Complexity shapes uniqueness: Neuropil volumes and synaptic clusters shape behavioural plasticity under challenging environments in the invasive Argentine ants

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Abstract:

Adaptation to new environments is key for organisms' survival, but also for their invasiveness in their introduced areas. Behaviour is considered the fastest phenotype allowing adaptation, but its plasticity can involve costs as neural development. Although individuals' investment in cognition was pointed out as unnecessary for colony behaviour in eusocial insects, recent studies are highlighting the behavioural dependence on neural traits in eusocial insects. The costs of producing behavioural and neural plastic offspring could exceed the investments of eusocial insects, in which one or certain reproductives must produce multiple offspring. Thus, we wanted to analyse the link between the neuroanatomy and the behavioural variability of *Linepithema humile*, an invasive species organised in supercolony units containing millions of individuals, to understand its adaptive mechanisms. We repeatedly tested same aged callow workers of *L. humile* in behavioural tests of increasing environmental complexity and analysed the volume of their brain functional areas (neuropils) and the synaptic clusters abundance in the mushroom body calices (information processing). Given the potential large cost of plasticity, we expected to find homogeneous interindividual neuronal structures and behavioural responses. Although *L. humile* is considered a monomorphic species, body size conditioned behavioural and neural traits and determined individuals efficiency in exploring simple environments. Contrary to our

expectations, the increase in environmental complexity revealed the behavioural plasticity of *Linepithema humile* workers as well as its correlation with neuropil volumes and synaptic clusters. Our results highlight the relevance of the central complex and the mushroom bodies on exploration efficiency rather than optic and olfactory lobes. Behavioural plasticity under complex environments relied on the synaptic connections of the olfactory processing area (dense lip), while individuals with higher number of synaptic connections on the visual processing area (collar) explored complex environments less efficiently. Our results suggest that behavioural differences that correlate with morphological traits might promote adaptive mechanisms in simple environments, whereas neurologically based plastic behavior may be necessary to adapt in complex environments.

Keywords: Argentine ant, social insects, personality, microglomureli, volumetry, Neuroethology.

Introduction

Species adaptation to the environment has been a revolutionary concept in the understanding of Biology since the publication of *On the Origin of Species* (Darwin, 1859). Gradual adaptations, often driven by the accumulation of mutations over many generations, allow physiological and morphological traits to evolve at the species level. However, evolutionary change can also occur rapidly, enabling organisms to adapt to sudden environmental shifts, such as those caused by climate change, or to establish themselves in new regions. A notable example of the latter is the introduction of non-native species, often a byproduct of globalization, which has become a significant ecological and economic challenge (*e.g.* Bertelsmeier, 2021; López et al., 2023). To become established in a new area, introduced species must cope with a range of diseases,

resources, competitors, and conditions. In the case of the alien invasive species, these adaptations not only allow them to survive in the new place, but to expand their range and colonize new areas (*e.g.* Prentis et al., 2008; Stapley et al., 2015).

One of the mechanisms that enable species to rapidly modify the way they interact with the environment is through their behaviour (reviewed in Snell-Rood, 2013). At the population level, these behavioural adaptations can arise from two different mechanisms. One of them consists of the difference of interindividual behavioural patterns (personality; Dall et al., 2004) and their match with the environmental conditions, which determines the fitness of the individuals. Another mechanism consists in the intraindividual changes in behaviour through processes and experiences (plasticity; Dingemanse et al., 2010). While these concepts may directly apply to solitary species, the proximate causes of behavioural variability and the fitness outcome of its dynamics differs in eusocial organisms. In such species, evolutionary pressures and selections do not act at the individual but at the colony level (*e.g.*, mole rats, Jacobs & Jarvis, 1996; ants, Delgado & Solé, 2000). On one hand, interindividual behavioural variability in eusocial organisms often relies on task speciation (Jeanson & Weidenmüller, 2014), which is frequently related with individuals' age (polyethism; reviewed in Beshers & Fewell, 2001), a form of intraindividual behavioural plasticity through development (also see Jeanson 2019). On the other hand, eusocial behaviour is strongly influenced by colony-level dynamics, as new behaviours often emerge as collective phenomena (reviewed in Lihoreau et al., 2012; Navas-Zuloaga et al., 2022).

However, in the last years a growing number of studies are demonstrating the importance of interindividual behavioural differences (*e.g.* Maák et al., 2020; Trigos-Peral et al., 2023) and

83 intraindividual behavioural plasticity (reviewed in Lucon-Xiccato et al., 2024) in the context of
84 coping with environmental challenges at the individual level in eusocial species. Whether due to
85 phenotype, task specialization or perceived experiences, proximal mechanisms such as
86 neuroanatomical development, are responsible for the variability of insect behavior. Although
87 insect morphology stops changing after metamorphosis, their brains show large plasticity in the
88 adult stage. For instance, the relative volumes of certain neuronal regions (*e.g.* Gronenberg et
89 al., 1996) or even the total volume of ants' brains can increase through maturation and
90 experience in the adult stage (*Camponotus* sp.. Gronenberg et al., 1996; Yilmaz et al., 2016b;
91 *Oecophylla smaragdina* and *Formica subsericea*, Kamhi et al., 2017). In polymorphic species,
92 larger workers show larger optical lobes - necessary when foraging - and smaller workers show
93 higher ratio of collars of the mushroom body to optic lobes, *i.e.* more processing than perception
94 of the visual cues (Arganda et al., 2020). The navigation under natural skylight involves the
95 increase of the synaptic buttons of visual projection neurons and volume of the central complex
96 in *Cataglyphus nodus* (Grob et al., 2017). When learning, *Acromyrmex ambiguus* shows a
97 temporal increase in the synaptic cluster (microglomureli) in the olfactory region rather than in
98 the optic region of the mushroom body (Falibene et al., 2015).

99 The production, development, and maintenance of neuronal plasticity (*e.g.* new or larger neural
100 routes) involves resource allocation and costs for the individual (Jeanson & Weidenmüller, 2014).
101 Behavioural processes such as memory and learning allow faster adaptation (Fawcett et al., 2013)
102 but involves the organisation of the brain functional areas (neuropils) in charge of processing
103 sensory information (mushroom bodies of insects; Aso et al., 2014; Li & Strausfeld, 1999). Some
104 of these costs could scale to the progenitor level, which might invest more resources to allow

individuals coping with neuroanatomic plasticity, leading to a trade-off related with the quantity and quality of the offspring production (Clutton-Brock et al., 1985; Einum & Fleming, 2004.). Therefore, we expected an inverse relationship between the behavioural plasticity and the colony size on eusocial animals, *i.e.* queens investing in workers' quality in small colonies but in workers' quantity in large colonies (Kramer & Schaible, 2013). Under this scenario, large colonies might rely more on the polyethism and the behavioural traits arised by the collective than in the individual behavioural properties.

The Argentine ant (*Linepithema humile*, Mayr 1868) could be one of the most representative examples to illustrate trade-offs between the benefits and costs of behavioural variability due to the millions of members that form its supercolonies (non-competitive colonies; reviewed in Moffett, 2012) and their necessity to adapt to the introduced areas in which they behave as invasive. We therefore used the Argentine ant to highlight the individual capacities to interact with the environment and its dependence on neuroanatomy. At present, we do not know how differences and neuroethological plasticity of workers cause or affect colony adaptation in invasive species. We expect that in a polygynous superorganism (Hölldobler & Wilson, 1990) in which workers are almost non-genetically related (Keller & Fournier, 2002) and with the dimension of the Argentine ant introduced supercolonies (reviewed in Angulo et al., 2024), the behavioural variability should rely more on genetic or developmental differences among and within the supercolony members than in the individual capacity to modulate behavioural responses. On the contrary, some characteristics of the *L. humile* workers such as the rapid consolidation of long-term memory (Wagner et al., 2023) and traits found in other ant species like learning (Czaczkes et al., 2013; Jeanson & Weidenmüller, 2014), personality and tool use

127 (Maák et al., 2020) or task syndromes (Trigos-Peral et al., 2023), suggest a high relevance of
128 individual cognition in problem-solving in eusocial organised species (also shown in bees,
129 Lemanski et al., 2021; bumblebees, Hill et al., 2023). Unraveling the underlying mechanisms
130 involved in resolving environmental challenges faced by the introduced *L. humile* supercolony
131 may help to prevent and control the continued expansion (reviewed in Angulo et al., 2024) and
132 long-term stabilisation (Baratelli et al., 2023) of this ant species.

133 We performed standardised laboratory experiments with different environmental complexities
134 to analyse the behavioural strategies used by the Argentine ants. We considered exploratory
135 behaviour as a basic mechanism to solve problems in new environments (open field test,
136 response to an object and solving maze). We focused on the relationship between the individual
137 neuroanatomy and the behavioural plasticity in the face of environmental challenges to
138 understand potential dependences of behaviour on the brain and thus, colony costs associated
139 with individual production. Argentine ants have managed to spread widely in their introduced
140 areas owing to their high activity, population density, and capacity to monopolise larger foraging
141 territories (Carpintero & Reyes-López, 2008; Human & Gordon, 1996), so we expected
142 experimental ants to show high level of exploratory behaviour. As a possible strategy of reducing
143 developmental costs in maintaining a large population (Sanmartín-Villar et al., 2021), we
144 expected ants showing low inter- and intraindividual behavioural variability when increasing the
145 environment complexity and sparse relationship between the neuroanatomy (microglomureli
146 organisation and brain region volumes). In other words, we expected that the colony interaction
147 with the environment will be a product of individuals' trial and errors and without cognitive
148 processes associated (Lihoreau et al., 2012).

Material and Methods

Sampling collection and laboratory maintenance

Linepithema humile colonies were collected in NW Spain between the 23rd and 28th of August 2022 in five locations separated by ca. 7 km (in latitudinal order; Catoira: 42°40'18"N, 8°43'41"; Rianxo: 42°38'43", 84°9'14"; Carril: 42°37'14", 8°46'21"; Chazo: 42°36'27", 8°51'25"; Vilanova: 42°34'00", 8°49'42"). Queens, workers, and larvae were transferred with the soil of their nests to plastic containers with the inner part of the walls covered by polytetrafluoroethylene (Fluon®) to prevent ants from escaping. The contents of each collected nest were spread in plastic pot bases (97 x 40 x 7 cm) also covered by fluon®. We added plastic tubes filled with water and cotton to provide humidity to the ants and food *ad libitum* following the recipe based on Bhatkar & Whitcomb (1970) and improved according to Csata *et al.* (2020). This food was used throughout the experiment. Over the course of five days, all ants successively entered into the tubes and were transferred to new plastic containers and transported, without soil, to the Museum and Institute of Zoology of Warsaw (Poland). Ant nests ("source nest" hereafter) were maintained in temperature chambers at 26°C with a 12:12h light:dark period, the optimal temperature from egg to adult development of *L. humile* (Abril et al., 2010). Food was replaced in the source nest three times per week until the end of the experiment.

To control the age of the focal individuals and avoid behavioural and neural differences due to polyethism (*e.g.*, Fahrbach et al., 1998; Kamhi et al., 2019; Tripet & Nonacs, 2004) and different experiences (*e.g.*, Weidenmüller et al., 2009), we followed the development of last instar larvae until their metamorphosis. To facilitate this process and to standardise the experiences lived by

170 focal individuals in their larval stage, we created homogeneous experimental groups by
171 transferring from the source colonies 20 workers and five last-instar larvae to Petri dishes (Ø=
172 5.7 cm) containing a 1.5 ml Eppendorf tube filled with water and cotton and the tube's lid filled
173 with food. Twenty experimental group replicates were initially isolated from each source nest. In
174 cases where two callow workers emerged in the same group, the newest callow worker was
175 transferred to fresh experimental groups. We reached a maximum of 50 and minimum of 20
176 experimental groups for the source nests. We checked each experimental group daily for new
177 emergences. One day after emerging, focal individuals were marked with pen markers (Edding
178 Creative 751©). Focal individuals were easily identified even one day after their emergence
179 because the colour of their cuticle was lighter than the one of their nestmates. Behavioural tests
180 started ten days after the emergence of focal individuals, a period that we considered optimal to
181 guarantee the development of the locomotive workers' function (see also Abril et al., 2010).

182 *Behavioural test*

183 To study inter- and intra individuals' behavioural variability, we exposed each focal individual (N=
184 172) to a series of three consecutive 10-minute tests. Five observations of the three different
185 tests, one per day for five consecutive days, were made (N= 860 tests) at 23.75±0.73 °C and
186 54.67±2.78 % of humidity, testing each individual over a similar time frame. Tests were always
187 conducted in the following order for each individual and observation: (i) open field test, focused
188 on the percentage of new area explored; (ii) object exposure test, focused on the interaction with
189 new objects; and (iii) maze test, focused on the decision making on whether to explore further in
190 a complex environment. A total of 13 individuals were not exposed to the objects and mazes, *i.e.*
191 they remained for 30 min in the open field (see below), to control the effect of the exposure to

192 new objects on the behaviour and brain structure. A total of 20 individuals were not exposed to
193 any of the behavioural tests as a control for possible inherent factors of our procedure such as
194 manipulation and isolation (Bernadou et al., 2018; Seid & Junge, 2016) that could cause some
195 changes to the brain.

196 Behavioural tests were performed in separate Petri dishes inside three 60 x 70 x 60 cm wooden
197 boxes with white walls to limit visual references and thus, without distal clues for the focal ants
198 (see Harris et al., 2010). Ants' behaviour was recorded from the top of the boxes with cameras
199 (two Sony FDR-AX53; one Canon Legria HF M56) and tracked with AnimalTA software (Chiara &
200 Kim, 2023). A maximum of 10 ants were recorded at the same time for a single camera. Before
201 each series of tests started, we transferred the experimental groups from the temperature
202 chambers and placed them in the wooden boxes for 10 minutes as pre-acclimatisation.

203 Open field test

204 Following the 10 min of acclimatisation, focal ants were carefully isolated into their respective
205 empty Petri dishes (Ø= 5.7 cm) using a brush. Petri dishes were covered with their lids after the
206 addition of each individual. These dishes possessed a red circle of 1 cm diameter drawn on the
207 centre and outer part of the bottom dish as a reference for placing the object in the following
208 test (see next paragraph). We assumed that ants did not perceive this circle due to its position
209 (no odour because it was out of the dish). Recordings started before the introduction of the first
210 ant and lasted 10 min after the introduction of the last ant in the experimental setup. By doing
211 so, we were able to register the behaviour of all focal individuals since their contact with the new

212 arena. This procedure was also applied to the other behavioural tests. Upon introducing the last
213 ant, we closed the wooden boxes with a lateral white wooden cover.

214 Object exposure test

215 Following the 10-minute open field test, we removed the cover of the wooden boxes and
216 introduced an object in each Petri dish. The objects were added in the centre of the arena,
217 matching its contour with the red circle designed. After adding the object to the last dish, the
218 cover of the wooden box was put back and we recorded the ants' behaviour for 10 mins. Objects
219 consisted of a three-level circular pyramid (Ø= 1 cm; Fig. 1A) designed on Tinkercad
220 (https://www.tinkercad.com/) and printed on resin (Anycubic Eco UV Resin White) using
221 Chitubox® and a 3-D printer (Elegoo Mars 2P). The objects were painted in blue (Pinty Plus Aqua;
222 Ref. 320). We consider that the shape, material, colour, and odour were uncommon in the focal
223 species environment and thus, relevant as a novelty stimulus. After the end of each test, the
224 objects were washed with hand soap and tap water and allowed to dry under sunlight.

225 Maze test

226 Following the 10 min object exposure test, ants were individually transferred to a new Petri dish
227 (Ø= 5.7 cm) respectively containing a white resin-printed maze (height 4 mm, Ø= 5.7 cm; Fig. 1B).
228 The maze consisted of a circular platform with two inner chambers (start and end) connected by
229 a concentric path of 3 mm wide. The path was composed of a sequence of five 1 cm segments.
230 Each segment ended in a Y-choice with the next two branches oriented 130˚ from the previous
231 path. Opposed at an angle of 100˚ to each of the segments, the maze possessed a dead-end 1
232 cm segment. In this way, the maze was composed of six Y-choices connected by the concentrical

233 path. Half of the mazes had dead-ends directed toward the left and the other half had dead-ends
234 directed toward the right. On each Y-choice, ants had the choice between selecting a dead-end
235 path or to continue advancing through the maze. The three vertices of the Y-choice were
236 expanded to the interior of the path as projections constraining it to 2 mm. This forced the focal
237 ants to face the two possible choices and avoid them selecting a choice based on the possible
238 thigmotaxis (wall-following) used to guide them through the previous path (see Czaczkes, 2018).
239 Focal ants were introduced to the ovoid start chamber of the maze (2.1 x 1 cm) with only one
240 exit towards the maze path. We covered the maze with a plexiglas layer to avoid ants escaping.
241 Ants that selected six times the same direction following the concentric path through the six
242 consecutive Y-choices encountered the ending reward chamber. This reward chamber was
243 circular (Ø= 1 cm) and positioned in the middle of the Petri dish (occupying the same place as the
244 red circle of the open field test and the object on the object exposure test). Before each test, a
245 drop of sucrose was added in this reward chamber (1M; see Czaczkes, 2018). The ending chamber
246 was the only section of the maze without resin floor, to allow a better removal of the sucrose
247 drop. The sucrose was added to stimulate the ants that found the end chamber for future trials,
248 although our aim was not to test learning (no training).

249 To analyse the variability of choice strategy under novel environments, focal ants were exposed
250 on the three first consecutive observations to mazes with a concentric path oriented in one
251 direction and to mazes oriented in the opposite direction on the last two observations. By this,
252 we could check if ants were habituated to the maze and how they reacted after a change, which
253 we assumed is a more demanding cognitive process (central integration; see Menzel & Giurfa,
254 2001) and thus, the opportunity to compare personality traits and cognition based on different

255 mechanisms (see Carere & Locurto, 2011). After 10 min of recording, ants were returned to their
256 original experimental group and then to the temperature chamber until the next day, in which
257 tests were repeated.

258 Ants belonging to the control group were maintained 30 minutes in empty arenas (open field) to
259 control the effect of experiencing new or/and complex environments (object, maze) while
260 considering the effects of experimentation (manipulation and isolation). Similar to the
261 experimental group, we made 5 observations over 5 consecutive days.

262 After 16 days of their emergence (immediately after the last test on the 5th day for focal ants),
263 individuals were isolated in 15 ml falcon tubes and placed in a thermally isolated box with ice
264 packs until brain extraction.

265 *Brain extraction and fixation*

266 Brain extraction was done following the protocol of Ott (2008). Focal and control ants (N=205)
267 were anaesthetised on ice. Immediately after decapitation, the eye span of each ant was
268 measured using a micrometre under a binocular lens (Olympus SZH10). Brain extractions were
269 performed in HEPES solution (150 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 25 mM sucrose, 10 mM
270 HEPES) in silicone-coated Petri dishes. Extracted brains were individually fixed in 1% Zn-PFA
271 solution overnight and continuously shaken (60 rpm; Thermo Fisher) at room temperature. The
272 brains were then rinsed in HEPES solution six times for 10 minutes each. Subsequently, the brains
273 were fixed in Dent's Fixative (1:4 DMSO-Methanol solution) at room temperature for 1 hour and
274 30 minutes. After fixation, the brains were stored in 100% methanol at -20°C until the

275 immunostaining procedure. Once all brains were collected, they were shipped to the University
276 Rey Juan Carlos (Spain) under cold conditions to ensure preservation.

277 *Immunohistochemical analysis and imaging*

278 To image the neuroanatomy of the extracted brains, they were rehydrated in 0.1 M Tris buffer
279 and seated in a block and permeabilization solution of normal goat serum (NGS) for 1 hour
280 (PBSTN solution: 5% NGS + 0.005% sodium azide in 0.2% Triton-X phosphate buffered saline
281 [PBST]) at room temperature on a shaker. After this step, brains were incubated on the shaker
282 during four nights at room temperature in the primary antibody solution (SYNORF1 –
283 AB_2315426, Developmental Studies Hybridoma Bank – diluted 1:30 in PBSTN). Then, brains
284 were washed six times (10 minutes/wash) in 0.2% PBST and incubated for another three nights
285 protected from light on a shaker in AlexaFluor488 (ThermoFisher) Goat anti-Mouse IgG (H+L)
286 Cross-Adsorbed ReadyProbes™ Secondary Antibody (1:500 in PBSTN). Once immunolabeled,
287 brains were washed again for six times in 0.2% PBST (10 minutes/wash) and dehydrated in a
288 series of ethanol:PBS solutions (10 minutes each in 30%, 50%, 70%, 95%, 100%, 100% solutions).
289 Then, they were either stored overnight at -20˚C or directly mounted as follows. To mount the
290 brains, they were cleared in methyl salicylate (removing the ethanol solution) and seated in wells
291 of stainless-steel slides containing methyl salicylate. Brains were imaged on an Olympus FV-3000
292 laser scanning confocal microscope. Whole brain images were taken with either a 20X (NA = 0.8)
293 or a 40X (NA = 0.95) objective, in z-steps of 2 µm thickness. Close ups of the right mushroom body
294 calyces (both lateral and medial) were taken with an oil immersion 100x objective, in z-steps of

295 0.5 µm thickness. We selected regions where the mushroom body calyces and the peduncle was
296 visible.

297 *Neuroanatomic analyses*

298 Volumetry

299 We aimed to quantify relative volumes of the following functionally distinct brain neuropils (Ito
300 et al., 2014): the optic lobe (with lamina excluded - because it was not always present after
301 dissection), the antennal lobe, the central complex (including central body, protocerebral bridge
302 and nodes), and the mushroom body, where both the medial and lateral calyces, the peduncle,
303 and lobules were separately identified. To avoid possible human bias and to save labelling time,
304 a statistical template of *L. humile* brains was created by co-registering ten of the extracted brains.
305 For these ten brains, the mentioned functional neuropils and the rest of the undifferentiated
306 central brain were manually labelled in Amira (FEI – Amira 3D 2022.2) in the right hemisphere of
307 each brain (only the central complex was labelled in both hemispheres, but only half of its volume
308 was considered in the analyses). These manually obtained labels were used to create consensus
309 labels in the template, allowing for automatic labelling of the rest of the registered brains.
310 Automatic labels were smothered with Amira and manually corrected when needed. To calculate
311 relative volumes of each neuropil, we calculated the volume of each region and of the whole
312 brain in Amira (module Material Statistics) and computed the ratio between them. The ratio
313 between primary (sensory) and secondary (processing) neuropils was calculated by dividing the
314 volume of the optic lobes and antennal lobes by the volume of the mushroom bodies and the
315 central complex.

316 Template construction and automatic labelling of brains

317 Following Arganda-Carreras et al. protocol (2017, 2018), the *L. humile* brain template was created
318 using diffeomorphic space. Diffeomorphisms, which are smooth and invertible mappings of 3-D
319 images, are commonly utilised in computational neuroimaging due to their topological properties
320 that facilitate brain co-registration and the measurement of structural variation (Arganda-
321 Carreras et al., 2017; Klein et al., 2009). The brain template was constructed from 3-D average-
322 shape brains derived from the co-registration of 10 original brains obtained through confocal
323 scans.

324 Initially, variations in illumination among the original brain images were corrected using
325 histogram matching (Yoo, 2004). Co-registration involved an initial affine transformation to
326 maximise mutual information between brain volumes, identifying common neuropil shapes. This
327 was followed by iterative refinement using local non-rigid transformations to maximise the cross-
328 correlation of voxel intensities among the co-registered brains. The final average shapes were
329 generated by computing the voxel-wise median of the co-registered brains.

330 After creating the anatomical template, we applied the same diffeomorphic transformations to
331 each label image of the original 10 brains, as performed on their corresponding brain anatomy.
332 This was followed by per-voxel majority voting across all deformed label images of the same brain
333 to produce consensus labels.

334 To automatically label new brain images, we registered individual brain images against the
335 template using the same two-step method described above: an initial affine registration
336 maximising mutual information, followed by a non-rigid registration optimising cross-correlation.

337 The inverse transformations were then applied to the template's regional labels, automatically
338 assigning label values to the individual grey value brain images registered against the template.

339 All steps were implemented using the Advanced Normalisation Tools (ANTs) software (Avants et
340 al., 2011) after converting grey and label images to the open NRRD format in Fiji (Schindelin et
341 al., 2012).

342 Microglomeruli

343 Microglomeruli numbers were counted manually in close-ups of the right mushroom body
344 calyces using Amira. Close-up images of the right mushroom body calyces were analyzed to
345 manually measure the number of microglomeruli. Using Amira, three cylindrical regions (each
346 spanning 5 µm along the z-axis) were selected for analysis within each calyx: one in the collar and
347 two in the lip. For the lip, regions were differentiated into the non-dense (volume: 256.288 μm^3)
348 and dense areas (volume: 97 μm^3). The medial region of the calyx was located using the
349 pedunculus as a reference, and circles were drawn on ten consecutive slices in this region.

350 Microglomeruli contours were analyzed in slice 5 of the ten marked slices. A masking threshold
351 of 700 was applied in Amira to identify microglomeruli contours, and the generated labels were
352 smoothed using the Smooth Label tool (size: 3). This step helped standardize the contours of the
353 microglomeruli within the cylindrical regions.

354 Images of slice 5, with the contours generated in Amira, were then imported into ImageJ for
355 counting. Using the Multi-Point Tool in ImageJ (Schindelin et al., 2012), microglomeruli were

356 marked within the defined contours. For validation, 3-D images in Amira were reviewed to
357 confirm that all labeled contours corresponded to microglomeruli.

358 *Statistical analyses*

359 All analyses were performed using R version 4.4.2 (www.r-project.org). Assumptions of normality
360 were checked for every variable using the *descdist* function. Non-parametric analyses were used
361 instead of data transformation. When comparing means, we used ANOVA (*aov*), Kruskal-Wallis
362 (*kruskal.test*), or Wilcoxon tests (*wilcox.test*) considering the dependency of the variables. Mean
363 values are shown with standard errors ($\bar{x}$±SE). When comparing regression lines, we used Linear
364 Mixed Models (LMMs) for models following Normal distribution (*lmer*) and Generalised Linear
365 Mixed Models (GLMMs) using Template Model Builder (*glmmTMB*) for those following Negative
366 Binomial (left-skewed and overdispersed continuous events) and Beta distribution (skewed
367 continuous proportions variables ranged between 0 and 1). Knowing the dependency on body
368 size of locomotion in ants (*e.g.*, Hurlbert et al., 2008), we used eye span as a proxy of body size
369 as a covariable for all the analyses. We tested the dependency of the measured behavioural
370 variables on body size (covariable) and across observations (fixed factor). We added the nest
371 source and the ant worker identification code as random factors to take into account the
372 potential variability among workers from different colonies (*e.g.* see colony personalities in
373 Pinter-Wollman, 2012; behavioural differences in the populations surrounding the selected
374 populations in Sanmartín-Villar et al., 2022) and to avoid pseudoreplication (individual repeated
375 measures across observations).

To analyse the underlying mechanisms driving the measured variables on the three behavioural tests, we first analysed the effect of the cited factors on the locomotive traits: the average moving speed (calculated by discounting stationary events) and the proportion of time moving on each observation. All the tracks were smoothed on AnimalTA with Salvitzky-Golay filter with window length 15 and polyorder 2 to reduce the noise of the tracks. We considered the ant moving if its speed was above 2mm/s.

We used exploration (percentage of the total area visited in 10 min) as a variable to analyse the ant efficiency (or willingness) (i) to cover an unknown area (open field test), (ii) to cover a known area (after 10 min of acclimatisation) under the presence of a new object (object exposure test), and (iii) to discover unvisited paths (maze test). Based on the measurements of 25 ants' sizes, we considered that ants were exploring an area of 5 mm^2 around them for these exploration analyses. The exploration and the use of the inner part of the arena (38.48 mm^2) were compared between experimental and control individuals by comparing the variables obtained in the open field (10 first min of observation) and the exposure to the object (10 next min) with the first 10 min and next 10 min of observation in a control open field.

We used the proportion of time spent interacting with the object as a proxy of neophobia/neophilia. We used the relative number of choices allowing maze exploration (those following the concentric path; after now, "ratio of choices to reward") as a proxy of ant exploration in complex scenarios. We consider that the ant made a choice if it crossed both the projections on each Y junction of the maze. To calculate the ratio of choices to reward, we first divided the total number of choices allowing maze exploration by two to avoid considering the data of individuals crossing backwards a choice point. In the case of obtaining decimal values, the

398 numbers were rounded to the next superior complete digit. For instance, a value of three units
399 meant that one ant crossed three times the same choice, two forward and one backwards. By
400 dividing by two (3/2= 1.5) and rounding to the next superior complete digit we obtain the number
401 of forward crosses (2). Later, we divided the outcome variable by the total number of choices
402 (total number of choices allowing maze exploration plus the total number of choices driving to a
403 dead-end segment). We analysed the direction (left or right) of the first path selected in the maze
404 to disentangle if this selection was conditioned by (i) lateralization preference (*e.g*. trend to select
405 left or right paths; see Wystrach et al., 2016); (ii) previous choices performed in the previous
406 observation (*e.g.* starting to the same direction as in the previous observation); (iii) the openness
407 of the path (assuming that focal ants were able to perceive the end of the path in the selection
408 moment, they could have a preference to select a path that allows continuing exploring or going
409 to a dead-end path).

410 To analyse if individuals showed different behavioural patterns across time (intraindividual
411 differences in behavioural plasticity) and if these patterns differed among individuals
412 (interindividual differences in behavioural plasticity) we compared the AIC values of models with
413 and without observations as random slopes for each individual intercept (see Sanmartín-Villar et
414 al., 2021). AIC values are shown as the difference (Δ) between $AIC_{without\ random\ slopes}$ - $AIC_{with\ random}$
415 $_{slopes}$. Models with random slopes were only selected under ΔAIC positive values higher than 2
416 units (the rule of thumb when selecting AIC models, *e.g.* Mazerolle, 2006). The marginal (R^2m;
417 variance explained by the fixed factors) and conditional (R^2c; variance explained by the full
418 model) coefficient of determination are given for the most explanatory model (lower AIC value).

419 To analyse the consistency of behaviours, we analyse unpredictability, repeatability, and
420 coefficients of variation. We calculated unpredictability following the residual individual standard
421 deviation (riSD), a standard deviation that "estimates variation around the expected values at
422 every time step instead of estimating the amount of variation around the average of all of the
423 observations" (Stamps et al., 2012). We selected this method among others (see Cleasby et al.,
424 2015) because its simplicity and the similarity of the produced results respect other methods that
425 we found in previous experiments (e.g., Sanmartín-Villar et al., 2021).

426 We calculated repeatability using the *rptGaussian* function (*rptR,* Stoffel et al., 2017; Suppl.
427 Material), which is based on the Intraclass Correlation Coefficient (see Nakagawa & Schielzeth,
428 2010). Due to criticisms of using this coefficient without taking into account the measurement
429 errors (see Dingemanse et al., 2022), we also calculated the coefficient of inter- and
430 intraindividual variation (formulae 4 and 5 in Dingemanse et al., 2022) for the behaviours that
431 we consider more explanatory of each test (exploration in opened field test, object interaction in
432 object exposure test, and the ratio of choices to reward in the maze test).

433 Behavioural syndromes (the correlation of personality traits; Bell, 2006) were analysed by adding
434 one behavioural measure as variable and other as covariable of GLMMs. We compared the
435 correlation between exploration in open fields and the interaction with the new objects and both
436 of them with the maze participation (ant willingness to enter into the maze after being added in
437 the starting area), the ratio of choices to reward, and the number of times that ants reached the
438 end of the maze.

To test the correlation between behaviours dependent of different information processing (see Carere & Locurto, 2011), we compared the proportion of time spent on the object for the first time (neophilia/neophobia) with the total number of choices and the ratio of choices to reward in the fourth maze test (maze performance after maze flip).

The behavioural relationship with neuropils relative volume and number of microglomeruli was analysed using MANOVA tests (*manova*), adding the behavioural means or variances as dependent variables and the neuropils relative volume or the number of microglomeruli from the measured brain areas as independent variables. We analysed by the same method the effect of worker size on the neuropil's relative volumetry and number of microglomeruli. We also performed Principal Components Analysis (PCA) to analyse which variables explain most of the variance in behaviour, neuropils relative volume and number of microglomeruli. We then used the resulting PCs to compare behaviour with the two brain traits. We could not compare the PCs from the two brain measures in the same analyses because of the quality of the images (some good for volume analyses but not for microglomeruli and *vice versa*).

Results

Behavioural variability

Test performance and individual differences

Body size affected locomotive traits. Larger ants were faster and spent more time moving in the behavioural tests (Table 1). The locomotive traits changed across observations. The ants' average

speed decreased across observations, but the proportion of time spent moving showed different patterns across time: they did not change in the open field, increased in the object exposure test, and decreased in the maze (Table 1).

Workers' exploration depended on body size but showed similar and consistent interindividual patterns across time. Larger ants explored a larger proportion of area in the open field and the maze, but arena exploration was not dependent on ant size in the object exposure test (Table 1). Individual's exploration pattern was constant through observations (no intraindividual differences) in the open field (60.60±0.69% of the area) and maze (26.81±0.74%) but it showed a marginal increase in object exposure tests (1st observation: 53.22±1.71%, 5th observation: 57.71±1.67%; Table 1). In the three behavioural tests, the exploration pattern through observations showed no differences among individuals (no interindividual differences; Table 2; Figure 1C).

The addition of the object represented a change in the exploration of the arena's areas and its interaction highlighted different behavioural patterns. Smaller ants used more the inner zone of the experimental dish when the object was absent (open field and control group in the object exposure test) but body size did not affect the interaction with the object in the object exposure test (Table 1). On the first observation, ants were more attracted to the new object (9.84±1.21% of the time interacting) than to the inner area of the dish in the open field during the 10 previous minutes (5.67±0.97%; V= 7816, p= 0.003). The proportion of time spent in the inner zone of the dish by the control group (object absent) was even lower (2.73±1.58%), but no statistical differences were found with the experimental group due to the sample size (W= 686, p= 0.258). The time spent interacting with the object increased through observations (5th observation:

481 18.28±1.24%; F= 25.13, p< 0.001). This increase was more pronounced than the increase of the
482 use of the inner zone in the open field or for control ants in the object exposure test (object
483 absent; 5th observation < 6.17±10.82%). The increase of the proportion of time interacting with
484 the object across observations differed among ants (interindividual differences; Table 2; Figure
485 1D). Unexpectedly, ants exposed to the object (focals) modified the use of the inner zone of the
486 dish in the open field (object absent) through observations, while those never exposed (controls)
487 did not change it (Table 1). After 10 min of test, *i.e.*, in the object exposure test, control individuals
488 (object absent) also increased the use of the inner zone of the dish though observations (Table
489 1), although this increase was lower than the observed in the experimental group and in the open
490 field test.

491 Focal workers showed different strategies toward the rewarded path. The ratio of choices for
492 rewarded path differed among focal workers (interindividual differences) both before
493 (28.26±1.16%; Figure 1E) and after flipping the maze direction (27.51±1.56%; Figure 1F).
494 Individual choices followed such different trends that it was not possible to statistically detect an
495 overall effect across observations (F< 0.20, p> 0.689). Ant size did not affect the ratio of choices
496 for rewarded path (Z= -0.35, p= 0.725). More detailed information about maze performance and
497 strategies of path selection are explained in Supplementary Material.

498 *Behavioural syndromes*

499 Behaviours measured in different tests were correlated within individuals. Ants that explored
500 more in the open field spent more time interacting with the new object (Table 1). This correlation
501 was not affected by the observation number (Table 1). Ants that explored more in the open field

502 participated more in the maze exploration (t= -4.37, df= 149, *p*< 0.001) and reached more times
503 the reward (χ^2_2= 18.18, *p*< 0.001), but they did not reach faster the reward of the maze after
504 having reached it once (Z= 0.61, *p*= 1). Ants' exploration or object interaction were not correlated
505 with the ratio of choices to reward (Table 1).

506 We found no behavioural syndromes involving different information processing. The first
507 interaction with the object was not correlated with maze participation ($F_{1;149}$= 0.075, p= 0.785)
508 or with the ratio of choices to reward of the fourth observation (first encounter of the flipped
509 maze) (S= 627247, p= 0.915, r= 0.01).

510 *Unpredictability*

511 Focal workers behavioural unpredictability was independent in the three behavioural tests, and
512 it was not affected by their behavioural performance. No correlations were found between the
513 unpredictability of the exploration in open field, object interaction, and the ratio of choices to
514 reward (t< [0.51], df= 154, *p*> 0.635). No differences in unpredictability were found between
515 control and focal individuals for their exploration in the open field ($F_{1;165}$= 0.12, *p*= 0.731). The
516 introduction of a new object decreased the unpredictability of the ants' use of the inner part of
517 the arena when the object was present - or after 10 min of test for the control group - (control-
518 focal: $F_{4;165}$= 4.75, *p*= 0.031), while the unpredictability to use the inner part did not change in
519 the exploration on the open field test ($F_{4;165}$= 0.12, p= 0.731). No differences in unpredictability
520 for the object interaction were found for individuals increasing or decreasing the interaction over
521 the observations (t= -0.53, df= 154, *p*= 0.592). The unpredictability of the ratio of choices to
522 reward was similar for those individuals increasing or decreasing this ratio considering all
523 observations or only those before maze flip (t< 1.36, df= 137.04, *p*> 0.177). Ant's exploration and

524 the ratio of choices to reward were similarly unpredictable according to the ant size (Table 1),
525 but the interaction with the new object was less unpredictable in larger ants (Table 1).

526 *Coefficients of variation*

527 The behavioural variability observed among focal and control ant workers was observed in the
528 object exposure tests, but not in the open field. The coefficient of intraindividual variation for
529 exploration was similar among control (21.52±3.23%) and focal ants (23.80±1.09%; $F_{1;165}$= 0.30,
530 *p*= 0.586). No differences were found for worker size (t= -1.63, *p*= 0.193). The coefficient of
531 intraindividual variation for the interaction with the inner area differed between control (without
532 object; 0.91±0.09%) and focal ants (with object; 13.67±0.80%; $F_{1;164}$= 5.40, *p*= 0.021) in the object
533 exposure test. Smaller ants showed higher variation (t= 12.70, *p*< 0.007). The coefficient of
534 intraindividual variation for the ratio of choices to reward (23.06±0.92%) did not depend on
535 worker size (t= -1.41, df= 154, *p*= 0.162).

536 The coefficient of interindividual variation was similar among the control (33.35%) and the focal
537 group (20.03%; Z= 1.31; critical value= 1.96) for exploration but strongly differed for the
538 interaction with the object (control: 33.16%; focal: 75.57%; Z= -3.63; critical value= 1.96). Ants
539 showed an interindividual variation of 43.17% for the ratio of choices to reward.

540 <u>Neuroanatomy</u>

541 *Volumetry*

542 The relative volume of the cited functional neuropils was only analysed in 38 focal workers (daily
543 exposed to the three behavioural tests over five days) due to logistics. Unfortunately, the relative

544 neuropils volumes of the control group (not exposed to behavioural tests) could only be analysed
545 in two individuals and were therefore discarded.

546 The relative volumes of the different neuropils were uncorrelated in most cases. No significant
547 correlation was found between the body size and the relative volume of the brain neuropils
548 measured ($F_{1;30} < 2.21$, $p > 0.148$). The relative volumes of the medial and lateral mushroom body
549 calyces were positively correlated (t= 5.09, df= 36, $p > 0.001$, r= 64.68%). The relative volume of
550 the central complex and the optic lobe was negatively correlated (t= -2.39, df= 36, $p = 0.022$). No
551 correlations were found between the relative volume of (i) the optic and antennal lobes, (ii) the
552 central complex and the mushroom body, (iii) and the mushroom body and the sensory (optic or
553 antennal) lobes (t< [1.37], $p > 0.181$, r< 22.19%).

554 *Microglomeruli*

555 The number of microglomeruli of the analysed regions (collar, dense lip, non-dense lip) were
556 positively correlated ($t > 3.75$, $p < 0.001$, $r > 54.65$). The number of microglomeruli present in the
557 collar showed a positive correlation with the body size ($F_{1;24} = 5.42$, $p = 0.029$). No significant effect
558 of worker size was found for the microglomeruli of the lip ($F_{1;24} < 0.69$, $p = 0.414$).

559 <u>Behaviour-brain correlation</u>

560 The relative volume of the neuropils conditioned the behavioural performance in complex
561 environments. Ant workers with higher relative optic lobe volumes moved faster in the presence
562 of the new object ($F_{1;29} = 7.43$, $p = 0.011$; Figure 2A). Ant workers with lower primary:secondary
563 neuropils ratio showed higher exploration when exposed to the object ($F_{1;29} = 4.84$, $p = 0.036$;
564 Figure 2B) and to the maze ($F_{1;29} = 5.30$, $p = 0.029$; Figure 2C). No other significant correlations

565 were found between the neuropils' relative volume and the mean and variance of speed or
566 exploration in the three behavioural tests performed ($F_{1;29}$< 3.02, *p*= 0.093). The regression slopes
567 of the ratio of choices to reward before the maze flip were negatively correlated with the relative
568 volume of the medial mushroom body calyx ($F_{1;29}$= 7.65, *p*= 0.010; Figure 2D; but not with the
569 lateral calyx: $F_{1;29}$= 2.68, *p*= 0.113) and positively correlated with the relative volume of the
570 central complex ($F_{1;29}$= 10.98, *p*= 0.002; Figure 2E). No significant differences were found when
571 comparing behavioural and brain volumetry PCs ($F_{1;35}$< 1.73, *p*> 0.196).

572 The number of microglomeruli was similar among control and focal ants and among those
573 showing different mean behaviours. No differences were found between the number of
574 microglomeruli of individuals exposed (N= 38) or not exposed (control; N= 5) to behavioural tests
575 in any of the three analysed brain regions ($F_{1;38}$< 1.64 , *p*> 0.209), but a trend suggested that
576 control individuals showed higher variance than the focal ones in the number of microglomeruli
577 present in the collar ($F_{1;38}$= 3.90, *p*= 0.056). The number of microglomeruli of the three regions
578 analysed was not correlated with the average speed ($F_{1;24}$< 1.20, *p*> 0.334) or the average
579 exploration ($F_{1;24}$< 1.17, *p*> 0.345) performed on the three behavioural tests. Nor were
580 correlations found with the mean object interactions and the mean ratio of choices to reward
581 before and after maze flip ($F_{1;24}$< 1.79, *p*> 0.189).

582 However, the number of microglomeruli differed among individuals showing different
583 behavioural patterns under complex environments. The number of microglomeruli of the three
584 regions analysed was not correlated with the variance of speed ($F_{1;24}$< 1.20, *p*> 0.334) or
585 exploration ($F_{1;24}$< 1.17, *p*> 0.345) performed on the three behavioural tests. Individuals with
586 higher number of microglomeruli on the dense lip showed higher variance ($F_{1;24}$= 6.50, *p*= 0.018;

587 Figure 3A), more unpredictability (riSD: $F_{1;24}$= 7.87, *p*= 0.010; Figure 3B) on the object interaction,
588 and higher increase of the ratio of choices to reward before maze flip ($F_{1;24}$= 9.10, *p*= 0.006; Figure
589 3C). No correlations were found between the object interaction variance and the number of
590 microglomeruli from the collar or non-dense lip ($F_{1;24}$< 1.23, *p*> 0.278), nor between the slopes
591 of the ratio of choices to reward before maze flip and the non-dense lip ($F_{1;24}$= 1.01, *p*= 0.326).
592 Individuals with higher numbers of microglomeruli in the collar showed decreased slopes of the
593 ratio of choices to reward before maze flip ($F_{1;24}$= 5.81, *p*= 0.024; Figure 3D). The variance after
594 maze flip was not correlated with the number of microglomeruli in any region ($F_{1;24}$< 0.85, *p*>
595 0.367). No significant differences were found when comparing behavioural and microglomeruli
596 PCs ($F_{1;13}$< 1.18, *p*> 0.339).

597

598 **Discussion**

599 The behavioral variability observed at the individual level suggests two mechanisms by which
600 *Linepithema humile* colonies manage to explore new environments: through efficiency
601 associated with body size and neural processing areas. On one hand, larger ants showed higher
602 speed, movement, exploration and more microglomeruli on the collar (visual perception) while
603 they did not show larger relative neuropil volumes. Smaller ants explored the arena differently,
604 visiting its inner zone more frequently. Behavioural differences linked to body size could increase
605 the efficiency of colony exploration due to different trial-and-error strategies. On the other hand,
606 individuals who invested more in processing than in perception were more efficient at exploring
607 complex environments, especially those who processed olfactory rather than visual stimuli.

608 Complex scenarios resolution may be favoured by this small number of individuals in whom the
609 correlation between behaviour and neuroanatomy was observed (see dots outside the dashed
610 line in Figure 2D-E, Figure 3C-D). This possible evolutionary strategies supports the hypothesis of
611 the colony's investment reduction in the behavioural and neural plasticity of its members in *L.*
612 *humile* (e.g., Sanmartín-Villar et al., 2021), limiting costs to cope with complex environments
613 through a neural demanding mechanism in only a few individuals, while in a non-neural
614 demanding mechanism in most them. Our results suggest that exploration at the colony level
615 might depend on the variability among-individuals, but interestingly, in the case of our study this
616 variability was not dependent on the individual adult development - as division of labour in
617 eusocial animals is often explained - because we analysed workers of the same age after
618 emergence. Future studies should corroborate in the field the consequences of the mechanisms
619 proposed here for local adaptation, invasiveness, and coping with climate change.

620 The expected similar behaviour among workers and lack of correlation between behaviour and
621 neuroanatomical traits were only observed at low environmental complexity. Individuals showed
622 different and dependent responses of their neuroanatomy when exposed to complex
623 environmental challenges, such as novel experiences and choices. Sensory neuropils hardly
624 affected behavior, only detecting faster exploration after object exposure in individuals with
625 larger visual lobes which support the navigation improvement through volume increase of
626 primary lobes as seen in other species of ants (*Cataglyphus nodus*; Grob et al., 2017, *Cataglyphis*
627 *fortis*; Schmitt et al., 2016, *Camponotus rufipus*; Yilmaz et al., 2017). Processing areas (central
628 complex and mushroom bodies) determined exploration and maze progress. Progress in the
629 maze was positively dependent on the number of synaptic clusters in the dense lip (olfactory

processing), but negatively dependent on those in the collar (visual processing). Individuals who showed more dense lip synaptic clusters showed higher behavioral variability (variance and unpredictability). These results highlight the relevance of high processing areas in the resolution of complex environments and the different functions of different brain regions. Specialized behavioral strategies could be expected from a higher neuropil volume due to the development of synaptic clusters, but also a lower number of these clusters due to pruning (the concentration of microglomeruli in a more volumetric button due to specialization; Changeux and Danchin, 1976; Cowan et al., 1984). The correlations found between exploration efficiency and the number of microglomeruli could highlight the diversity of behavioral strategies (variance, unpredictability) rather than learning processes, although they coil highlight long term memory formation due to the increase of microglomeruli density (Falibene et al., 2015).

In contrast to the expected, *L. humile* workers showed low percentage of exploratory performance, exploring an average of 60% ca. of the open field, interacting with the object up to 18% of time, and selecting rewarded paths in 27% ca. of cases. Although *L. humile* is considered a monomorphic species, body size conditioned behavioural and neural traits. The allometry for speed and body size is a common phenomenon in ants due to the higher motion of individuals with longer legs and higher metabolic rate (Hurlbert et al., 2008), but we did not expect to see such a strong difference within a monomorphic species. Assuming size-dependent differences, we expected that body size correlate with risk-taking decisions as it occurs in fish (e.g. Darby & McGhee, 2019; but see Brown & Braithwaite, 2004), turtles (Ibáñez et al., 2013), or snakes (Mayer et al., 2016). Therefore, we expected a body size dependency on the interaction with the novel object (but see the mismatch between neophobia and boldness in Carter et al., 2012), the use of

652 the inner part of the arena (assumed as a bolder trait for some authors; Carlson & Langkilde,
653 2013; Detrain et al., 2019; Sneddon, 2003; Valle, 1970; Walsh & Cummins, 1976), and the
654 participation on the maze. However, none of these traits correlated with body size, suggesting
655 that *L. humile* workers may not evaluate the environment challenges according to their body
656 characteristics.

657 The interaction with the object was less variable and unpredictable for larger workers, suggesting
658 a more homogeneous behaviour. In addition, larger workers reached more times the reward
659 inside the maze but decreased their ratio of choices to reward through observations. Although
660 we could not determine the overall volume changes in the brains based on experience (see
661 Gronenberg et al., 1996) due to our methodology, our results support the relationship between
662 body size, brain development and foraging behaviour shown on *Atta cephalotes* (Arganda et al.,
663 2020), highlighting our findings that larger individuals were better in simple tasks like exploration
664 and navigation but not in complex tasks like in the maze and also being associated with larger
665 volume ratio of primary to secondary neuropil. All these results suggest that large workers
666 succeeded to better solve environmental complexity (higher exploration and maze-solving, more
667 constant object interaction) due to their higher locomotive traits, but not due to the
668 neuroanatomy that might underlie their cognitive skills (no correlation was found between speed
669 or exploration or body size and neuropil relative volumes or microglomeruli abundance). In fact,
670 our results suggest that large workers only had more microglomeruli in the collar than smaller
671 workers (therefore, no more microglomeruli on the lip than smaller workers). We consider
672 improbable that larger workers could be able to better solve the tests in which they were exposed
673 due to their higher secondary processing of visual stimuli in comparison with the chemical stimuli

674 (more microglomeruli on the collar, but no more on the lip; Falibene et al., 2016; Gordon et al.,
675 2018, 2019; Gordon & Traniello, 2018; Seid & Wehner, 2008) due to the lack of visual references
676 and similitude among choices. In any case, our results support the idea that body size can
677 influence environmental exploration in monomorphic species as it was shown in other species
678 such as *Lasius niger* (*e.g.*, Grześ et al., 2016; Okrutniak et al., 2020). Therefore, future behavioural
679 studies should control for the effect of body size in monomorphic ant species but also analyse
680 whether worker body size is related to the spatial complexity of the environment as an
681 evolutionary strategy.

682 Focal workers acclimatised to the experimental setup, reducing their average speed, increasing
683 the interaction with the object and decreasing their mobility in the maze over the observations.
684 This suggests that ants were able to modify their behaviour according to previous experiences,
685 as was previously shown in ants of the same species (Wagner et al., 2023). However, we cannot
686 discard that the obtained outcomes depend on other factors such as fatigue or developmental-
687 related changes. Previous works done with repeated observations on open fields showed
688 different patterns (reduction of locomotion in a ten repetition test in *L. niger*, Sanmartín-Villar et
689 al., 2021; increase of locomotion in a five repetition test in *L. humile*, Sanmartín-Villar et al.,
690 2021). If ants recognise the object from the previous tests, the object introduced could only be
691 considered as novel on the first observation. Despite the lack of novelty and apparently lack of
692 functionality of the object (except for climbing), the focal workers interacted more with it
693 through the observations, especially the workers that showed higher exploration, while the
694 opposite happened in the maze, in which workers could find a reward. Note that we did not
695 starve focal individuals, and a lack of food motivation could interfere with performance in the

696 maze. Still, we expected that ants would increase their maze performance while losing the fear
697 to navigate through it and especially after the reduction of their speed through observations,
698 which may could be due to the speed-accuracy trade-off and its dependency on neural structures
699 (reviewed in Standage et al., 2014).

700 The increase of the environmental complexity through the addition of an object in a relatively
701 known arena (experienced through 10 minutes) triggered behavioural differences among
702 workers and modified the apparently characteristic homogeneous locomotion pattern of the *L.*
703 *humile* workers in open fields (also seen in Sanmartín-Villar et al., 2021). The unpredictability of
704 the object interaction, *i.e.*, its deviation through the expected pattern, correlated with the neural
705 area in charge of processing chemical stimuli (dense lip) and the mean and variance of the object
706 interaction correlated with the relative volume of the central complex, which is in charge of
707 navigation (Honkanen et al., 2019; reviewed in Webb & Wystrach, 2016). The increase of the
708 object interaction and movement (marginally exploration) could fit with the expected result of
709 environmental enrichment (reviewed in Newberry, 1995), in which individuals improve their
710 cognitive skills after the addition of objects. Individuals exposed to the object used more the
711 inner zone of the area in posterior tests when the object was not present while control individuals
712 showed this pattern with a delay of 10 min, suggesting an improvement of the area exploration
713 after previous stimuli. However, we cannot discard that the behaviours observed were caused
714 by the stress produced when adding a new element in a known area. Considering the correlation
715 trend of the object interaction with the central complex (navigation centre), we propose a third
716 explanation in which workers used the object as a spatial reference to which they recurred to
717 orient in an arena lacking other spatial clues.

718 The difficulty of the maze test highlighted the relation between neuroanatomy and behaviour.
719 Within the mushroom bodies (in charge of information processing, memory and learning), the
720 maze performance before the flip was only correlated with the relative volume of the medial
721 calyx, and not with the lateral calyx. As medial and lateral calyces are considered to perform the
722 same function, our results might be a suggestion for further research trying to disentangle
723 distinctive information processing among them. The relative volume of the central complex
724 (navigation centre) also correlates with maze performance before the flip. The difficulty of the
725 maze due to its design and the lack of references accomplished the aim of exposing ants towards
726 an exercise focused on exploration rather than learning (we did not train the ants before the
727 tests). Workers with more microglomeruli in dense lip showed higher ratio of choices to reward
728 before maze flip, which suggests that the presence of more synaptic routes - but smaller - in this
729 region responsible for chemical processing allowed individuals to improve their efficiency on the
730 maze. We discarded that the experience on the maze was responsible for the synaptic cluster
731 development during the tests because of the similar microglomeruli found between control and
732 focal workers, although more research must be performed to conclude it. In contrast, the number
733 of microglomeruli of the collar negatively correlated with the ratio of choices to reward before
734 maze flip. In addition, the relative volume of the central complex (more relevant in the maze
735 performance) was negatively correlated with the relative volume of the optic lobes. We interpret
736 these results as a conflict between the role of primary and higher order processing regions in the
737 studied behaviours: one region in charge of perceiving and processing of visual stimuli (optical
738 lobes), and two in charge of the higher order processing of stimuli (the chemical information in
739 the non-dense lip in the mushroom body calyces, and the visual, chemical and mechanosensory

740 information required for orientation in the central complex). Individuals that relied more on
741 visual stimuli were probably not able to disentangle the orientation on the maze due to the exact
742 symmetry of all the choices. However, individuals relying more on the chemical stimulus could
743 better interpret their own pheromones or other odors present in the maze (although backwards
744 behaviour was uncommon). Surprisingly, no correlations were found with the antennal lobes,
745 which we expected to have a higher relevance in the ants' orientation.

746 The relevance of the microglomeruli of the dense lip in behaviour (unpredictability of the object
747 interaction, increase of rewarded path choice) opposes the results found in honey bees (Van Nest
748 et al., 2017), in which only the microglomeruli of the non-dense lip (also olfactory) were related
749 with foraging behaviour. Thermal fluctuations affect the microglomeruli density in the non-dense
750 lip in the mushroom body calyx of *Camponotus mus* but were less evident in dense lip and visual
751 collar (Falibene et al., 2016). If the correlations between these experiments are comparable, this
752 would highlight phylogenetic differences in the neuroethology of the species, with behaviour
753 being dependent on different neural structures across insect and ant species. However, all these
754 experiments highlight the expected relevance of the olfactory stimulus over the visual one to
755 drive behaviour.

756 Our results suggest that individual variability depends on neuronal development and highlight
757 behavioural plasticity and the strategies to cope with environments with different degree of
758 challenge in *Linepithema humile*, skills likely related with its adaptation in introduced areas.
759 Although we could not observe the associated neuronal changes with each exposure to
760 behavioural tests, likely to decipher in detail, the mechanisms involved in their behavioural
761 patterns, we provide the first neuroethological clue to understand the invasive strategies of this

species. We attempted to dissect and correlate the behavioural and neuronal plasticity of an invasive species to cope with novel scenarios, providing a framework for understanding the dynamics for colony fitness in the introduced areas. We are convinced that the present results could be the basis for future control and management programs dealing with *L. humile* invasion. For instance, neural disruptors could affect the behaviour of worker ants differently depending on their size and on the complexity of their environment.

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783 and S.A. collected the brain-related data; I.S.V. analysed the data; S.N., M.E.V., S.A., I.A.C., and
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787

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1084 **Table 1**. Statistical values (*Value*: t, F or Z) for the behavioural variables measured. *Ratio choices*

1085 *reward*: relative number of times ants selected the path connecting to the reward instead of the

1086 dead-end paths of the maze.

Variables	**Test**	**Value**	**DF (F)**	***P***
Speed × Size	Open field	26.78	1;154.36	**<0.001**
	Object exposure	31.55	1;148.50	**<0.001**
	Maze	6.69	1;151.23	**0.011**
Speed × Observation	Open field	11.83	1;621.48	**<0.001**
	Object exposure	9.04	1;600.23	**0.003**
	Maze	17.10	1;605.13	**<0.001**
Time Moving (%) × Size	Open field	11.17	1;153.78	**0.001**
	Object exposure	5.86	1;154.07	**0.017**
	Maze	2.83		**0.005**
Time Moving (%) × Observation	Open field	3.29	1;621.71	**0.070**
	Object exposure	10.67	1;622.01	**0.001**
	Maze	-2.92		**0.004**
Exploration × Size	Open field	3.46		**<0.001**
	Object exposure	1.76		0.078
	Maze	2.72		**0.007**
Exploration × Observation	Open field	0.45		0.656
	Object exposure	1.90		0.057
	Maze	1.45		0.141
Object interaction × Size	Object exposure	-0.93		0.354
Object interaction × Observation	Object exposure	8.25		**<0.001**
Inner arena use × Size	Open field	3.33		**<0.001**
	Object exposure (control)	-2.82		**0.005**
Inner arena use × Observation	Open field	3.03		**0.002**
	Open field (control)	0.57		0.572
	Object exposure (control)	2.27		**0.026**
Maze participation × Size	Maze	1.91	1;149	0.169
Maze participation × Observation	Maze	2.62	1;603	0.106
Ratio choices reward × Size	Maze	0.51		0.608
Ratio choices reward × Observation	Maze	-0.35		0.725
Exploration × Object interaction	Open field & Object exposure	2.88		**0.004**
Exploration × Object interaction × Observation	Open field & Object exposure	0.29		0.773
Exploration × Ratio choices reward	Open field & Maze	1.25		0.212
Object interaction × Ratio choices reward	Object exposure & Maze	1.34		0.182

Exploration unpredictability × Size	Open field	1.91	1;152	0.169
Object interaction unpredictability × Size	Object exposure	5.44	1;152	**0.021**
Rewarded path choice unpredictability × Size	Maze	0.00	1;152	0.926
Exploration unpredictability × Object interaction unpredictability	Open field & Object exposure	0.48	1;154	0.635
Exploration unpredictability × Ratio choices reward unpredictability	Open field & Maze	-0.09	1;154	0.930
Object interaction unpredictability × Ratio choices reward unpredictability	Object exposure & Maze	0.51	1;154	0.611

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1088 **Table 2**. Intraindividual differences. *Ratio choices reward*: relative number of times ants selected

1089 the path connecting to the reward instead of the dead-end paths of the maze.

Variable	**Test**	ΔAIC	R^2m	R^2c
Exploration	Open field		0.06	0.49
	Object exposure		0.01	0.11
	Maze		-0.22	-2.82
Object interaction	Object exposure	9.02	0.16	1.60
Ratio choices reward before flip	Maze	73.55		
Ratio choices reward after flip	Maze	52.72		

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1091 **Figure 1**. Behavioural tests and patterns of *L. humile* workers (regression models) across
1092 observations. **A-B**: Schematic representation of the novel object (A) and maze (B) used in the
1093 behavioural tests. Both objects can be downloaded for printing from
1094 https://www.tinkercad.com/things/2lNxDsoOoAD-neophobia-object-miiz and
1095 https://www.tinkercad.com/things/7IELVyTIwXr-maze-miiz. **C**: Exploration in the open field test.
1096 **D**: Interaction with the object. **E**: Relative number of times ants selected the path connecting to
1097 the reward instead of the dead-end paths of the maze (*Ratio choices reward*) before maze
1098 direction flip. **F**: Relative number of times ants selected the path connecting to the reward instead
1099 of the dead-end paths of the maze (*Ratio choices reward*) after maze direction flip.

1100 **Figure 2**. Correlation between brain volumetry and behaviour of *L. humile* workers: correlation
1101 between the relative volume of the optic lobe and the mean speed performed (**A**); correlation
1102 between the ratio primary:secondary neuropils and the mean exploration performed in presence
1103 of the object (**B**), on the maze (**C**), and the slopes for the relative number of times ants selected
1104 the path connecting to the reward instead of the dead-end paths of the maze (*Ratio choices*
1105 *reward*) before flip (**D**); correlation between the relative volume of the central complex and the
1106 slopes for the relative number of times ants selected the path connecting to the reward instead
1107 of the dead-end paths of the maze before flip (**E**). Continuous red: linear regression between the
1108 two variables. Dashed orange: zero value to facilitate the visual comparison of negative and
1109 positive slopes. F: different sections of a *L. humile* brain showing the neuropils analysed (synapsin
1110 in green).

1111 **Figure 3**. Correlation between the number of microglomeruli and behaviour of L. humile workers:
1112 correlation between the number of microglomeruli of the dense lip and the variance (**A**) and

1113 unpredictability (**B**) of the object interaction, and the slopes of choices before maze flip (**C**);

1114 correlation between the number of microglomeruli of the collar and the slopes of choices before

1115 maze flip (**D**).

Figure 1

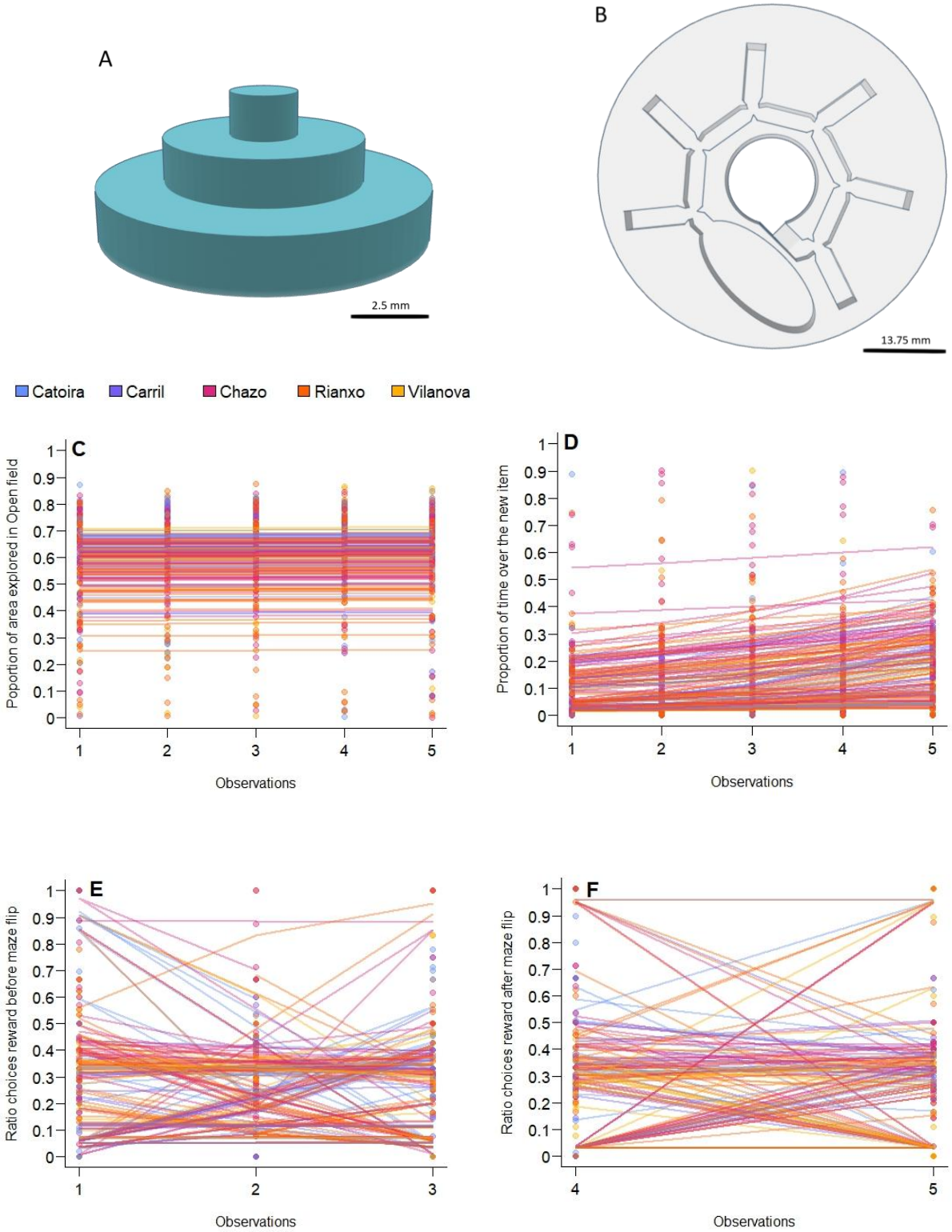


Figure 2

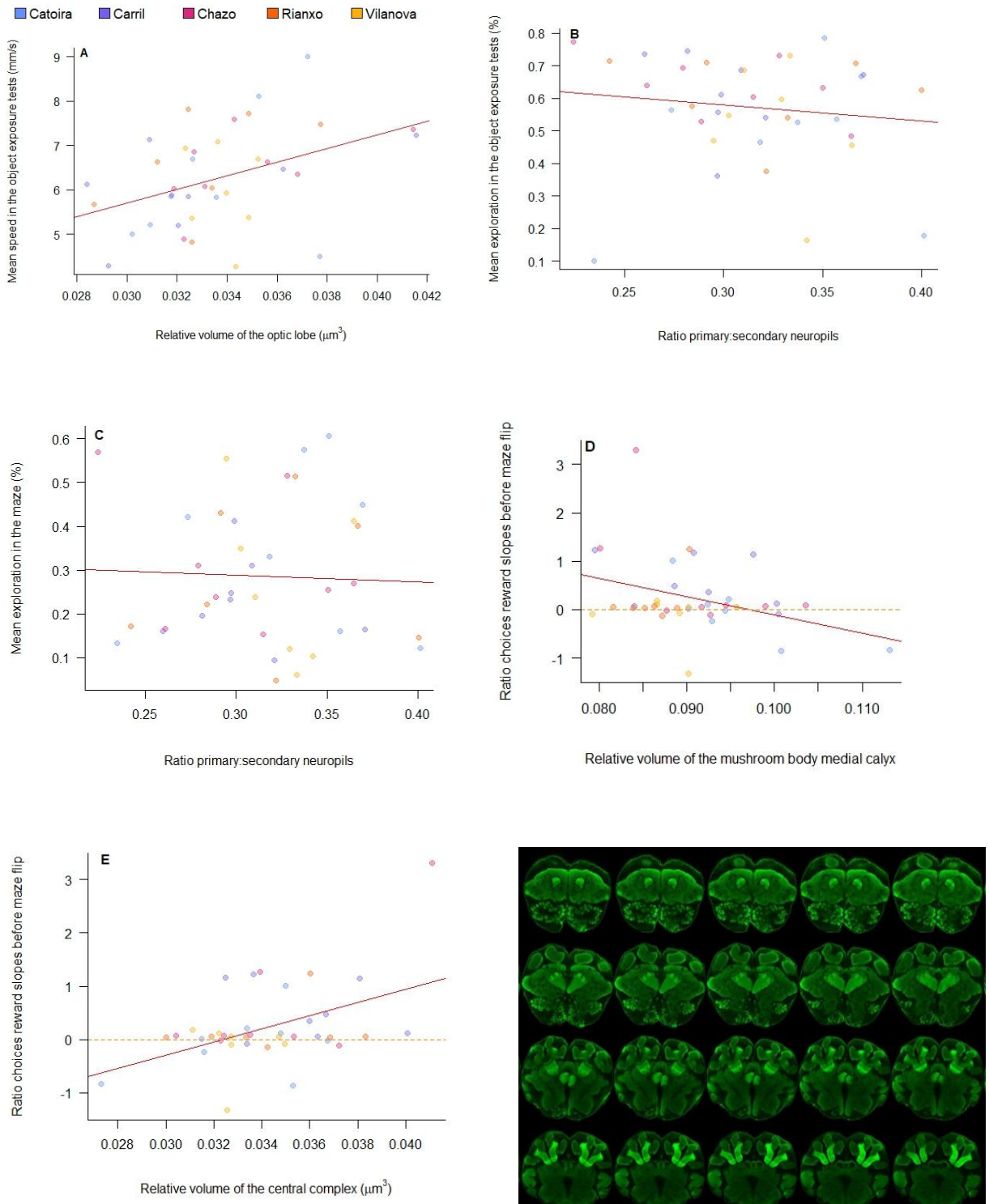
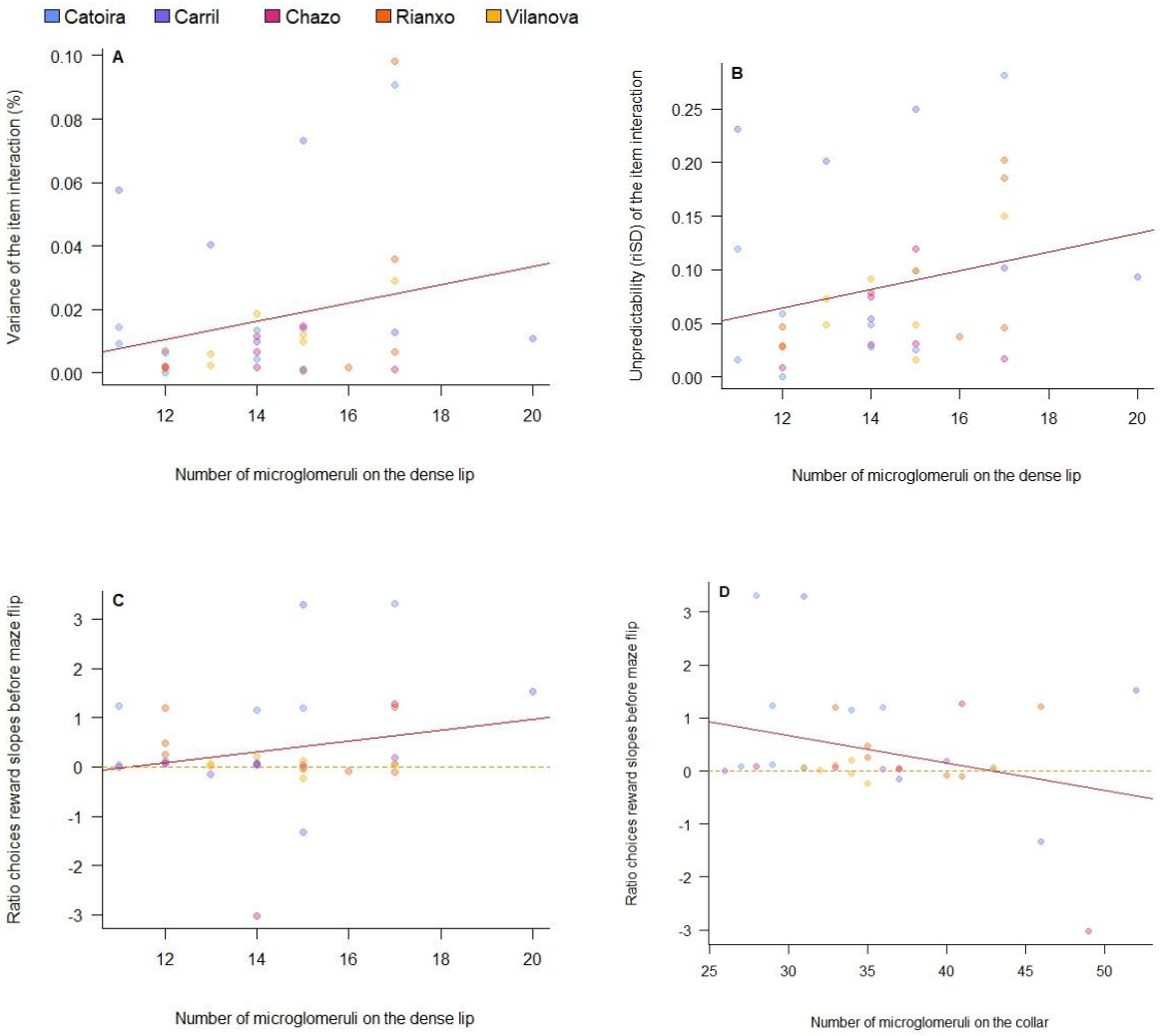

Catoira
Carril
Chazo
Rianxo
Vilanova
A
Mean speed in the object exposure tests (mm/s)
Relative volume of the optic lobe (μm3)
B
Mean exploration in the object exposure tests (%)
Ratio primary:secondary neuropils
C
Mean exploration in the maze (%)
Ratio primary:secondary neuropils
D
Ratio choices reward slopes before maze flip
Relative volume of the mushroom body medial calyx
E
Ratio choices reward slopes before maze flip
Relative volume of the central complex (μm3)

Figure 3



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