

1 **Number neurons in the nidopallium of young domestic chicks**

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21 **Abstract**

22 Numerical cognition is ubiquitous in the animal kingdom. Domestic chicks are a
 23 widely used developmental model for studying numerical cognition. Soon after
 24 hatching, chicks can perform sophisticated numerical tasks. Nevertheless, the neural
 25 basis of their numerical abilities has remained unknown. Here, we describe for the
 26 first time number neurons in the caudal nidopallium (functionally equivalent to the
 27 mammalian prefrontal cortex) of young domestic chicks. Number neurons that we
 28 found in young chicks showed remarkable similarities to those in the prefrontal
 29 cortex and caudal nidopallium of adult animals. Thus, our results suggest that
 30 numerosity perception based on the labeled-line code provided by number neurons
 31 might be an inborn feature of the vertebrate brain.

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33 **Significance**

34 Numerosity, i.e. the number of items in a set, is a significant aspect in the perception
 35 of the environment. Behavioural and in silico experiments suggest that number
 36 sense belongs to a core knowledge system and can be present already at birth.
 37 However, neurons sensitive to the number of visual items have been so far
 38 described only in the brain of adult animals. Therefore, it remained unknown to what
 39 extent their selectivity would depend on visual learning and experience. We found
 40 number neurons in the caudal nidopallium (a higher associative area with functional
 41 similarities to the mammalian prefrontal cortex) of very young, numerically naïve
 42 domestic chicks. This result suggests that numerosity perception is possibly an
 43 inborn feature of the vertebrate brain.

44 **Introduction**

45 Be it a number of conspecifics in a group (Balestrieri et al. 2019), a number of food
 46 items (Hunt et al. 2008), or a number of motifs in a song (Templeton et al. 2005),
 47 correct estimation of quantities is of vital importance for animals. Several behavioural
 48 studies have confirmed that numerical competence is not a prerogative of human
 49 beings, but is a widespread phenomenon in the animal kingdom (reviewed by Nieder
 50 2019, Lorenzi et al. 2021). Mammals (Davis and Albert 1986, Ward and Smuts 2007,
 51 Beran et al. 2008), birds (Lyon 2003, Templeton et al. 2005, Rugani et al. 2009),
 52 reptilians (Gazzola et al. 2018), amphibians (Stancher et al. 2015), fishes (Potrich et
 53 al. 2015), and invertebrates (Bortot et al. 2021), although evolutionary distant, all can
 54 spontaneously assess quantities using an approximate number system (Brannon
 55 and Merritt 2011).

56 For the approximate number system, which is based on the Weber-Fechner law
 57 (Nieder 2016), the perception of cardinal numbers resembles the perception of
 58 continuous physical stimuli. As a consequence, discrimination of quantities is
 59 imprecise and depends on the numerical distance between stimuli. In other words, it
 60 is easier to tell apart 5 and 10 than 9 and 10. Moreover, discrimination of quantities
 61 becomes increasingly difficult with the numerical size. For a given numerical distance
 62 (e.g., 1) it is easier to discriminate between numbers with low magnitudes (1 vs. 2),
 63 than with high magnitudes (9 vs. 10).

64 Recent research has uncovered that the approximate number system relies on the
 65 activity of a specific neuronal population. Neurons that respond to abstract
 66 numerosity irrespective of objects' physical appearance (shape, colour, size) have
 67 been found in the forebrain of human and non-human primates (Nieder 2012, Kutter

et al. 2018) and in crows (Ditz and Nieder 2015). In mammals, numerical responses were recorded in the parietal and the prefrontal cortices (PFC, Nieder 2012). In birds, similar neurons have been described in the caudolateral nidopallium (NCL, Ditz and Nieder 2015). The NCL is believed to be an analogue of the PFC in the avian brain (Güntürkün et al. 2021) and is involved in a variety of cognitive processes, including memory formation (Diekamp et al. 2002, Hahn et al. 2021), abstract rule learning (Veit and Nieder 2013), and action planning (Veit et al. 2015).

Both monkeys and crows are among the most evolutionary advanced species of their phylogenetic groups. They independently developed sophisticated intellectual capacities (Nieder et al. 2020) and both possess enlarged forebrains (Olkowicz et al., 2016). The neural representation of numerosities described in these species also shares remarkable similarities (Nieder et al. 2002, Ditz and Nieder 2015, Viswanathan and Nieder 2013, Wagener et al. 2018). In both species, the number neurons tuned to a preferred numerosity show a gradual decrease in firing rate to other numerosities (numerical distance effect). Their tuning curves are skewed towards larger numerosities and become progressively broader (less selective) with increasing numerosities (numerical size effect). However, it is unclear whether the presence of similar number neurons in these two species emerges as a consequence of their elaborate cognitive skills and enlarged forebrains. To understand the evolution of the number sense we need to explore its neural correlates in distant bird species with more ancestral traits.

Moreover, until now, number neurons have been described only in adult animals (e.g. Nieder et al. 2002, Sawamura et al. 2002, Viswanathan and Nieder 2013, Ditz and Nieder 2015, Wagener et al. 2018). At the same time, behavioural data from human infants (Izard et al. 2009) and young domestic chicks (Rugani et al. 2008,

2009) indicate that some core numerical abilities might be an inborn or spontaneously emerging (Kim et al. 2021, Nasr et al. 2019) property of the vertebrate brain. Testing the presence of number neurons in young and untrained organisms is crucial to verify this hypothesis.

In our study, we aimed to describe the neural correlates of the number sense in domestic chicks (*Gallus gallus*), which belong to a sister group of modern Neoaves (Prum et al. 2015). The domestic chick is a well-established developmental model for studying numerical cognition. Soon after hatching these birds are already capable of discriminating quantities (Rugani et al. 2008, 2013) and even performing basic arithmetic operations (Rugani et al. 2009). It has also been shown that young chicks represent numbers across the mental number line (Rugani et al. 2015), a cognitive ability that had been previously attributed only to humans.

We hypothesised that neural processing of numerical information in young untrained chicks might be similar to crows, despite them having been evolving independently over the last ~ 70 million years (Prum et al. 2015). In a domestic chicken, the NCL is morphologically different from that of corvids (von Eugen et al. 2020), but it is unclear whether this reflects any functional difference. Therefore, we decided to search for neural responses to numerical stimuli in the NCL of domestic chicks. For this purpose we habituated young chicks to a computer monitor, where numerical stimuli were presented (Fig. 1A). We explored neural responses to numerosities from 1 to 5. To control for non-numerical parameters we presented three different categories of stimuli: “radius-fixed”, “area-fixed”, and “perimeter-fixed” (Fig. 1B).

Results

We recorded the activity of 471 units in the NCL of young domestic chicks and examined the mean firing rate of each unit during stimulus presentation (Fig. 2A-B). Among these units, 53 (11%) responded to numerosity irrespective of the stimulus type (henceforth “number neurons”, see Nieder 2016). This was revealed by a two-way ANOVA with the factors “numerosity” (5 levels: 1-5) and “stimulus type” (3 levels: “radius-fixed”, “area-fixed”, “perimeter-fixed”). The unit was considered as a number neuron only if the main effect of the factor “numerosity” was highly significant ($p < 0.01$), but not the effect of the “stimulus type” or the interaction between the two factors. The numerosity that elicited the strongest neural response was defined as the preferred numerosity for this number neuron (after Ditz and Nieder 2015). Five examples of number neurons tuned to different numerosities are shown in Fig. 2C-G. The corresponding statistics are summarised in Table 1. The responses of all 53 number neurons are summarised in Fig. 3A (for statistical results see Table S1 in the supplementary materials, for an example of trials with numerical responses see Video S1 in the supplementary materials).

Most of the number neurons were tuned to the numerosity 1 (30%, $N = 16$) and 5 (32%, $N = 17$). However, we found neurons responsive to other numerosities as well (2: 11%, $N = 6$; 3: 9%, $N = 5$; 4: 17%, $N = 9$), covering the whole range of tested numerosities (Fig. 2B).

When grouped by their preferred numerosity, the tuning curves of number neurons were asymmetric and increasingly wider towards larger numerosities (Fig. 3B). To quantify this numerical magnitude effect, we plotted neural filter functions of single neurons on four different scales: a linear scale, a power function with an exponent of

0.5, a power function with an exponent of 0.33, and a logarithmic (log2) scale. The neural filter functions became significantly more symmetric on a non-linear scale (Friedman test: $X^2(3) = 10.653$; $p = 0.014$, Fig. 4A), with the linear scale significantly different from the power 0.33 and logarithmic scales (Nemenyi test: $p = 0.022$ and $p = 0.028$, respectively), but not from the power 0.5 scale (Nemenyi test: $p = 0.176$). The sigma of the Gaussian fit increased with numerosity only when plotted on a linear scale (Fig. 4B, slope of the linear fit = 0.17) but not with other non-linear scales (Fig. 4B, power 0.5: slope = -0.003, power 0.33: slope = -0.01, log2: slope = -0.03).

To evaluate the probability of finding false positive numerical responses in our dataset, we used the trial-shuffle method (1000 times for every recorded unit). After each shuffling we selected false-positive number neurons by the same statistical criteria described above. The proportion of real number neurons (11 %; 53 out of 471) was significantly higher than the proportion of false-positive units (0,95%; 4488 out of 471000) obtained by the analysis of randomly shuffled trials (proportion test: $X^2(1) = 512,56$, $p < 0.001$).

We further compared the tuning curves between the false positive number neurons and the real number neurons. For this, for each numerical category we randomly selected the same number of corresponding false-positive neurons as the actually recorded number neurons in that category (Fig 2B). We compared the tuning curves of the real and false-positive neurons (Fig 4C) computing a two-way ANOVA with the factors “absolute numerical distance” (5 levels: 0-4) and “data type” (2 levels: real/false positive). The response of real neurons decreased gradually with numerical distance, meaning that closest numerosities were more likely to trigger the number neurons. In contrast, the tuning curve of the false-positive neurons, which by chance happened to have higher firing rates for a random numerical stimulus, was

markedly different from real number neurons (interaction for the factor “numerical distance * data type”: $F_{(1,526)} = 6.275$; $p = 0.01$).

We further described the selectivity of number neurons for unique numerosities by performing a post hoc analysis and comparing the response between the most preferred and other numerosities (Table 2). 9 out of 53 number neurons showed statistically different firing rates between the most-preferred and closest neighbouring numerosities. As expected from the numerical size effect, neurons tuned to lower numerosities are generally more selective than neurons coding for larger numerosities. 6 out of 16 neurons with the preferred numerosity 1 significantly decreased their firing rate in response to the numerosity 2. At the same time, out of 17 neurons that preferentially responded to the numerosity 5, only 1 neuron showed significantly lower response to the numerosity 4. The number neurons generally better differentiated between numerosities with increasing numerical distance (GLM for absolute numerical distance: $X^2(18) = 3.935$, $p = 0.047$; GLM for the log of the numerical distance: $X^2(18) = 6.544$, $p = 0.011$). This effect was more prominent when the numerical distance was calculated on the logarithmic scale: the GLM for the log of the numerical distance had a better fit than the GLM for absolute numerical distance ($\Delta BIC = 6.36$). For the summary of all post hoc results, see TableS2 in the supplementary materials.

Discussion

We recorded number neurons in 8-12 day old chicks, the youngest animals in which number neurons have been described so far. In the NCL of young domestic chicks, 11% of neurons showed a strong selective response to numerical stimuli, confirming

the role of this structure in avian numerical cognition. Moreover, our chicks were not trained in any numerical discrimination task. Instead, they were simply paying attention to the screen, where the numerical stimuli were presented (passive fixation, Hussar and Pasternak 2009). Thus, our young chicks can be considered numerically naïve (see Viswanathan and Nieder 2013).

This result is in line with previous studies on numerically naïve adult animals of distant lineages: 14% of neurons in the prefrontal cortex (PFC) of adult monkeys (Viswanathan and Nieder 2013) and 12% of neurons in the NCL of adult crows (Wagener et al. 2018) have shown numerical response. It is important to note that in any of these studies including our own, we cannot completely exclude a potential unsupervised learning effect due to a repeated exposure to numerical stimuli. Indeed, recent studies have found that in a deep neural network designed to analyse images, a similar amount of number detectors can emerge spontaneously (Nasr et al. 2019), even without pre-training (Kim et al. 2021). However, the hypothesis that number neurons can spontaneously emerge in the biological visual system has not been directly tested so far.

Our data, together with behavioural evidence from newly hatched domestic chicks (Rugani et al. 2008, 2009) and newborn infants (Izard et al. 2009), suggest that numerosity detection might be an inborn or spontaneously emerging feature of the brain. Our results certainly should not be automatically extrapolated to other newborn organisms, since the domestic chicken is a precocial species with a very rapid development after hatching. Nevertheless, even in 2-weeks-old chicks, the functional organization of the brain still remains immature and flexible (Zappia and Rogers 1987). Thus, the presence of number neurons in young chicks, which were not trained to discriminate any specific numerosity, supports the idea of an inborn

number sense. Moreover, since behavioural studies (Rugani et al. 2009, 2013) show that newly hatched chicks perceive numerical information, we might expect to find number neurons already at the first day after hatching.

The number neurons in domestic chicks showed very similar features to those observed in primates and crows (Viswanathan and Nieder 2013, Ditz and Nieder 2015, 2016). First, the number neurons we observed were tuned to specific numerosities, in accordance with the labelled-line code shown for the number neurons in the NCL of crows (Ditz and Nieder 2015, 2016). Second, chicks' number neurons showed a specific decay in response to non-preferred numerosities, which was similar to number neurons in other species and could not have been obtained by chance (see false-positive neurons in Fig. 4C). Third, we observed the numerical magnitude effect: the tuning curves of number neurons became wider with increased numerosity and more symmetrical when plotted on the non-linear scale (Fig. 3B). Also the selectivity of the neural response to non-preferred numerosities increased with the logarithm of the numerical distance, rather than with the absolute numerical distance.

The overall low selectivity to specific numerosity at a single-cell level, as revealed by the post hoc analysis (Table 2), appears to be similar to what was observed in other species (Viswanathan and Nieder 2013; Ditz and Nieder 2015, 2016). How brains can depict precise numerosities with such a noisy system may be explained by a population rate code (Nieder 2016). Single cells respond to every trial in a probabilistic way and only on average show increased firing rates to a given numerosity. Smooth tuning curves only emerge after the neural filter functions of many single cells tuned to the same numerosity are pooled together (Fig. 3B). For an animal to immediately assess numerosity, independent responses of several number

neurons should be averaged simultaneously over a large population. Thus, at the neural population level, stimulation by a specific numerosity would result in a distinct activation pattern. This hypothesis can be tested only by implementing methods that allow recording of single-cell activity over a large neural population.

Given these striking similarities between number neurons in the NCL of crows and chicks, it seems a reasonable guess that they share the same evolutionary origin. This, in turn, would mean that the neural mechanism of the number sense is not an advanced evolutionary adaptation of a few highly intelligent species. Number neurons in the NCL are likely an ancestral feature in avian species, since domestic chicks belong to the sister group of modern Neoaves (Prum et al. 2015).

While it is very tempting to go one step further and discuss the idea of the common evolutionary origin of number neurons for all vertebrates, this would be too speculative. The NCL in birds and the prefrontal cortex in mammals do share similar functions, but are not homologous structures (Güntürkün and Bugnyar 2016, Preuss and Wise 2021). Birds independently developed cortical brain regions, including the NCL, that enabled their high cognitive functions (Güntürkün et al. 2021) based on similar to mammalian pallial architecture (Stacho et al. 2020). The same is likely true also for the Dc (dorsal-central) region in the telencephalon of zebrafish, which has been recently shown to process numerical information (Messina et al. 2021). The possibility of a direct homology between the Dc and either the PFC or the NCL can only be addressed at a macroscopic level. These three regions likely belong to larger neocortex homologues in the respective species (Briscoe and Ragsdale 2019, but see Puelles 2017). However, given the developmental and morphological differences between these areas, new cortical subregions with similar functions must have appeared multiple times during independent evolution of these structures.

Two hypotheses can be put forward on the evolution of number sense in vertebrates. The first one is that number perception evolved independently several times in different phylogenetic groups, although our data strongly suggest that number neurons in the nidopallium are an ancestral trait of birds. This mechanism, however, still can be an adaptation that evolved in parallel to mammalian number neurons. In this case, the identical labelled-line coding scheme for numerosities adopted by birds and mammals might be computationally advantageous and, therefore, evolved independently in both groups (Ditz and Nieder 2015). The second hypothesis is that numerosity processing can be based upon an ancient core neural circuit shared among all vertebrates. In this case, we would expect to find numerosity responses in other, evolutionary conserved brain regions homologically shared among vertebrates (Lorenzi et al. 2021). Indeed, some indirect evidence suggests that at least a coarse estimation of quantity, i.e. more vs. less, might be present already at the subcortical level in humans (Collins et al. 2017) and in the midbrain of birds (Gusel'nikov et al. 1971) and zebrafish (Preuss et al. 2014).

These hypotheses are, however, not mutually exclusive. The putative ancestral neural circuit might be dedicated to assess continuous physical parameters normally associated with the numerosity, like total area of the stimulus. On the contrary, higher order brain processing leading to estimation of cardinal numerosities at a more abstract level may have developed several times, together with the independent evolution of new cortex homologue brain areas in distant phylogenetic groups (Striedter and Northcutt 2019).

Summing up, our study provides the first step in addressing a complex evolutionary and developmental aspect of numerical cognition. By implementing a simplified training procedure, we could, for the first time, demonstrate the existence of number

neurons in young numerically naïve domestic chicks. In the future, this method might be easily adopted for studying the neural correlates of numerical cognition in other brain regions, as well as in other species.

Materials and methods

Subjects

12 domestic chicks (*Gallus gallus domesticus*) of both sexes from the Aviagen ROSS 308 strain were used. Fertilized eggs were obtained from a local commercial hatchery (CRESCENTI Società Agricola S.r.l.–Allevamento Trepola–cod. Allevamento 127BS105/2). Eggs were incubated and hatched within incubators (Marans P140TU-P210TU) at a temperature of 37.7 °C, with 60% humidity in a dark room. After hatching in dark incubators, chicks were isolated and housed individually in metal cages (28 cm wide × 32 cm high × 40 cm deep) with food and water available *ad libitum*, at a constant room temperature of 30–32 °C and a constant light–dark regime of 14 h light and 10 h dark. All experimental protocols were approved by the research ethics committee of the University of Trento and by the Italian Ministry of Health (permit number 745/2021-PR).

Experimental setup

The setup consisted of a rectangular shaped arena (28 X 40 X 32 cm; W X L X H) with metal walls that were grounded. In the centre of one of the shorter walls there was a circular opening (diameter 12 cm). A computer screen (AOC AGON AG271QG4, 144Hz) used for stimuli presentation was positioned directly behind the circular opening. Within the rectangular arena was a small wooden box 14 X 13 X 22

(W X L X H) cm whose frontal wall was made of a metal grid in. The box was placed 25 cm in front of the circular opening with the screen (Fig. 1A). During the experiments chicks remained inside the box from where they could observe the stimuli. Stimulus presentation was controlled by the PsychoPy toolbox (Pierce et al. 2019).

Habituation procedure

The habituation occurred between the 3rd and the 6th day after hatching. On the 3rd day post hatching, chicks learned to peck on mealworms. During the day 4 after hatching, chicks were, first, habituated to the experimental setup and then to the number stimuli appearing on the screen. The birds received mealworms every time after the stimulus appeared on the screen, which motivated them to pay attention to the moment when any stimulus would appear. The stimuli were presented and rewarded randomly, so that chicks would not associate any particular numerosity with the reward. During the days 5 and 6 post hatching we gradually decreased the reward rate, so that birds would still pay attention to the screen even without getting a mealworm. This procedure allowed us to minimize rewarding during actual recording sessions.

Surgery and recordings

On the 7th day after hatching chicks were fully anaesthetized using Isoflurane inhalation (1.5 – 2.0% gas volume, Vetflurane, 1000mg/g, Virbac, Italy) and placed in the stereotaxic apparatus with a bar fixed at the beaks' base and tilted 45° to ear bars. Local anaesthesia (Emla cream, 2.5% lidocaine + 2.5% prilocaine, AstraZeneca, S.p.A.) was applied to the ears and skull skin before and after the surgery. Metal screws were placed into the skull for grounding and stabilisation of

the implant. A small craniotomy was made in the skull on the right hemisphere above the NCL (1.0 mm anterior to the bregma, 4.5 mm lateral to the midline). For extracellular recordings we used self-wired tetrodes made out of formvar-insulated Nichrome wires (17.78 μm diameter, A-M Systems, USA), which were gold-plated to reduce the impedance to 300 – 400 kOm (controlled by nanoZ, Plexon Inc., USA). Then, a commercially available Halo-5 microdrive (Neuralynx, USA) was assembled according to the producer instructions, where four single tetrodes were put into polymicro tubes (inner diameter 0.1 mm) and glued to the plastic shuttles. The microdrive was implanted and fixed first with quick adhesive silicone (Kwik-Sil, World Precision Instruments, USA) and then with dental cement (Henry Schein Krugg Srl, Italy). To increase the probability of finding number-responsive units, we did not glue the electrode tips within the tetrodes. We, thus, considered each tetrode as a brush-like arrangement of four single electrodes. Since in this brush arrangement the positions of the electrode tips can vary, some of the electrodes may have recorded signals from the same neurons. Hence, for sorting and subsequent analyses we chose only the best electrode from each tetrode at every recording position to avoid double-counting of the same neurons.

After the surgery, the chicks were left to recover until the next day in their home cages. Between the 8th and the 12th day after hatching we recorded neural responses to numerical stimuli in the NCL of chicks. Before every recording session the microdrive was connected to the Plexon system (Plexon Inc., USA) via a QuickClip connector and an omnetics headstage (Neuralynx, USA). After every recording session the tetrodes were manually advanced by 60 – 100 μm .

Signals were pre-amplified with a 16-channel head-stage (20 \times , Plexon Model number: PX.HST/16V-G20-LN) subsequently amplified 1000 \times , digitalised and

filtered (300 Hz high-pass filter, 3 kHz low-pass filter and 50 Hz noise removal). Common average referencing (CAR) method (the averaged signal across channels) of the PlexControl system was used for referencing. Spikes were detected with the PlexControl software with an automatic 4-sigma threshold from the average noise level. Subsequently, spike sorting was performed manually in the Plexon Offline Sorter (see Figure S1 in supplementary information for examples of the raw signal and spike sorting).

Stimuli

As numerical stimuli we used red dots outlined with a thin black line that appeared in the centre of the screen in a white background circle 6 cm in diameter. The size of stimuli ranged from 0.25 cm to 1.4 cm ($0.6 - 3.2^\circ$, Schmid and Wildsoet 1998). We explored neural responses to numerosities from 1 to 5. To control for visual parameters that might interfere with numerosity perception, during every recording session we presented three different types of stimuli (Fig. 1B). “Radius-fixed” type of stimuli consisted of dots with a fixed radius, meaning that area and perimeter increase with numerosity. “Area-fixed” stimuli have constant total area over all numerosities, while the total perimeter of the dots increases with numerosity. “Perimeter-fixed” stimuli have constant total perimeter over all numerosities, while total area of these stimuli decreases with numerosity. To further control that neurons do not respond to other visual parameters except for quantity, the inter-distance interval between dots varied randomly. Moreover, for every day of recording we created a new batch of stimuli consisting of 30 unique images for each numerosity/stimulus type combination. Numerosity stimuli were created using GeNEsIS software (Zanon et al. 2021).

During recording sessions we randomly presented stimuli for 500 ms with 2000 ms of inter-stimulus interval. Experiments were video-recorded using CineLAB system (Plexon Inc., USA). To enhance the motivation of birds to pay attention to the screen, random trials were occasionally rewarded. These trials were subsequently discarded from the analysis.

Histological analysis

After the last neural recording birds were overdosed with the ketamine/xylazine solution (1:1 ketamine 10 mg/ml + xylazine 2 mg/ml). Electrolytic lesions were made at the recording sites by applying a high-voltage current to the tetrodes for 10-15 seconds. Then, the birds were perfused intracardially with the phosphate buffer (PBS; 0.1 mol, pH=7.4, 0.9% sodium chloride, 5 °C) followed by 4% paraformaldehyde (PFA). Brains were incubated for at least two days in PFA and further two days in 30% sucrose solution in PFA. Coronal 60 µm brain sections were cut at -20 °C using a cryostat (Leica CM1850 UV), mounted on glass slides, stained with the Giemsa dye (MG500, Sigma-Aldrich, St. Louis, USA), and cover slipped with Eukitt (FLUKA). Brain sections were examined under the stereomicroscope (Stemi 508, Carl Zeiss, Oberkochen, Germany) to estimate the anatomical position of recording sites.

Data analysis

Based on the analysis of video-recordings, we selected only those trials, when birds were not rewarded. Since in birds there is an almost complete decussation of the optic fibres we recorded from the right hemisphere and we selected only trials, when birds looked at the stimulus with both eyes or with the contralateral (left) eye. For each recorded unit, we excluded trials with a firing rate of less than 1 Hz. For the

final analysis we considered only those units that were recorded for at least 7 trials for each numerosity and stimulus type (on average 23 trials per numerosity/stimulus type).

The neural activity of recorded units was analysed as the mean firing rate over 500 ms of stimulus presentation. To find numerosity-responsive neurons we performed two-way ANOVA with the numerosity (1 to 5) and the stimulus type (“radius-fixed”, “area-fixed”, “perimeter-fixed”) as factors. We considered the recorded neuron as numerosity-responsive if only the effect of the “numerosity” factor was highly significant ($p < 0.01$), but not of the stimulus type or the interaction between the two factors. The numerosity that elicited the strongest neural response was defined as a preferred numerosity for this neuron.

For every number neuron we performed a post hoc analysis (Tukey’s test) to compare the neural response between the most preferred and other numerosities. To test for the numerical distance effect we applied a GLM for binomial data with the logit-link function. In the model, the proportion of neurons that significantly differentiate between given numerosities was taken as the response variable, and either the absolute numerical distance or the logarithm of the numerical distance was taken as a factor. We then compared the goodness-of-fit of these two models based on the difference in the Bayesian Information Criterion (ΔBIC), where the lower is the BIC value, the better is the model’s fit.

To validate the stability of our recordings we performed a cross-validation analysis. For each numerosity-responsive unit we calculated the preferred numerosity for the first and the second half of all trials separately. If the neural response to number stimuli was stable across the recording, we expected the Pearson’s correlation

between the first and the second half of the trials to be close to 1 for the whole population of number neurons. The cross-validation analysis showed a strong correlation of 0.82 ($p < 0.001$) between the preferred numerosity in the first and the second half of the trials, confirming the stability of our recordings.

For every number neuron we normalised neural activity by setting the firing rate in response to the preferred numerosity at 100% and to the least preferred numerosity at 0%. The resulting neural filter functions were averaged by group based on the preferred numerosity, thus, creating numerosity tuning curves for e.g. neurons preferring numerosity 1, numerosity 2, etc.

To evaluate the chance level of finding false-positive numerical responses in our dataset, for every recorded neuron we shuffled all the trials for 1000 times and performed an ANOVA each time to select false-positive number neurons. We compared the proportion of false-positive and real number neurons with the proportion test. We further compared the tuning curves between the false-positive number neurons and the real number neurons. For this, we randomly sampled from the false-positive neurons the same number of neurons as the one of actually recorded number neurons. We compared the tuning curves of real and false-positive neurons performing a two-factor ANOVA with the interaction of “numerical distance” and “data type” (real/false positive).

According to the Weber-Fechner law, the perception of sensory stimuli (including quantities) is proportional to a logarithmic (Fechner 1860) or power scale (Stevens 1961) of stimulus magnitude. Therefore, when plotted on a linear scale, one might expect tuning curves to become increasingly asymmetric and wide with increasing numerosity. These properties are usually referred to as a numerical distance effect

and a numerical magnitude effect respectively. To evaluate the symmetry and the width of the neural filter functions we fitted the Gaussian function to the curves (MatLab Curve Fitting Toolbox) plotted on four different scales: linear, a power function with an exponent of 0.5, a power function with an exponent of 0.33, and a logarithmic (log2) scale (Ditz and Nieder 2016). The symmetry of the Gaussian fit was estimated based on R-squared (r^2) values, i.e. the higher is r^2 the better and more symmetric is the fit. The width of the Gaussian fit was reflected by its sigma (σ). The four scaling methods were compared based on the r^2 values by the Friedman's test for non-parametric data with repeated measures with the post-hoc pairwise comparison with the Nemenyi test. The relationship between the numerosity and the sigma of the Gaussian was tested by an ANOVA for different scaling methods separately.

All statistical analyses and visualization of the data was performed in R (R Core Team 2020) with packages "tidyverse", "ggplot2", and "PMCMRplus" and in MATLAB using custom-made scripts and the Curve Fitting Toolbox.

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Author contributions

D.K, U.M. and G.V. designed the experiment. D.K. and U.M. established the procedure for behavioural training and electrophysiology in freely moving chicks. D.K. performed the experiment and analysed the data. M.Z. developed the numerical stimuli and contributed to apart of the data analysis. D.K. wrote the original draft of the manuscript and implemented the comments of all authors. U.M. and G.V. revised and edited the manuscript.

Availability of data and material: A summary table including the mean firing rates for all numerosity-responsive neurons for all trials is included as a supplementary data file. Any additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability: The custom codes that were used for data analysis are available from the corresponding author upon reasonable request.

Competing financial interests: The authors declare no competing financial interests.

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Figures

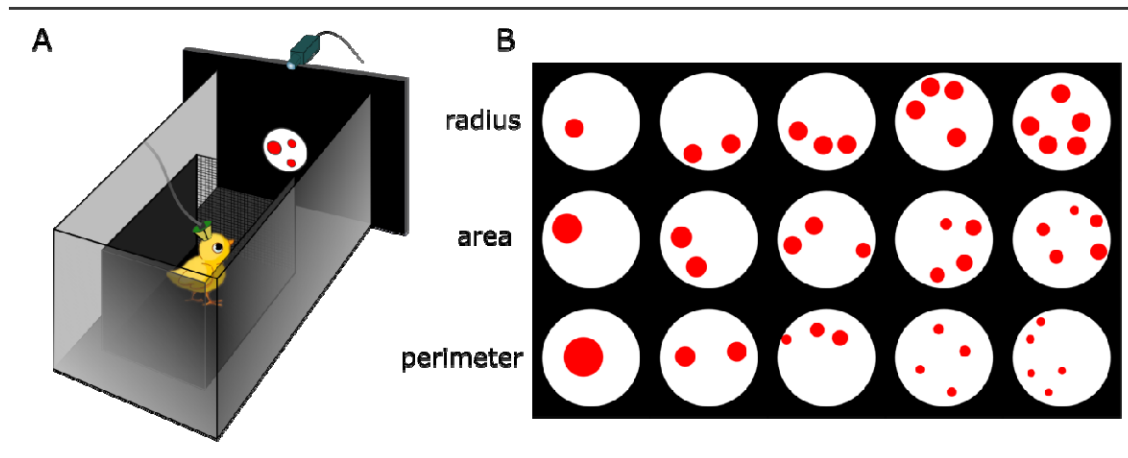


Figure 1. Experimental design. (A) Schematic drawing of the experimental setup. Young chicks were placed in a small wooden box in front of the screen, where numerical stimuli appeared. They were trained to pay attention to the stimuli without any further discrimination between different numerosities. (B) Examples of different types of numerosity stimuli that we presented by every neural recording: “radius-fixed”, “area-fixed”, and “perimeter-fixed”.

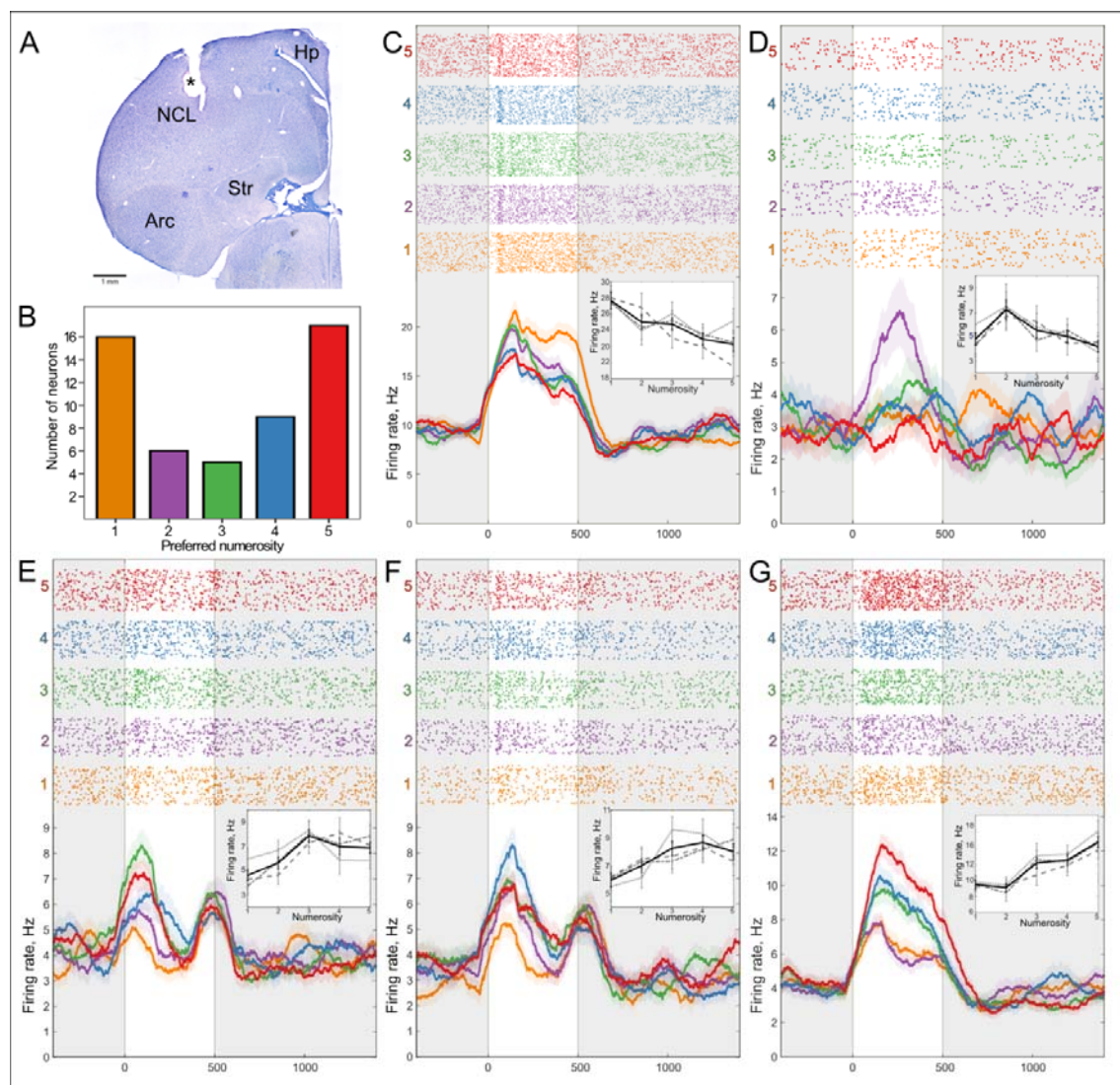


Figure 2. Neurons in the NCL of chicks responding to numerosity. (A) An exemplary coronal section of the chicken forebrain showing the recording site in the NCL (electrolytic lesion is marked by an asterisk). Arc: arcopallium, Hp: hippocampus, NCL: caudolateral nidopallium, Str: striatum. (B) Distribution of neurons that preferred each numerosity stimulus. Examples of neurons that were tuned to numerosity 1 (C), 2 (D), 3 (E), 4 (F), or 5 (G). Top: raster plots representing neural activity, where each line corresponds to one trial and each dot corresponds to a spike. Trials are grouped by numerosity. The 500-ms duration of the stimulus is marked by a transparent window. Bottom: averaged spike density functions

(smoothed by a 100 ms Gaussian kernel; SEM is plotted as a shaded area along the lines). Insert: average firing rate in response to numerosities of each stimulus type. Grey dotted line corresponds to “radius-fixed”, dashed line – to “perimeter-fixed”, dot-dashed line – to “area-fixed” stimuli, and black solid line – to an average. Error bars correspond to SEM.

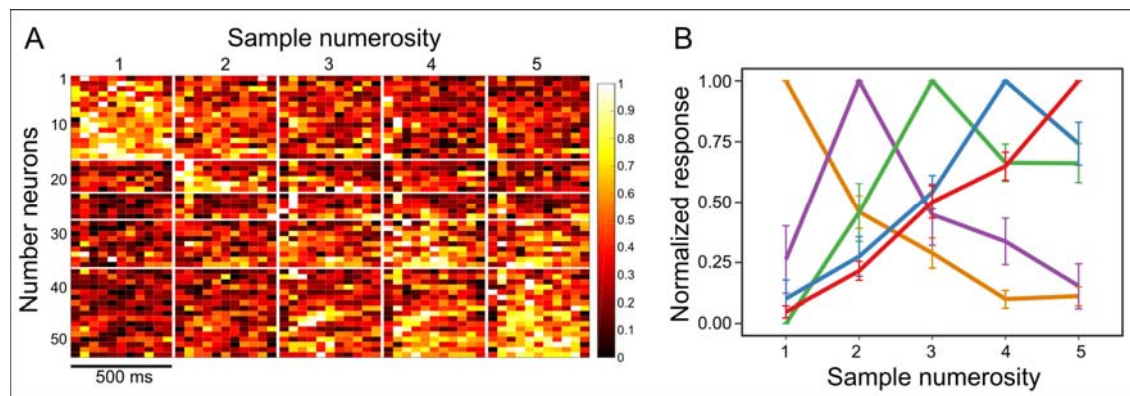


Figure 3. (A) Neural response of all recorded number neurons to numerosity stimuli. Heatmap values represent the mean firing rate during the stimulus presentation (binned by 50-ms), normalised [0, 1] for the corresponding neuron in each row. Values are further grouped by the numerosity stimuli from 1 to 5 (vertical white lines), and by the numerical preference of recorded neurons (horizontal white lines) from neurons that preferred numerosity 1 (top) to neurons that preferred numerosity 5 (bottom). (B) Average tuning curves of numerosity selective neurons. The neural activity of neurons is first normalised [0 = response to the least-preferred numerosity, 1 = response to the most-preferred numerosity] and then grouped by their most preferred numerosity. Error bars correspond to SEM.

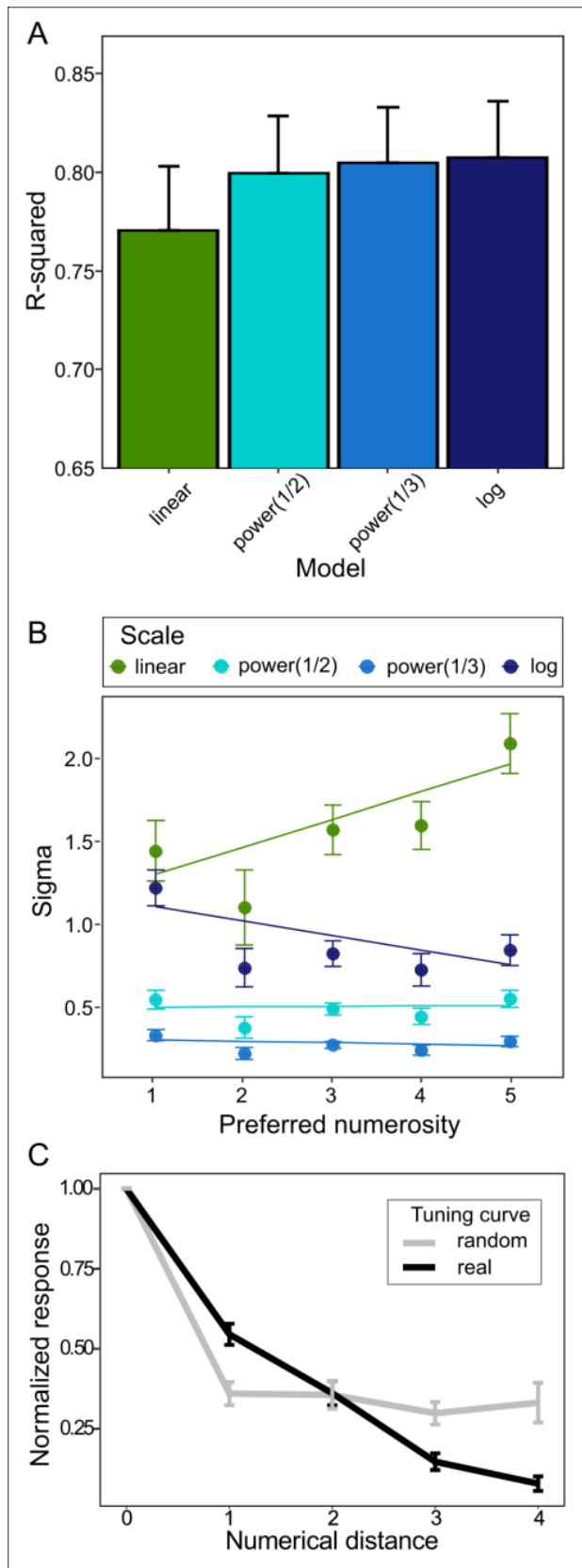


Figure 4. (A-B) Comparison of different scaling schemes for the tuning curves. (A) R-squared, a measure of goodness-of-fit reflects symmetry of the tuning curves plotted on the four different scales. The tuning curves of number neurons become more symmetric when plotted on the non-linear scale. (B) Sigma of the Gaussian fit for neurons preferring different numerosities. When plotted on the linear scale, the tuning curves become wider with increased numerosity. Error bars correspond to SEM. (C) Averaged normalised activity of all numerosity selective neurons compared to the random tuning curve (see Materials and methods section for details). The neural activity was normalised [0 = response to the least-preferred numerosity, 1 = response to the most-preferred numerosity] and then plotted as a function of absolute numerical distance from the most preferred numerosity. Neural response of numerosity selective neurons (black line) gradually decreased with the numerical distance. The slope of this tuning curve is notably different from the random tuning curve (grey line) of false-positive neurons obtained by random shuffling of trials. Error bars correspond to SEM.

Tables

Preferred numerosity	ANOVA (Firing rate ~ Stimulus type * Numerosity)		
	Numerosity	Stimulus type	Interaction
num1	F(4,251)=5.7273, p < 0.001	F(2,251)=0.9275, p = 0.396	F(8,251)=1.2411, p = 0.273
num2	F(4,253)=3.5881, p = 0.007	F(2,253)=0.2355, p = 0.79	F(8,253)=0.454, p = 0.887
num3	F(4,496)=5.8789, p < 0.001	F(2,496)=0.1179, p = 0.889	F(8,496)=1.4538, p = 0.173
num4	F(4,308)=4.2385, p = 0.002	F(2,308)=0.1141, p = 0.892	F(8,308)=0.9124, p = 0.506
num5	F(4,355)=13.4938, p < 0.001	F(2,355)=1.832, p = 0.162	F(8,355)=0.3219, p = 0.958

Table 1. Results of the two-way ANOVA for five example number neurons shown in the Figure 2 (C-G). Preferred numerosity: numerosity eliciting the strongest response. ANOVA results (F-statistics and p-value) for the factor “stimulus type” (“radius-fixed”, “area-fixed”, “perimeter-fixed”), “numerosity” (numerosity 1 to 5), or interaction between them.

Summary		Number of neurons				
Sum	Group	num1	num2	num3	num4	num5
16	num1		6	9	12	14
6	num2	4		2	1	3
5	num3	5	1		0	0
9	num4	6	2	1		1
17	num5	15	12	6	1	

Table 2. The summary of the post hoc analysis. For each group based on their preferred numerosity we calculated the number of neurons that showed significant difference between the most-preferred and the given numerosity.

Supplementary information

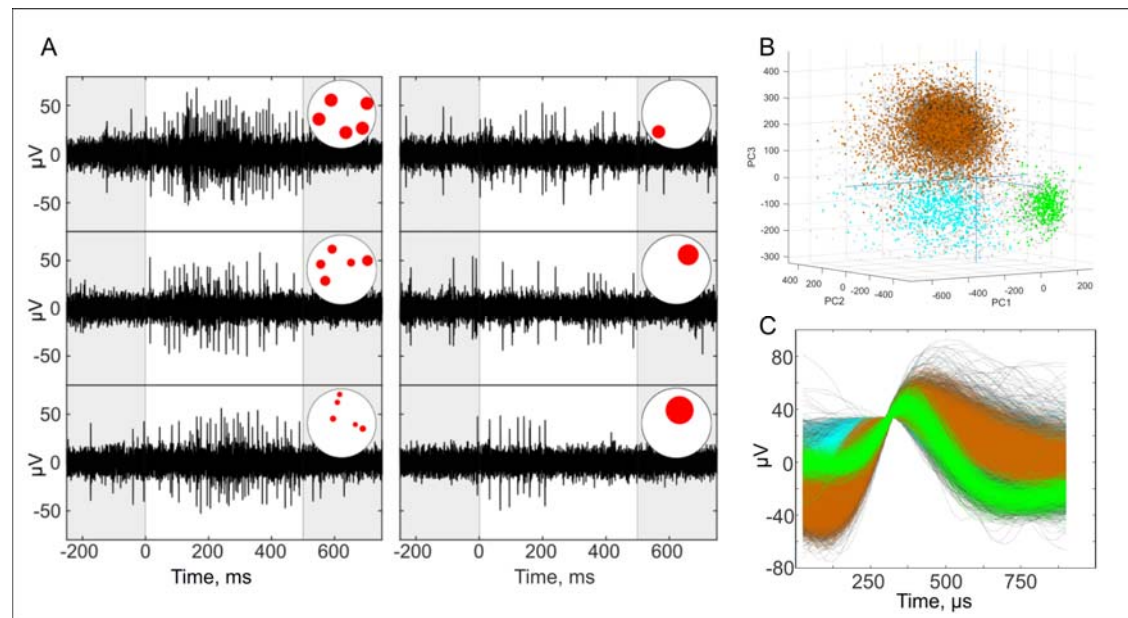


Figure S1. (A) Example of raw spike trains: electrical signal is shown after the high-pass filter was applied. Examples of single trials, representing neural response of the unit to numerosity 5 (left column), irrespective of the stimulus appearance (top: radius-fixed, middle: area-fixed, bottom: perimeter-fixed). Note the decreasing neural response to the numerosity 1 (right column). (B) The PCA clustering of the corresponding recording with waveforms of different units shown by different colours. The waveforms of the number-responsive unit are shown in orange, unsorted waveforms are shown in grey. (C) Spike waveforms of corresponding units isolated from the recording of one electrode.

Unit	Preferred numerosity	ANOVA (Firing rate ~ Stimulus type * Numerosity)		
		Numerosity	Stimulus type	Interaction
1	num1	F(4,546)=4.0327, p = 0.003	F(2,546)=0.5228, p = 0.593	F(8,546)=1.4242, p = 0.183
2	num1	F(4,197)=3.5672, p = 0.008	F(2,197)=0.4656, p = 0.628	F(8,197)=0.8763, p = 0.537
3	num1	F(4,202)=4.0268, p = 0.004	F(2,202)=1.7732, p = 0.172	F(8,202)=1.1967, p = 0.303
4	num1	F(4,565)=4.2588, p = 0.002	F(2,565)=0.0012, p = 0.999	F(8,565)=0.7852, p = 0.616
5	num1	F(4,270)=5.3565, p = 0	F(2,270)=1.2038, p = 0.302	F(8,270)=0.9519, p = 0.474
6	num1	F(4,270)=5.4507, p = 0	F(2,270)=0.0475, p = 0.954	F(8,270)=0.7056, p = 0.687
7	num1	F(4,239)=3.7861, p = 0.005	F(2,239)=0.4114, p = 0.663	F(8,239)=1.6597, p = 0.109
8	num1	F(4,419)=4.9175, p = 0.001	F(2,419)=1.0111, p = 0.365	F(8,419)=1.0988, p = 0.363
9	num1	F(4,399)=7.4226, p = 0	F(2,399)=2.8675, p = 0.058	F(8,399)=2.1769, p = 0.028
10	num1	F(4,366)=4.2597, p = 0.002	F(2,366)=0.9588, p = 0.384	F(8,366)=1.4233, p = 0.185
11	num1	F(4,364)=6.4075, p = 0	F(2,364)=0.2249, p = 0.799	F(8,364)=1.2606, p = 0.263
12	num1	F(4,490)=3.9053, p = 0.004	F(2,490)=1.1902, p = 0.305	F(8,490)=0.6286, p = 0.754
13	num1	F(4,251)=5.7273, p = 0	F(2,251)=0.9275, p = 0.396	F(8,251)=1.2411, p = 0.273
14	num1	F(4,186)=3.5545, p = 0.008	F(2,186)=1.1998, p = 0.304	F(8,186)=0.6334, p = 0.749
15	num1	F(4,233)=4.4063, p = 0.002	F(2,233)=1.138, p = 0.322	F(8,233)=0.3761, p = 0.933
16	num1	F(4,316)=3.6533, p = 0.006	F(2,316)=1.4135, p = 0.245	F(8,316)=0.8235, p = 0.582
17	num2	F(4,177)=4.5348, p = 0.002	F(2,177)=0.2742, p = 0.761	F(8,177)=0.6772, p = 0.711
18	num2	F(4,176)=4.1623, p = 0.003	F(2,176)=0.8853, p = 0.414	F(8,176)=0.8814, p = 0.533
19	num2	F(4,147)=3.6633, p = 0.007	F(2,147)=0.929, p = 0.397	F(8,147)=0.5183, p = 0.841
20	num2	F(4,145)=3.7461, p = 0.006	F(2,145)=0.514, p = 0.599	F(8,145)=0.5375, p = 0.827
21	num2	F(4,298)=3.4934, p = 0.008	F(2,298)=0.7184, p = 0.488	F(8,298)=1.0979, p = 0.364
22	num2	F(4,253)=3.5881, p = 0.007	F(2,253)=0.2355, p = 0.79	F(8,253)=0.454, p = 0.887
23	num3	F(4,213)=3.4856, p = 0.009	F(2,213)=0.6473, p = 0.524	F(8,213)=0.5014, p = 0.854
24	num3	F(4,184)=3.6183, p = 0.007	F(2,184)=0.5629, p = 0.571	F(8,184)=0.6608, p = 0.725
25	num3	F(4,472)=5.1242, p = 0	F(2,472)=2.8061, p = 0.062	F(8,472)=0.809, p = 0.595
26	num3	F(4,410)=3.7563, p = 0.005	F(2,410)=4.5228, p = 0.011	F(8,410)=1.3146, p = 0.233
27	num3	F(4,496)=5.8789, p = 0	F(2,496)=0.1179, p = 0.889	F(8,496)=1.4538, p = 0.173
28	num4	F(4,176)=3.926, p = 0.004	F(2,176)=1.1196, p = 0.329	F(8,176)=0.6298, p = 0.752
29	num4	F(4,178)=3.9175, p = 0.004	F(2,178)=0.4244, p = 0.655	F(8,178)=0.6897, p = 0.7
30	num4	F(4,267)=4.5852, p = 0.001	F(2,267)=0.6043, p = 0.547	F(8,267)=0.8609, p = 0.55
31	num4	F(4,371)=3.6503, p = 0.006	F(2,371)=1.9928, p = 0.138	F(8,371)=0.5894, p = 0.787
32	num4	F(4,430)=3.4615, p = 0.008	F(2,430)=0.3739, p = 0.688	F(8,430)=0.513, p = 0.847

33	num4	F(4,420)=4.4972, p = 0.001	F(2,420)=1.1432, p = 0.32	F(8,420)=1.1135, p = 0.353
34	num4	F(4,459)=6.5488, p = 0	F(2,459)=2.8234, p = 0.06	F(8,459)=1.3186, p = 0.232
35	num4	F(4,333)=3.4399, p = 0.009	F(2,333)=3.3226, p = 0.037	F(8,333)=1.5331, p = 0.145
36	num4	F(4,308)=4.2385, p = 0.002	F(2,308)=0.1141, p = 0.892	F(8,308)=0.9124, p = 0.506
37	num5	F(4,323)=5.0184, p = 0.001	F(2,323)=1.9782, p = 0.14	F(8,323)=0.3722, p = 0.935
38	num5	F(4,188)=3.5707, p = 0.008	F(2,188)=1.1655, p = 0.314	F(8,188)=0.5456, p = 0.821
39	num5	F(4,307)=5.1489, p = 0	F(2,307)=0.0322, p = 0.968	F(8,307)=0.4935, p = 0.861
40	num5	F(4,260)=5.1953, p = 0	F(2,260)=0.945, p = 0.39	F(8,260)=0.682, p = 0.707
41	num5	F(4,234)=4.65, p = 0.001	F(2,234)=0.7971, p = 0.452	F(8,234)=0.8709, p = 0.542
42	num5	F(4,232)=3.7216, p = 0.006	F(2,232)=0.0802, p = 0.923	F(8,232)=1.3969, p = 0.199
43	num5	F(4,484)=3.6509, p = 0.006	F(2,484)=0.1205, p = 0.887	F(8,484)=0.7686, p = 0.631
44	num5	F(4,458)=12.8202, p = 0	F(2,458)=0.7574, p = 0.469	F(8,458)=0.4219, p = 0.908
45	num5	F(4,192)=4.4432, p = 0.002	F(2,192)=2.0198, p = 0.135	F(8,192)=1.8229, p = 0.075
46	num5	F(4,415)=3.8044, p = 0.005	F(2,415)=1.618, p = 0.2	F(8,415)=0.5316, p = 0.832
47	num5	F(4,685)=14.1207, p = 0	F(2,685)=1.9687, p = 0.141	F(8,685)=1.4958, p = 0.156
48	num5	F(4,371)=6.7422, p = 0	F(2,371)=0.6683, p = 0.513	F(8,371)=1.6257, p = 0.115
49	num5	F(4,359)=4.1619, p = 0.003	F(2,359)=0.1355, p = 0.873	F(8,359)=0.4252, p = 0.906
50	num5	F(4,355)=13.4938, p = 0	F(2,355)=1.832, p = 0.162	F(8,355)=0.3219, p = 0.958
51	num5	F(4,354)=6.4972, p = 0	F(2,354)=0.6433, p = 0.526	F(8,354)=0.9821, p = 0.45
52	num5	F(4,288)=3.3897, p = 0.01	F(2,288)=2.1069, p = 0.123	F(8,288)=1.2445, p = 0.273
53	num5	F(4,460)=5.4865, p = 0	F(2,460)=1.4027, p = 0.247	F(8,460)=0.7622, p = 0.636

775

776 Table S1. Summary of the two-way ANOVA for every recorded unit. Unit: id of the
777 recorded unit. Preferred numerosity: numerosity stimulus that elicited the strongest
778 response in the corresponding unit. ANOVA results (F-statistics and p-value) are
779 summarized for the factor “Stimulus type” (radius-fixed, area-fixed, perimeter-fixed),
780 “Numerosity” (numerosity “one” to “five”), or interaction between them.

781

782

Unit	Preferred numerosity	Post hoc analysis, p-value				
		num1	num2	num3	num4	num5
1	num1		0.016	0.022	0.019	0.007
2	num1		0.081	0.019	0.011	0.021
3	num1		0.988	0.903	0.303	0.003
4	num1		0.974	0.315	0.002	0.209
5	num1		0.890	0.650	0.000	0.027
6	num1		0.176	0.063	0.001	0.001
7	num1		0.035	0.048	0.006	0.020
8	num1		0.031	0.001	0.001	0.023
9	num1		0.788	0.174	0.000	0.000
10	num1		0.465	0.002	0.009	0.169
11	num1		0.033	0.000	0.000	0.001
12	num1		0.036	0.013	0.090	0.004
13	num1		0.221	0.120	0.001	0.000
14	num1		0.147	0.142	0.061	0.004
15	num1		0.031	0.002	0.084	0.004
16	num1		0.304	0.048	0.008	0.014
17	num2	0.447		0.986	0.362	0.001
18	num2	0.001		0.027	0.025	0.206
19	num2	0.023		0.945	0.065	0.063
20	num2	0.013		0.017	0.826	0.608
21	num2	0.973		0.181	0.125	0.011
22	num2	0.040		0.312	0.075	0.004
23	num3	0.005	0.807		0.760	0.123
24	num3	0.006	0.094		0.504	0.899
25	num3	0.001	0.913		0.998	0.796
26	num3	0.005	0.059		0.370	0.857
27	num3	0.000	0.024		0.798	0.683
28	num4	0.003	0.282	0.120		0.016
29	num4	0.020	0.051	0.213		0.999
30	num4	0.710	0.001	0.028		0.103
31	num4	0.007	0.369	0.813		0.998
32	num4	0.059	0.059	0.936		0.999
33	num4	0.065	0.007	0.864		1.000
34	num4	0.000	0.092	0.419		0.968
35	num4	0.004	0.628	0.616		0.850
36	num4	0.003	0.194	0.981		0.928
37	num5	0.002	0.002	0.007	0.054	
38	num5	0.022	0.287	0.995	0.999	
39	num5	0.000	0.148	0.653	0.803	
40	num5	0.000	0.026	0.525	0.214	
41	num5	0.001	0.021	0.226	0.012	
42	num5	0.008	0.150	0.010	0.431	
43	num5	0.004	0.097	0.111	0.725	
44	num5	0.000	0.000	0.750	0.851	
45	num5	0.015	0.032	0.001	0.080	
46	num5	0.070	0.002	0.428	0.069	

47	num5	0.000	0.000	0.016	0.868
48	num5	0.000	0.003	0.251	0.901
49	num5	0.003	0.394	0.940	0.997
50	num5	0.000	0.000	0.034	0.078
51	num5	0.000	0.009	0.014	0.801
52	num5	0.074	0.023	0.988	0.798
53	num5	0.006	0.004	0.276	0.998

783

784 Table S2. The post hoc analysis of the two-way ANOVA based on the Tukey-Kramer
785 method summarising p-values for every pairwise comparison between the most-
786 preferred and other numerosities. Significant p-values <0.05 are highlighted in bold.

787