variables during the insertion phase.

A further methodology, probably more effective, is that of sensory transfer. When an animal acquires a response to a stimulus in a specific sensory modality (for example, visual, such as two light flashes) and subsequently generalizes the same response to a stimulus presented in a different sensory modality but of the same numerosity (for example, auditory, such as two sounds), one can reasonably conclude that the animal used numerosity to solve the task, as it is the only common characteristic between the two stimulations. An example of a study is that conducted by Jordan et al. (2005), in which untrained monkeys showed a preference for watching a dynamic video display that depicted a number of conspecifics corresponding to the number of vocalizations they had previously heard.

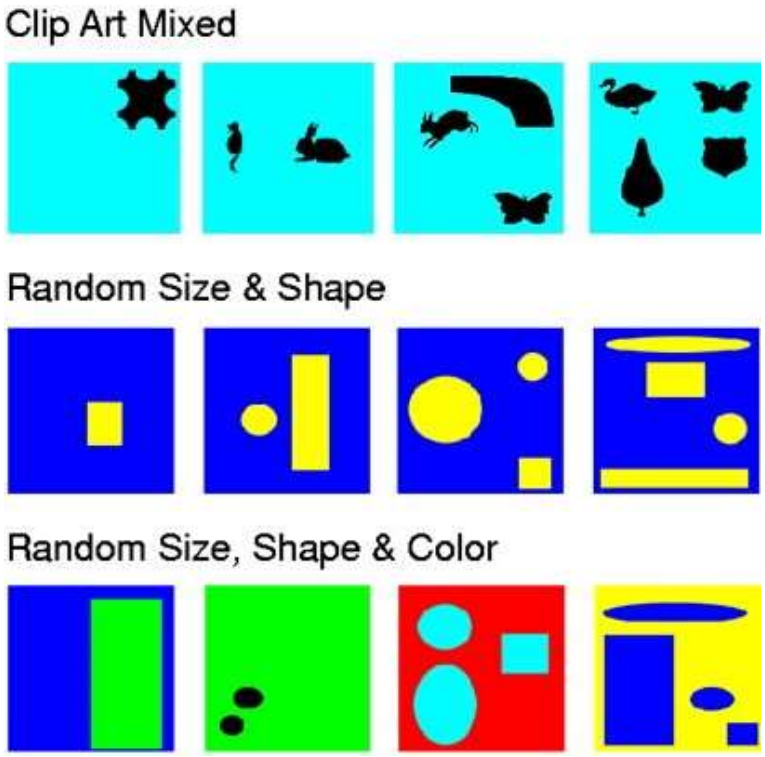

Figure 1.2. Examples of stimuli used in a study on quantity ordering abilities in rhesus monkeys (adapted from Brannon & Terrace, 1998). As can be observed, the numerosity of the stimuli ranged from 1 to 4, and continuous variables were randomized to control for non-numerical cues.

1.3.3. Numbers in laboratory and in nature

A work by Davis & Pérusse (1988) reports the hypothesis that animals rely on discrete numerosity only in the absence of continuous cues, as its processing would be very cognitively demanding. This hypothesis seems supported by studies on humans, since they seem to rely on continuous information when counting is prevented (Durgin, 1995; Gebuis & Reynvoet, 2012), but also by studies on animals. For example, in a study on salamanders, it was seen that when presenting 2 groups of prey of different numerosity they were able to choose the more numerous one, but when the density of movement was equalized they were no longer able to make the discrimination (Krusche et al., 2010), indicating that the subjects used the latter information to make the decision and that they were not able to use discrete numerosity in this specific experimental protocol.

However, other studies seem to prove the opposite; for example, evidence on primates suggests that discrete information is processed automatically even if continuous information is available, such as the study by Cantlon & Brannon (2007) conducted on macaques. These proofs are not limited to primates but also to phylogenetically more distant groups from humans such as fish (Agrillo et al., 2011).

There are also other types of evidence against the last resort hypothesis, namely: Even insects, with therefore relatively simple nervous systems, are able to make numerical discriminations (see Section 1.1.1); even neural networks with few units are able to spontaneously extrapolate discrete information (as we will see in Section 1.4.3); finally, there is evidence from psychophysiology regarding the phenomenon of sensory habituation. Regarding this last point, it has been seen that if we are exposed for 30 seconds to a stimulus composed of numerous dots in a portion of the visual field, we tend to underestimate the number of points subsequently presented in the same portion of the retina (Burr & Ross, 2008). This would indicate that the visual system would be able to extract numerical information and that numerosity would be another primary visual property of the stimulus, as much as color or brightness.

Three reasons can be hypothesized underlying these discordant results (Agrillo, 2012, p. 52–53): A first possible explanation is possible interspecific differences based on different evolutionary pressures, for example in an amphibian the visual system sensitive to movement might find this factor of the stimulus more salient (Krusche et al., 2010). A second explanation could be that the salience of numerical information is stimulus dependent; in fact, it is reasonable to think that regarding a food choice, the total quantity of food is more important than the discrete number, since 2 particularly large apples satiate more than 3 small apples. A third reason could be that the salience of numerical information is context dependent, so even studying the same species with the same types of stimuli could give different results for example based on the numerical ratio of the quantities to be discriminated.

1.4. Numerosity processing systems

When we observe the performance of different species in numerical tasks, a constant pattern can be noted: while with numerosities greater than 4, performance depends on Weber's law (thus on the numerical ratio between the two numerosities to be discriminated), with lower numerosities performance seems instead not to depend on this ratio. This pattern seems to exist for both humans and other animals, as for example in the study by (Agrillo et al., 2012) where the performance of fish was compared with that of humans.

This evidence has led to theorizing two distinct systems of numerical processing: the Object Tracking System (OTS), precise and up to 3-4 elements, and the Approximate Number System (ANS), approximate and for numerosities greater than 4.

The perceptual phenomenon that allows to quickly and accurately estimate the numerosity of a set formed by less than 5 units is called subitizing (Kaufman et al., 1949) and thus allows, for example, to immediately extract the numerosity "3" when seeing 3 pens on the table. What would allow this phenomenon is the OTS system, a cognitive mechanism originally evolved to keep track in parallel of different objects in motion (up to 4 units) and store them in working memory. The estimation of numerosity would thus represent only a secondary utility of this system. This functional peculiarity would explain why discriminating between 3 and 4 elements is not more difficult than discriminating between 1 and 4, although the second discrimination should be simpler both for the ratio between the two quantities (0.75 in the first and 0.25 in the second), and for the absolute difference between the two (1 in the first and 3 in the second).

Regarding larger quantities instead, numerosities would exceed the parallel tracking capacity of this system and one would thus rely on ANS (Gallistel & Gelman, 1992; Nieder, 2005) whose precision depends on Weber's law, that is, on the ratio between the quantities presented and not on the absolute difference between the quantities. Each object would be represented by an impulse from the central nervous system, then summing the individual impulses together and transferring the information to working memory. This can be represented as a graduated container where each drop poured can represent an object or an event. This implies that there is no precise representation of the quantity and that therefore even the discrete type quantity would be treated by the nervous system as if it were a continuous dimension (Vallortigara & Panciera, 2014). This would explain why making a discrimination like 10vs20 is equally easy as making the discrimination 100vs200, although the absolute difference between the two quantities is 100 in the second case and only 10 in the first case, the ratio in fact remains 0.5 in both cases.

However, there are also various empirical proofs that seem to go against the idea of the 2 systems, in which the performance of animals depends on the numerical ratio even with small numbers that should fall within the OTS, as for example has occurred in dogs (Ward & Smuts, 2007) and birds (Al Aïn et al., 2009), thus their existence is still debated.

1.5. Neurobiology of number

1.5.1. Neuropsychology of number

To talk about the neurobiological bases of these processes, we will start by talking about humans first. Returning to Dehaene's cognitive model, seen previously (see Section 1.2), it presents certain neuro-anatomical substrates for each of the three codes (see Figure 1.3). The verbal code is located in the peri-sylvian areas of the left hemisphere, where in fact language is located and which in fact also includes Broca's and Wernicke's areas, while the Arabic code and the analogic code are located bilaterally, respectively in the temporal lobe and in the parietal lobe (Butterworth & Cipolotti, 1997). Since we are interested in numerical processing from a comparative point of view, we will focus on the neuroanatomical correspondents of the analogic code.

There are various studies that have demonstrated the involvement of the parietal lobe, for example (Roux et al., 2008), through cortical electrostimulation in patients who had to undergo surgery for brain tumors, had the purpose of mapping the areas in which electrostimulation interfered with arithmetic tasks. In this study, the highest number of calculation disorders was observed precisely with parietal stimulation.

Many studies seem to affirm that a fundamental role in the representation and manipulation of quantities is played in particular by the intraparietal sulcus (IPS) (Simon et al., 2002).

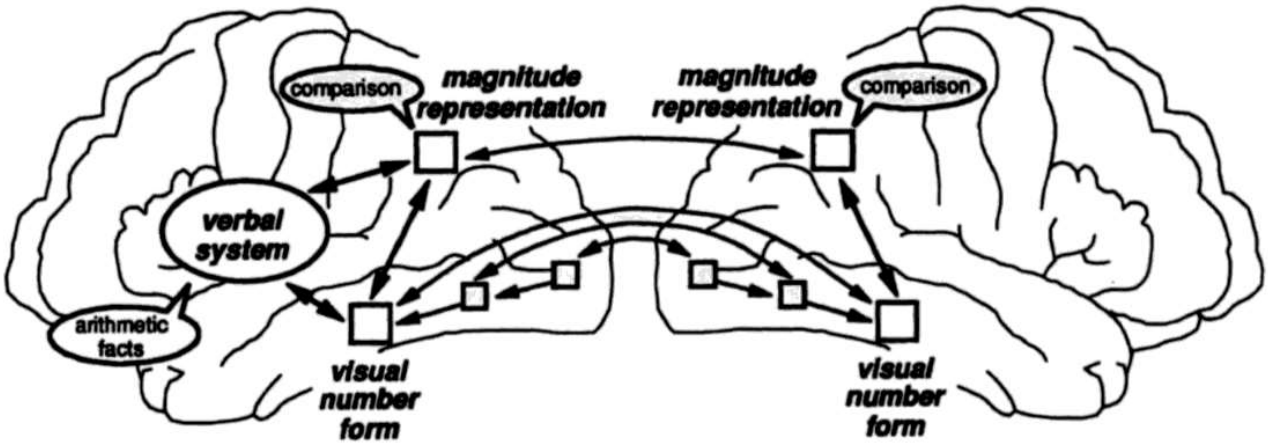

Figure 1.3. Anatomical distribution of the three number representation in the left and right hemisphere (From Dehaene & Cohen, 1996).

1.5.2. Other cortices

Regarding other mammals apart from humans, neurons have been discovered in macaques that seem to respond specifically to discrete numerosity, independently of spatial, temporal factors and the sensory modality involved (Nieder, 2013, see Figure 1.4). In the study, the activity of neurons was recorded while the subjects performed a task where they had to establish whether a certain numerosity that was presented to them was the same or different from a numerosity presented a few moments before.

The discharge activity of these neurons is modulated on the numerosity of the stimulus, with for example the neuron tuned to the numerosity "3" that will activate with three trees, three light flashes, or 3 movements of a limb. These neurons are located in a portion of the posterior parietal cortex, namely in the ventral intraparietal area (VIP) and in the lateral intraparietal area (LIP).

These neurons show maximum activity for a specific numerosity and decreases with increasing distance from the latter; this means that for example the neuron tuned to the numerosity "3" will have the maximum discharge frequency with this numerosity, decreasing in part with numerosity "2" and "4" and decreasing further with numerosity "1" and "5" and so on.

The precision of the neuronal response tends to decrease with larger numerosities compared to smaller ones. For example, a neuron tuned to the numerosity "2" will greatly decrease its discharge frequency when exposed to the numerosity "1" (one unit less). In comparison, a neuron tuned to the numerosity "30" will maintain a relatively high discharge frequency when exposed to the numerosity "29" (still one unit less). This happens despite in both cases the numerical difference being identical (one unit).

This is in line with what can be observed in behavior, that is, the probability of giving a judgment of parity in the case of 2vs1 decreases greatly compared to the presentation of 2vs2, while it decreases relatively less in the case of 30vs29 compared to the presentation of 30vs30. So with equal numerical distance between the two numerosities to be compared (one unit less in both cases of the example), the probability of erroneously giving a judgment of parity is greater with greater numerosities.

Interestingly, in these areas the response occurs regardless of whether the animal must make a discrimination of numerosity.

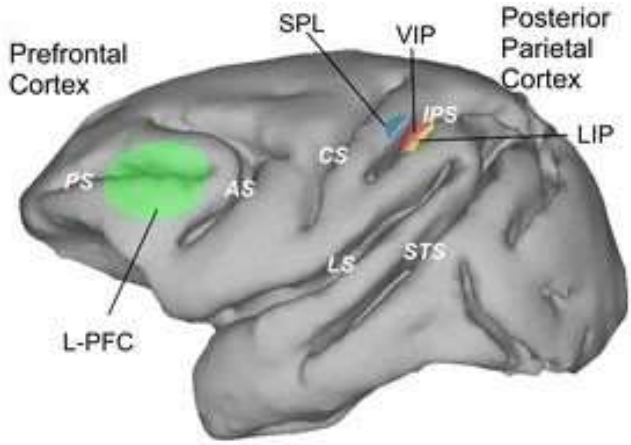

Figure 1.4. Dorso-lateral view of a rhesus macaque brain. The colored areas represent regions in which neurons that respond to numerosities have been identified via single-cell recordings (from Nieder, 2013).

1.5.3. Numbers without cortex

Neurons selective for discrete numerosity have also been documented in numerically naive birds, particularly in the endbrain of corvids (Ditz & Nieder, 2015; Wagener et al., 2018) and in the caudal nidopallium (a higher associative area functionally similar to the prefrontal cortex of mammals) of chicks at only 8-12 days of life (Kobylkov et al., 2024).

It would therefore seem not to be necessary the mammalian cortex, however a similar structure seems to have been documented also in the brain of birds (Stacho et al., 2020). The study reveals a neuroarchitecture of the avian sensory forebrain composed of canonical circuits organized iteratively within tangentially organized lamina-like entities and column-like entities positioned orthogonally.

However, as we have seen, we also possess many proofs, at least behavioral, of numerical abilities also in other animals lacking laminated cortex such as reptiles, amphibians, and fish (see Section 1.1.1); it is therefore likely that it is not a necessary type of structure. This seems to be supported also by studies on invertebrates or on neural networks.

Bees, for example, have a relatively simple nervous system, of about just one cubic millimeter in volume and composed of 960,000 neurons (Vallortigara & Panciera, 2014, p. 53), yet they are endowed with numerical capabilities (see Section 1.1.1).

Regarding neural networks, a study seems to demonstrate that for making simple numerical comparisons, even just 35 neurons are sufficient (Stoianov & Zorzi, 2012). The neural model was formed by 2 layers of hierarchically organized units, which received as input images formed by a different number of elements but of equivalent total area. The network was trained to respond to the sensory input without having any specific information about numerosity, but subsequently 35 of the neurons in the second layer responded specifically to numerosity and with the same response properties as the neurons in the LIP area of the monkey's parietal cortex (Vallortigara & Panciera, 2014, p. 53–54).

1.6. Aim of the study

1.6.1. Hypothesis of early numerical processing

The present research aims to adapt an experimental protocol previously validated in zebrafish (*Danio rerio*; Messina et al., 2020, 2022) to a phylogenetically distinct animal model, namely the chick (*Gallus gallus domesticus*). This comparative approach aims to investigate the neural substrates of numerical processing across different classes of vertebrates, testing the hypothesis that the processing of discrete numerosities may occur early in primary sensory areas, in addition to brain regions associated with higher-order cognitive functions (Lorenzi et al., 2021, 2024; Vallortigara et al., 2022).

1.6.2. Methodological Background

The original methodology employed in studies on zebrafish combined the habituation/dishabituation paradigm to evoke the processing of numerosity change with molecular biology techniques to detect the correlated neural activation.

In the first study, distinct groups of zebrafish were subjected to a habituation phase with repeated presentations of 3 or 9 red dots, associated with food reward. The stimuli systematically varied in size, position, and density across different trials, keeping both numerosity and overall area constant. During the subsequent dishabituation phase, the subjects were exposed to a variation in number (from 3 to 9 or vice versa, with total surface invariant), in shape (keeping surface and numerosity constant), or in size (preserving shape and numerosity). A control group was exposed to the same stimuli presented during habituation.

Neural activation was evaluated by analyzing the expression of immediate early genes (IEGs). The results showed that the telencephalon and thalamus presented the most consistent modulation of IEGs expression in response to numerical variation, while the retina and optic tectum responded mainly to dimensional variations of the stimuli.

The second study, conducted with a methodologically similar procedure, deepened the analysis of pallial activation, focusing on the different subdivisions of the dorso-central region of the telencephalon. The modulation of IEGs expression and in situ hybridization revealed a selective activation of the caudal portion of this area in response to the change in numerosity.

1.6.3. Adaptation to the Avian Model

Based on these results, we proceeded to adapt the experimental paradigm to the avian model, focusing the investigation on three specific brain regions. The first is the nucleus rotundus, a thalamic structure involved in visual processing, considered partially homologous to the lateral geniculate nucleus of mammals (Zeigler & Bischof, 1993). The selection of this area is motivated by the thalamic activation observed in zebrafish. The others are the Wulst and the Entopallium, primary visual processing areas considered in part analogous to the striate cortex (V1) of mammals (Atoji & Karim, 2014; Medina et al., 2000). These regions were selected by virtue of the pallial activation (Dc) found in zebrafish. The detailed methodology of the experimental implementation will be presented in the following section.

2. MATERIALS AND METHODS

2.1 Ethical statement

The experiment performed in the present study complies with all the applicable European Union and Italian laws and guidelines for animals' care and use. All the experimental procedures were approved by the Ethical Committee of the University of Trento OPBA and by the Italian Health Ministry (permit number 791/2019).

2.2 Animals

80 newly-hatched females of domestic chicks (*Gallus gallus domesticus*) of the Ross 308 strain were used. Only females were used because they are more active and attentive toward visual stimuli soon after hatching and show stronger affiliative tendencies compared to males (Vallortigara, 1992). Eggs were obtained from a local commercial hatchery (Passirano, Brescia (BS) – Italy) and incubated in our laboratory at standard temperature and humidity until two days before hatching. Eggs were then separated in individual compartments in total darkness at a constant temperature of 37.7°, with 60% humidity until hatching. The same conditions were maintained after hatching until imprinting on post-natal day 1. In order to avoid any visual experience before the experiment, all the procedures until imprinting were performed in complete darkness.

2.3 Behavioral part

In this study, a similar procedure previously established with zebrafish was employed a similar procedure previously established with zebrafish (*Danio rerio*; (Messina et al., 2020, 2022), in which an habituation-dishabituation behavioral paradigm was adapted and integrated with biomolecular techniques (see Section 3.4) to investigate the neural basis of ANS.

2.3.1 Experimental paradigm

The habituation-dishabituation paradigm is a behavioral procedure well-suited for investigating sensitivity to change, it consists of an initial phase (habituation), where a stimulus is presented, followed by a subsequent phase (dishabituation), where a second stimulus is presented, differing from the habituated one in a single key characteristic. In our case, the key characteristic for the experimental condition is discrete numerosity, while other continuous dimensions are used in control conditions, further details of stimuli (see Section 2.3.3) and conditions (see Section 2.3.2) employed will follow.

2.3.2 Conditions

During the habituation phase, the filial imprinting phenomenon was exploited to fasten and strengthen the learning process. Filial imprinting is defined as a series of behavioral modifications through which a young animal establishes an attachment toward a maternal figure; it typically occurs immediately after hatching and produces a very stable attachment that is difficult to modify (Manning & Dawkins, 2012). Two groups of chicks were exposed and imprinted to either a stimulus presenting 3 or 9 red dots (see Section 3.3.3).

During the dishabituation phase each chick was single exposed to either one of 5 different conditions, two experimental conditions that showed a change in number and three control conditions, one that showed no change with respect to the habituation stimulus and two that showed a change in the continuous variable area (see Table 2.1). In the two experimental conditions, the numerical value changed compared to the habituation phase; thus, if chicks were habituated with 3, 9 was used for the dishabituation, and vice versa. In one of these conditions, the dishabituation stimuli were balanced with respect to the habituation one for the total area, while in the other condition, they were balanced for the total perimeter. The three control conditions maintained all the same numerical value as the habituation phase. In one control condition a stimulus from the habituation phase was used (no change), in a second control condition the total area occupied from the dots was three times bigger than during habituation, and in the third condition the total area was three times smaller than during the habituation.

Habituation phase (Imprinting)	**Dishabituation phase (Test)**
Numerosity → 3 (N=40)	*Change numerosity → 9 total area balanced (N=5)
	*Change numerosity → 9 total perimeter balanced (N=5)
	Same numerosity → 3 no changes (N=10)
	Same numerosity → 3 area x3 (N=10)
	Same numerosity → 3 area ÷3 (N=10)
Numerosity → 9 (N=40)	*Change numerosity → 3 total area balanced (N=5)
	*Change numerosity → 3 total perimeter balanced (N=5)
	Same numeosity → 9 no changes (N=10)
	Same numerosity → 9 area x3 (N=10)
	Same numerosity → 9 area ÷3 (N=10)

Table 2.1. Summary table of the 10 experimental conditions, with the number of subjects for each condition indicated in parentheses. The experimental conditions are marked with an asterisk (*).

2.3.3 Visual stimuli

In the present study, two numerosities, 3 and 9, were used, represented by red dots (single element size of mean radius 1,16 mm and 0.56 mm) inside a circular white arena of radius 1.5 cm (see Figure 2.1). The colour red was selected to enhance the attractiveness of the stimuli for chicks (Salzen et al., 1971). Stimuli were created using the software GeNEsIS (Zanon et al., 2022), a Matlab program that allows to create numerical collections of stimuli controlled for several non-numerical magnitudes.

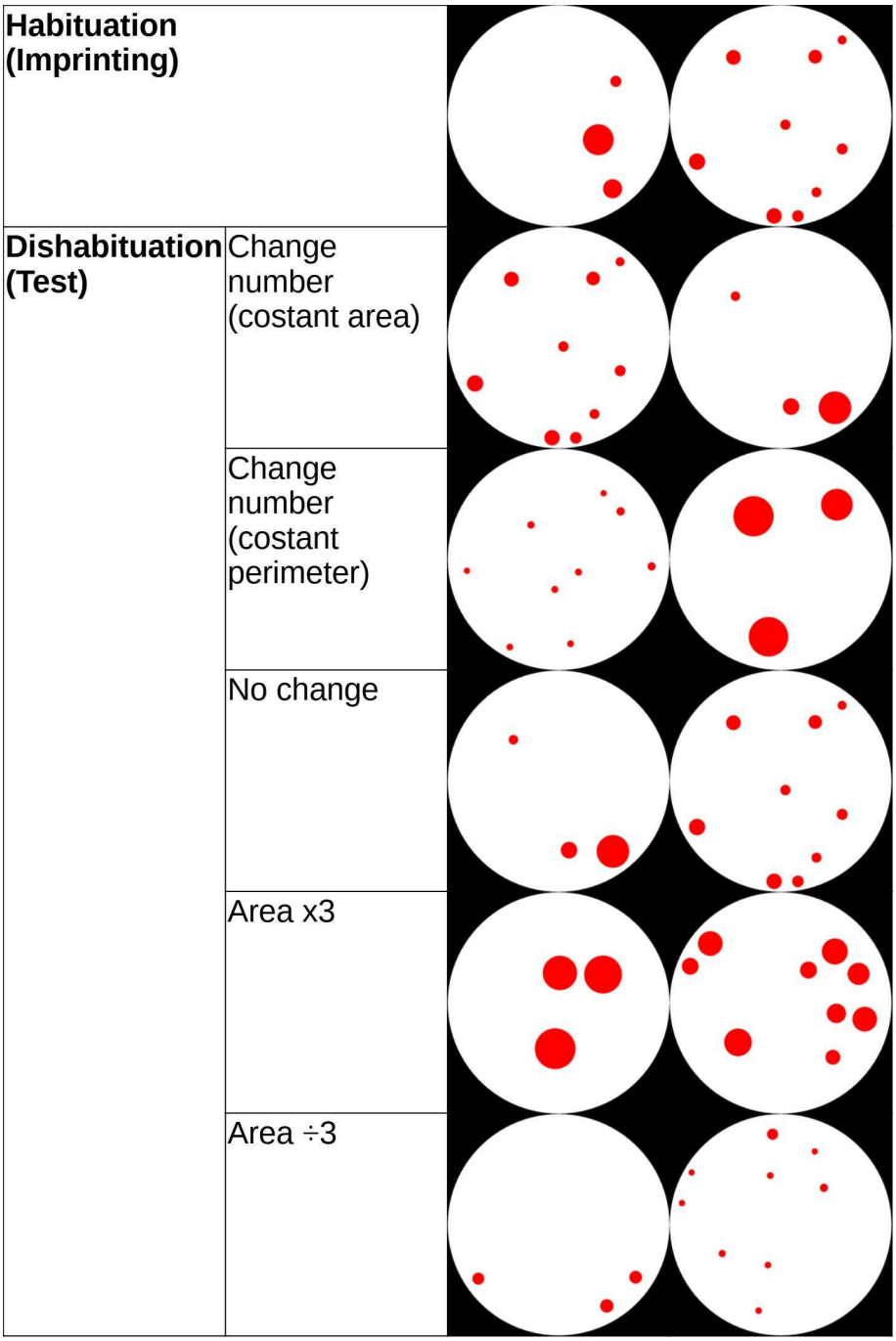

Figure 2.1. Example of stimuli used in the habituation and dishabituation phases and their relative different conditions.

Stimuli were presented consecutively using Psychophysics Toolbox Version 3 package in MATLAB R2019b. Stimuli changed every minute during the 12-hour habituation phase and during the 5-minutes dishabituation phase, while maintaining constant the numerosity and the total area occupied from dots within each session.

A ratio of 1:3 between stimuli was used as in previous studies in the same species (Lorenzi et al., 2024; Rugani et al., 2010). The use of changing stimuli also served to prevent the possible involvement of OTS (see Introduction).

Stimuli with numerosities of 3 and 9 were selected for several reasons. Numerosity 1 was excluded, as it could be interpreted as the simple categorization of a single object, while numerosity 2 might be perceived as a line. Additionally, using numerosities of 4 or higher would have required, in order to maintain a 1:3 ratio, the use of opposing stimuli with numerosities of 12 or greater. This, in turn, would have created individual elements that were too small.

2.3.4 Experimental apparatus

The apparatus (see Figure 2.2) consisted of a box (44x58x49 cm) with a high-frequency video screen (ASUS MG248Q, 24'', 120 Hz) which constituted one of the longer sides. The inner glass box (12 × 12 × 20 cm) was positioned at the center of the screen at 15 cm away from it only during the dishabituation phase. A video-camera was placed above the apparatus and recorded the animals' behaviour during both phases.

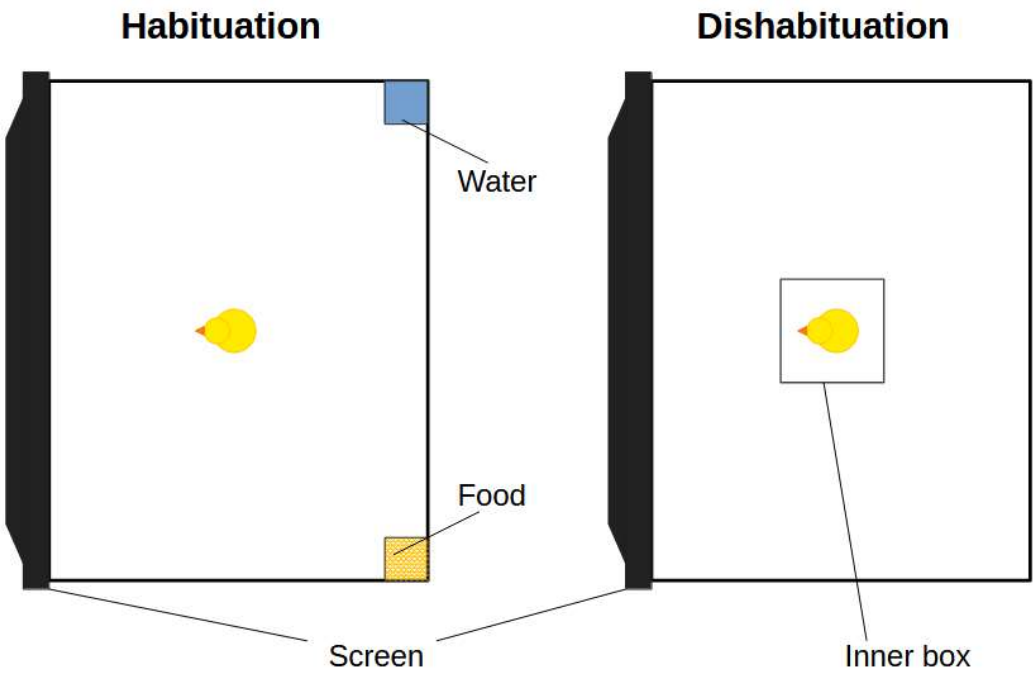

Figure 2.2. Illustration of the apparatus in the habituation (left) and dishabituation (right) phases.

2.3.5 Experimental procedure

The subjects were hatched in individual cardboard compartments in the darkness to avoid any form of visual numerical experience, allowing the exclusion of experiential factors as confounding variables. The habituation phase occurred on the first day of life, during which each subject spent 12 hours freely exploring the experimental apparatus alongside the numerical stimulus presentation. During this habituation phase, food and water were provided ad libitum to ensure normal physiological conditions. Half of the subjects were exposed to 3 and the other half to 9. Subsequently, they underwent a 5-minute dishabituation phase in which each subject was assigned to one of 5 conditions (see section 2.3.2). During the habituation phase, subjects were free to move throughout the entire apparatus; however, during the dishabituation phase, subjects were confined in the inner box (see section 2.3.4) to maximize attention toward the stimulus. After a 30-minute interval following the dishabituation phase, to allow for gene expression to reach the peak, the subjects were sacrificed for molecular analyses using CO_2 for five minutes.

2.4 Biomolecular part

2.4.1 Tissue acquisition

The brains were removed from the skulls using a stereotaxic apparatus following the procedure described in the chick brain atlas Kuenzel & Masson (1988). Care was taken to ensure that the coronal brain sections were oriented at 45°, as specified in the atlas. The brain hemispheres were separated, frozen in OCT, and stored at −80 °C.

Prior to sectioning, the OCT blocks were acclimated to -20°C. A cryostat (Leica CM 1520) was used to produce coronal sections, in the rostro-caudal direction, with a thickness of 60 µm, which were then transferred onto slides. Three sets of slides were prepared for each cerebral hemisphere: the first was designated for tissue collection for qPCR analysis, the second was stained using Giemsa (MG500, Sigma-Aldrich) and served as a reference for precise localization of the punch sites in the first set, and the third served as a backup.

Using a stereotaxic microscope (Zeiss Stemi 305 8-40X EDU WiFiCam), the Giemsa-stained series was used as a reference to identify and isolate with a pipette tip the three regions of interest (see Figure 2.4) for each hemisphere . According to the chicken brain atlas of Kuenzel & Masson (1988), 10 sections per hemisphere were selected from each of the following regions: Wulst between coordinates A14.8-7.6, L3.0-0.2, D8.0-4.4; Entopallium between coordinates A11.8-8.6, L5.8-3.0, D7.2-4.4; and Nucleus Rotundus between coordinates A7.2-5.6, L3.0-1.8, D3.2-1.6.

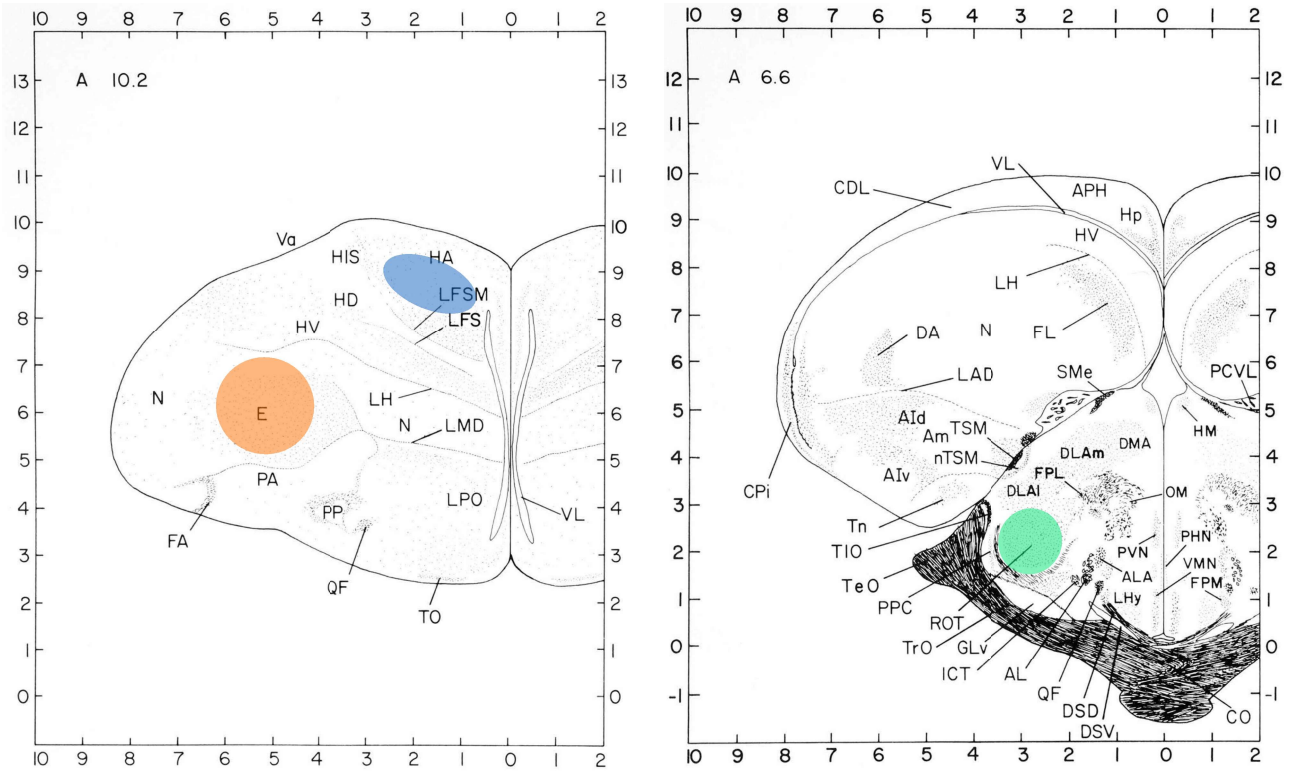

Figure 2.4. Representative coronal sections of the three regions of interest): the left panel shows the Wulst (in blue, labeled HA) and Entopallium (in orange, labeled E). Right panel shows the Nucleus Rotundus (in green, labeled ROT). Pictures modified from the stereotaxic atlas of Kuenzel and Masson (1988).

2.4.2 RNA extraction and quantification

Total RNA was extracted from the sampled tissues using the PicoPure RNA Isolation Kit (Life Technologies). On-column digestion with the RNase-Free DNase Set (Qiagen) was performed to remove any potential genomic DNA contamination.

A measure of RNA concentration and purity (A260/A280 and A260/A230 values) was obtained with the NanodropTM spectrophotometer (NanodropTM Onec; ThermoFisher Scientific, USA). Each sample was then stored at -20°C.

2.4.3 Reverse transcriptase-polymerase chain reaction

In order to be able to quantify the expression levels of specific RNA, Retro Transcriptional PCR (RT-PCR) was performed using a reverse transcriptase enzyme to produce reverse transcription of single stranded RNA to double-stranded complementary DNA (cDNA) while preserving relative concentration ratios of different RNAs. For this procedure, SuperScriptTM VILOTM cDNA Synthesis Kit (Invitrogen, ThermoFisher Scientific, USA) was used following manufacturer's instructions. For a final solution of 15 µL, 1,5 µL of enzymes, 3 µL of buffer solution and 10,5 µL of RNA were used. The cDNA was then diluted to a concentration of 2.2 ng/µL in order to get 10 ng of cDNA in 4.5 µL of solution. This reaction was performed with C100 TouchTM Thrmal Cycler (Bio-Rad, USA).

2.4.4 Quantitative Polymerase Chain Reaction

Quantitative PCR (qPCR) was performed to analyze the expression of the immediate early gene (IEG) *c-fos* (NM_205569) and ribosomal protein large subunit 13 (*RPL13*), which was used as an internal reference for each sample, using specific forward and reverse primers (see Table 3.2). This allowed for the standardization of results by accounting for variations in mRNA and cDNA quantity and quality. qPCR assays were conducted in triplicate using PowerUp™ SYBR™ Green Master Mix (2X) and run on a CFX96™ Real-Time System (Bio-Rad, USA). 5 µL of Master Mix, 0.5 µL of primers (forward + reverse) and 4.5 µL of cDNA were used at a concentration of 2,2 ng/4.5 µL. The ΔCq method was used for expression quantification (Kovas et al., 2009). Data were normalized to the expression of the reference gene *RPL13* (ΔCq), and the relative expression of each target gene was calculated.

Gene primer	Sequence
c-fos Fw	GTGTTCCTGGCAATATCGTG
c-fos Rw	TCAGACCACCTCAACAATGC
RPL13 Fw	TCGTGCTGGCAGAGGATTC
RPL13 Rw	TCGTCCGAGCAAACCTTTTG

Table 3.2. Gene sequences of the primers (forward and reverse) used in the RTqPCR.

2.5 Statistical analyses

To assess differences between conditions, a mixed model ANOVA was conducted with two within-subject factors: Hemisphere (2 levels: Left, Right) and Area (3 levels: nucleus Rotundus, Wulst, Entopallium), and two between-subject factors: Habituation (2 levels: 3, 9) and Dishabituation (5 levels: Change_N (A), Change_N (P), No_change, Area_x3, Area_÷3). The assumption of normality was tested using the Shapiro-Wilk test.

For post-hoc analyses, two-tailed paired *t*-test were used to compare habituation conditions, Bonferroni was used to correct for multiple comparisons.

3. RESULTS

The present study aimed to investigate the role of three visual areas - Wulst, Entopallium and nucleus Rotundus - in response to changes in discrete numerosity. The distributions of the data for each combination of factors are graphically represented in Figure 3.1.

The mixed-model ANOVA revealed a main effect of Area ($F(2,396)=4.218$, $p=0.0154$) and Test ($F(4,396)=2.418$, $p=0.0481$), while nor any significant interaction (see Table 4.1). For descriptive purposes and completeness, the complete set of data is reported in Table 3.1.

Main effect or interaction	**F(df1,df2)**	**p-value**
Imprinting	F(1,396)=0.011	p=0.9161
Hemisphere	F(1.396)=0.048	p=0.8273
Area	F(2,396)=4.218	p=0.0154*
Test	F(4,396)=2.418	p=0.0481*
Imprinting * hemisphere	F(1,396)=0.166	p=0.6842
Imprinting * area	F(2,396)=0.028	p=09725
Hemisphere * area	F(2,396)=0.018	p=0.9818
Imprinting * test	F(4,396)=1.826	p=0.1229
Hemisphere * test	F(4,396)=0.526	p=0.7169
Area * test	F(8,396)=0.703	p=0.6893
Imprinting * hemisphere * area	F(2,396)=0.051	p=0.9502
Imprinting * hemisphere * test	F(4,396)=0.158	p=0.9593
Imprinting * area * test	F(8,396)=0.303	p=0.9646
Hemisphere * area * test	F(8,396)=0.176	p=0.9941
Imprinting * hemisphere * area * test	F(8,396)=0.367	p=0.9377

Table 3.1. Summary of the ANOVA results for the effects of Imprinting, Hemisphere, Area, Test, and their interactions on the dependent variable. The table reports the F-statistics with corresponding degrees of freedom and p-values for each main effect and interaction.

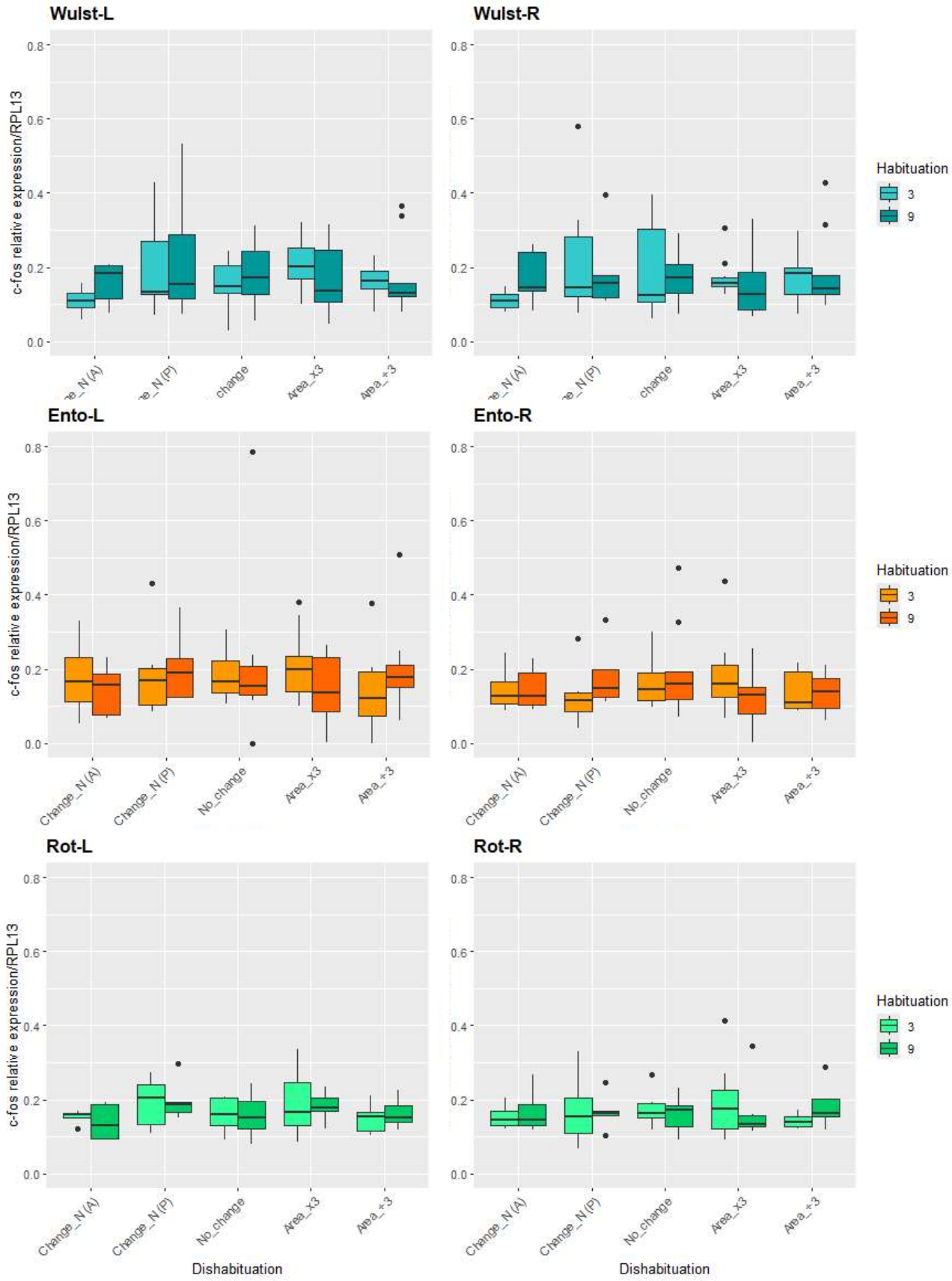

Figure 3.1. Plots report *c-fos* relative expression/*RPL13* for every brain Area (Wulst – blue, Entopallium – orange, nucleus Rotundus – green) and Hemisphere (Left and Right) in each Habituation condition (3 or 9). Every single plot reports a different Dishabituation condition. Boxplots represent the median (thick black line), interquartile range (box), maximum and minimum values (whiskers) and outliers (black circles).

Therefore, data for subsequent analyses considered as factors only Test (Figure 3.2) and Area (Figure 3.3), while Habituation conditions and Hemispheres were pooled together. No significant difference was found between Areas (see Table 3.2 and Figure 3.2) , while a marginally non-significant difference was found when comparing the two experimental conditions (p=0.079), with the perimeter balanced condition showing more *c-fos* expression overall in the brain (0.1911±0.0137) than the area balanced condition (0.1494±0.0080; see Table 3.3 and Figure 3.2).

Area_1	**Area_2**	**Mean ± SE (Area_1)**	**Mean ± SE (Area_2)**	**p-value**
Wulst	Ento	0.1783 ± 0.0075	0.1711 ± 0.0081	p=1.00
Wulst	Rot	0.1783 ± 0.0075	0.1681 ± 0.0045	p=0.89
Ento	Rot	0.1711 ± 0.0081	0.1681 ± 0.0045	p=1.00

Table 3.2. Pairwise comparisons of *c-fos* expression levels across brain areas. The table reports mean *c-fos* expression levels (± SEM) for each pairwise comparison between the Wulst, Entopallium (Ento), and Rotundus (Rot) regions. No statistically significant differences were found between regions. All p-values were adjusted for multiple comparisons using the Bonferroni correction.

Dishabituation_1	**Dishabituation_2**	**Mean ± SE (Dishabituation_1)**	**Mean ± SE (Dishabituation_2)**	**p-value**
Change_N (A)	Change_N (P)	0.1494 ± 0.0080	0.1911 ± 0.0137	0.079
Change_N (A)	No_change	0.1494 ± 0.0080	0.1768 ± 0.0088	0.524
Change_N (A)	Area_x3	0.1494 ± 0.0080	0.1763 ± 0.0073	0.550
Change_N (A)	Area_÷3	0.1494 ± 0.0080	0.1640 ± 0.0071	1.000
Change_N (P)	No_change	0.1911 ± 0.0137	0.1768 ± 0.0088	1.000
Change_N (P)	Area_x3	0.1911 ± 0.0137	0.1763 ± 0.0073	1.000
Change_N (P)	Area_÷3	0.1911 ± 0.0137	0.1640 ± 0.0071	0.423
No_change	Area_x3	0.1768 ± 0.0088	0.1763 ± 0.0073	1.000
No_change	Area_÷3	0.1768 ± 0.0088	0.1640 ± 0.0071	1.000
Area_x3	Area_÷3	0.1763 ± 0.0073	0.1640 ± 0.0071	1.000

Table 3.3. Pairwise comparisons of *c-fos* expression levels across dishabituation conditions. The table presents post hoc t-test comparisons of mean *c-fos* expression levels (±SE) between all pairs of experimental conditions in the dishabituation test. No statistically significant differences were observed among the pairwise comparisons. All p-values were corrected for multiple comparisons using the Bonferroni method.

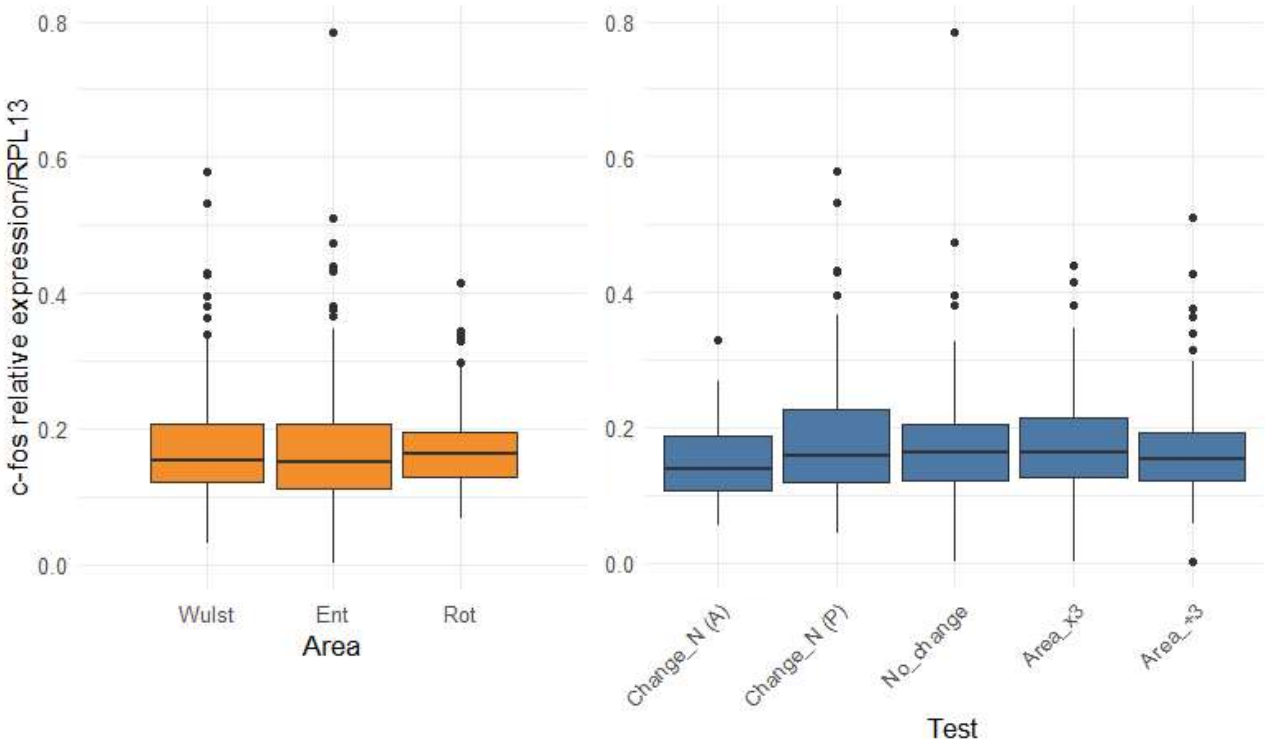

Figure 3.2. *c-fos* expression, represented on the vertical axis, across the different levels of Area (Left figure) and Dishabituation (Right figure). Boxes depict the means and bars the standard error of the mean (SEM).

4. DISCUSSION

4.1 Interpretation of main results

The present study aimed to adapt an experimental protocol, previously validated in zebrafish (*Danio rerio*; Messina et al., 2020, 2022), to investigate the neural substrates of numerical processing in a phylogenetically distinct animal model, the chick (*Gallus gallus domesticus*). This research is part of a broader context of comparative studies that aim to test the hypothesis that discrete numerosity processing might occur early in primary sensory areas, in addition to brain regions associated with higher-order cognitive functions (Lorenzi et al., 2021, 2024; Vallortigara et al., 2022).

Following exposure to numerical stimuli (first habituation and then dishabituation), subjects were sacrificed and areas of interest (Wulst, Entopallium, Nucleus Rotundus) were collected. Analysis of immediate early gene expression (in our case *c-fos*) was then performed using RT-qPCR as a marker of brain activation.

The results of *c-fos* gene expression analysis in early visual pallial areas (Wulst and Entopallium) and in the Nucleus Rotundus (visual thalamic nucleus) revealed a main effect of Area and dishabituation test condition, but no significant interaction between these factors. Despite this, subsequent post-hoc analyses did not show significant differences in *c-fos* expression among the three brain regions examined or between dishabituation test conditions. This would seem to suggest that there is no particular involvement of any of these areas in quantity processing.

A particularly interesting finding appears to be a marginally significant difference between the two experimental conditions: change in numerosity with constant area and change in numerosity with constant perimeter, despite the reduced sample size compared to other conditions (n=5 instead of n=10). One might have expected to find no differences between these two groups and thus be able to aggregate them in subsequent analyses, making all groups of equivalent size. In particular, there is a tendency toward greater *c-fos* expression in the condition of numerical change with balanced perimeter compared to the condition with balanced area. However, given the small sample size, it would be appropriate to repeat the study with an adequate size also for both experimental groups. If a significant difference were found, it would indicate greater processing for area change compared to perimeter change; however, this was not observed in the control conditions where area was increased or decreased.

The absence of significant results might also reflect the inappropriateness of the paradigm used for newly-hatched visually naïve chicks. It might be possible that changes in different quantities might not have been so salient for chicks, that were probably imprinting on the set of red dots.

On the other hand, it could also be that differences related to the perception of changes in quantities might be found in other brain regions such as, for example, the caudolateral nidopallium, the hippocampal formation and the intermediate medial mesopallium (Ditz & Nieder, 2015; Kobylkov et al., 2024; Lorenzi et al., 2024). Indeed, the hippocampus is involved in novelty detection (Corrales Parada et al., 2021; Morandi-Raikova & Mayer, 2021), the caudal nidopallium contains numerical neurons in chicks (Kobylkov et al., 2022) and corvids (Ditz & Nieder, 2015), while the

intermediate medial mesopallium appears to be partially involved in the initial formation of recognition memory for imprinting (McCabe, 2013).

Lastly, the absence of positive results might also reflect the limitations of the technique used. IEGs' expression is commonly used as an indirect measure of neuronal activity, as for instance *c-fos* is a proto-oncogene that is expressed only within some neurons following depolarization. This aspect limits the estimation of neuronal activation to the only neuronal populations expressing *c-fos*.

The experimental paradigm adopted in the present study is based on the work of Messina and collegue (2020, 2022) conducted on zebrafish, who found selective activation of the telencephalon and thalamus in response to numerical variations and of the retina and the optic tectum primarily in response to dimensional variations of stimuli. In our research on chicks, we did not detect a clear functional dissociation between the different brain areas examined.

This discrepancy could be attributed to several reasons. First, anatomical and functional differences between the nervous systems of fish and birds might influence the localization and organization of neural circuits responsible for numerical processing. Second, the experimental protocol might require further adaptations to fully capture the complexity of numerical processing in the avian model. Indeed, the ability to detect rapid changes in numerosity has been already documented in visually naïve chicks at birth (Lorenzi et al., 2024). Oddly, the single exposure to rapid numerical changes at birth evoked changes in *c-fos* expression also in the visual Wulst, that we did not find differentially involved in the present work.

4.2 Conclusions

In conclusion, the present study represents a first attempt to adapt an experimental protocol previously validated in zebrafish to investigate the neural substrates of numerical processing in chicks. The analysis results did not show clear activation in the areas of interest across the various stimulation conditions. This could be due to the need for a different adaptation of the procedure validated in zebrafish.

Additionally, it would be appropriate to repeat the procedure considering the two experimental sub-groups two proper conditions and therefore, having the same sample size for all the experimental groups.

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