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Segment-specific and State-dependent Targeting Accuracy of the Stick Insect

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Abbreviations:

fCO – femoral chordotonal organ

dist. – distance

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35 **Abstract**

36 In its natural habitat, *Carausius morosus* climbs on the branches of bushes and trees. Previous
37 work suggested that stick insects perform targeting movements with their hind legs to find
38 support more easily. It has been assumed that the animals use position information from the
39 anterior legs to control the touchdown position of the ipsilateral posterior legs. Here we
40 address the questions if not only the hind but also the middle leg performs targeting, and if
41 targeting is still present in a walking animal when influences of mechanical coupling through
42 the ground are removed. If this were the case, it would emphasize the role of underlying
43 neuronal mechanisms. We studied whether targeting occurred in both legs, when the rostral
44 neighboring leg, i.e. either middle- or front leg, was placed at defined positions relative to the
45 body, and analyzed targeting precision for dependency on the targeted position. Under these
46 conditions, the touchdown positions of the hind legs show correlation to the position of the
47 middle leg parallel and perpendicular to the body axis while only weak correlation exists
48 between the middle and front legs, and only in parallel to the body axis. In continuously
49 walking tethered animals targeting accuracy of hind and middle legs parallel to the body axis
50 was barely different. However, targeting became significantly more accurate perpendicular to
51 the body axis. Our results suggest that a neural mechanism exists for controlling the
52 touchdown position of the posterior leg but that the strength of this mechanism is segment-
53 specific and dependent on the behavioral context in which it is used.

54 **Introduction**

55 If terrestrial animals want to walk through any kind of environment, they need to know how
56 to move their legs to reliably find foothold. This information becomes particularly relevant
57 when navigating through an unknown or irregular terrain. For cats and humans it is known
58 that targeting of leg movements is primarily mediated by visual information which is captured
59 on average two steps ahead (cat: McVea and Pearson, 2007; McVea et al., 2009; Wilkinson
60 and Sherk, 2005; human: Mohagheghi et al., 2004; Patla and Vickers, 2003). Likewise Niven
61 et al. (2010) could show that locusts visually target their front legs towards the position of a
62 ladder rug and information about the position of the rug is acquired before leg swing is
63 initiated. However, how do animals find appropriate foothold when visual information is not
64 available? In the same study, Niven et al. (2010) also observed that placement of the middle
65 leg in locusts was not visually guided. For this purpose, the control system not only has to
66 have information about the environment, but also on the actual positions of the aiming and the
67 targeted leg. This information can be provided by several kinds of sense organs. Cats, for
68 example, use information from muscle receptors and cutaneous receptors in the skin to match
69 sensory information from different joints and reliably represent the position of the limb
70 relative to the body in the dorsal root ganglia (Stein et al., 2004). This information is also
71 transferred to area 5 in the posterior parietal cortex where it is integrated with memorized
72 visual information in order to perform appropriate leg movements (McVea et al., 2009) which
73 are in turn generated in the local networks of the spinal cord (for review, see, e.g., Grillner
74 and Jessell, 2009; Kiehn et al., 2010). Similarly, it is known from work on stick insects that
75 proprioceptive inputs of several sensory structures in the leg influence the protraction
76 endpoint of all legs (Wendler, 1964; Bässler, 1977; Dean and Wendler, 1983; Cruse et al.
77 1984).

78 In their natural habitat, stick insects (*Carausius morosus*) live in a complex three dimensional
79 maze of twigs and leaves to which they have to constantly adapt their locomotor behavior. As
80 nocturnal animals they primarily rely on mechanosensory information from the antennae, and
81 do not use vision to guide their front legs towards an appropriate foothold (Dürr, 2001,
82 Bläsing and Cruse, 2004, Schütz and Dürr, 2011). How the stick insect guides its hind legs
83 towards an appropriate foothold has also been the focus of several earlier investigations (e.g.
84 Cruse, 1979; Cruse et al., 1984; Dean, 1984; Dean, 1989; Dean and Wendler, 1983), in which
85 it was shown that the touchdown position of the hind leg depends on the position of the
86 standing middle leg (Cruse, 1979). The sense organs that appear to be primarily responsible

87 for targeting parallel to the body axis are hair rows and hair fields on the coxa (Cruse et al.,
88 1984; Dean and Wendler, 1983), while targeting information perpendicular to the body axis
89 seems to originate primarily from the femoral chordotonal organ (fCO) (Cruse et al., 1984).
90 Information about posture of the middle leg is transmitted via the ipsilateral connective
91 (Dean, 1989), and Brunn and Dean (1994) described three interneurons, each signaling the
92 angle of one leg joint, that together could be able to encode the tarsus position. This has led to
93 the inclusion of targeting into coordination rules, which assume targeting of all legs during
94 walking in the stick insect (Cruse, 1990; Cruse et al., 1995).

95 However, it is not known if stick insects guide their middle legs towards an appropriate
96 foothold equally well, and if they also use position information from the front legs. In
97 addition, it is still unclear how information from sense organs that detect angular positions
98 and velocities of joints is incorporated into a reference frame for motor control. In recent
99 years, more and more evidence has suggested that the behavioral state of an animal is
100 important for the effectiveness of sensory processing onto the motoneurons (Clarac et al.,
101 2000; Duysens et al., 2000; Pearson, 1993; Akay et al., 2007; Hellekes et al., 2012; for
102 review, see, e.g. Büschges and El Manira, 1998) but it is not known to what extent movement
103 of the anterior leg, limb joint constraints or effects of mechanical coupling through the ground
104 influence the targeting accuracy of the middle or hind leg, and at which time point the
105 information used for targeting is sampled.

106 We have therefore investigated the placement of middle and hind legs towards their anterior
107 neighbor in the stick insect *Carausius morosus* to study spatial coordination of the legs and
108 foot placement without visual guidance under two behavioral conditions, either the first step
109 after standing or during continuous walking. We measured the targeting accuracy of the two
110 legs and compared their performance with each other, and under the two behavioral
111 conditions, to find out if there were segment-specific and state-dependent differences. By
112 tethering the animal above a slippery surface we could reliably remove mechanical coupling
113 of leg movements through the ground (Gruhn et al., 2006). Targeting in the continuously
114 walking animal under these conditions would emphasize the role of neuronal control
115 mechanisms underlying this behavior.

116 **Materials and Methods**

117 *Animals*

118 All experiments were performed on adult female stick insects (*Carausius morosus*). Animals
119 were reared in the animal facility of the institute in a 12-h/12-h light/dark cycle at 23–25°C
120 and were fed with blackberry leaves (*Rubus fruticosus*) *ad libitum*.

121 *Experimental setup*

122 The general setup is an adaptation of the setup described in detail in Gruhn et al. (2006). In all
123 experiments, animals were tethered above a 13.5 x 13.5 cm polished nickel-coated brass plate.
124 To allow unimpeded stationary stepping or walking under tethered conditions and minimize
125 mechanical coupling between the legs, the plate was covered with a lubricant composed of
126 95% glycerin and 5% saturated NaCl. The animals were glued ventral side down on a 3 x 5 x
127 100 mm [W x H x L] balsa rod using three droplets of dental cement (ProTempII, ESPE,
128 Seefeld, Germany) along the length of the thorax such that the legs and head protruded from
129 the rod and all joints were unrestrained. Animal height above the substrate was adjustable, but
130 was typically set to 10 mm, measured from the coxae of front and hind legs on both sides.
131 Experiments were performed in a darkened Faraday cage at room temperature.

132 In the continuous walking sequences, walking was elicited by projecting a progressive striped
133 pattern (pattern wave length 21°) onto two 13.5 cm diameter round glass screens placed at
134 right angles to each other and at a 45° angle to the walking surface, about 6-7 cm away from
135 the eyes of the animal (Scharstein, 1989; for a detailed description see Gruhn et al., 2006).
136 Reflections on the polished brass plate further increased the field of view. Alternatively, a
137 single white stripe on dark background (toward which the animals orient with straight walking
138 sequences) was placed in front of the animal. If the animal did not begin locomotion
139 spontaneously, walking was elicited by light brush strokes to the abdomen. In all sequences
140 with the previously positioned, standing anterior leg, stepping of the posterior leg was also
141 elicited by light brush strokes to the abdomen.

142 To analyze the precision of first steps, we carefully placed tarsus of the anterior leg on a small
143 5mm x 10mm cardboard platform with a particularly rough surface. This small platform was
144 attached to a brass tube which was connected to a micromanipulator, similar to Cruse (1979).
145 Exact positioning of the anterior leg was achieved by moving the platform to one of seven
146 aiming positions. In all experiments the tarsus was not artificially fixed to the platform. The

147 location of these positions was defined by the central position (No. 5) directly underneath the
148 femur-tibia joint when the tibia was perpendicular to the surface, and the femur perpendicular
149 to the body (see figure 1). The other six tested positions were arranged around position No. 5
150 as follows: positions 1 and 2 were 5 mm posterior, while positions 8 and 9 were 5 mm
151 anterior. Positions 1 and 4 were 5 mm central, while positions 6 and 9 were 5 mm distal of
152 position 5. Positions 3 and 7 could be taken up by the anterior leg, but very often caused the
153 animal to re-position the anterior leg. Therefore we focused our analysis on the remaining
154 positions. The standing position of the anterior leg was randomly changed to a different
155 position after each step of the posterior leg. Figure 1 gives a schematic representation of the
156 stationary stick insect with the seven aiming positions relative to the body, shown for the
157 stepping hind and stationary middle leg. The same general setup was used for the front leg. A
158 smaller version of this representation is also given as insets in figures 2 and 4. For the
159 supplementary data, the tarsus was glued to the platform with dental cement (s.a.), and
160 successively moved between positions 1-9.

161 *Optical recording and digital analysis of leg movements*

162 Optical recordings of the steps were performed and analyzed as in Gruhn et al. (2009a). In
163 brief, walking sequences were recorded with a high-speed video camera (Marlin F-033C;
164 Allied Visions Technologies, Stadroda, Germany) that was externally triggered at 100 fps.
165 Insect head, thorax, and legs were marked with fluorescent pigments (Dr. Kremer Farbmühle,
166 Aichstetten, Germany) mixed with dental cement. During the recording of walking sequences,
167 the animal was illuminated with blue light-emitting diode arrays (12 V AC/DC; Conrad
168 Electronic, Berlin). The video files were analyzed using motion-tracking software
169 (WINanalyze 1.9; Mikromak Service, Berlin). Position values are always given in millimeters
170 in the form xx.x; yy.y (s.d.x; s.d.y). A virtual 0 line was drawn across the animal at the level
171 of the coxa of the anterior leg (figure 1). Positive and negative x-values indicate points
172 anterior and posterior to this coxa, respectively; y-values are given with respect to the axis
173 perpendicular to the length of the animal. Larger y-values denote more distal, smaller values
174 more central points. Figure 1 shows a schematic drawing of the stick insect with the tracked
175 reference points for the analysis of leg kinematics marked as yellow dots and the standing
176 positions of the anterior leg. All steps were transposed to reflect walking as a left leg
177 regardless of which leg was being recorded.

178 *Data analysis and figure preparation*

179 Leg positions were measured with their x and y coordinates in mm. Care was taken to choose
180 intact animals of the same size (average animal length: 77.2 mm, s.d. 2.8). The number of
181 animals used for a given condition (N) and the number of steps evaluated (n) are given in the
182 figures. The sample size for the kinematic analysis of continuous walks was N = 8, for the
183 standing front leg or standing middle leg it was N = 6, respectively.

184 For statistical analyses, Mann-Whitney U test, Hotellings T² test and Pearson's correlation test
185 were used (Matlab, Statistics toolbox; The MathWorks, Natick, MA). Statistical significance
186 was assumed at values of P < 0.05 (*), P < 0,01 (**), and P < 0,001 (***)).

187 **Results**

188 *Targeting accuracy of the hind leg towards the middle leg*

189 First, we analyzed whether the hind legs of *Carausius* target the position of the ipsilateral
190 middle leg during first steps. We tethered the animals above a slippery surface and placed one
191 middle leg onto one of seven pre-defined standing positions. Each position was used ten times
192 in a randomized succession. The touchdown position of the first step by the hind leg was
193 recorded, after initiating walking of the animal through a brush stroke to the abdomen.
194 Sequences in which the middle leg moved before the hind leg had finished its swing phase
195 were not evaluated.

196 The plot with the positions of the standing middle leg and the respective touchdown position
197 of the stepping ipsilateral hind leg (Fig. 2) shows that all seven investigated positions of the
198 middle leg were within reach of the hind leg (dotted semi circle shows calculated average
199 maximum range of fully stretched hind legs). The touchdown position of the hind leg was
200 often anterior to the position of the middle leg coxa (vertical dotted line). Only when the
201 middle leg was standing at positions one or two, did the hind leg rarely touch the ground
202 anteriorly to the middle leg coxa. Taking the position of the middle leg coxa (dotted vertical
203 line) as a reference, it becomes apparent that the touchdown positions of the hind leg were
204 more anterior for farther anterior standing positions of the middle leg. Similarly, for more
205 lateral standing positions of the middle leg, the touchdown positions of the hind leg were on
206 average also more laterally.

207 We quantified these qualitative observations, by testing if the target (middle leg) and the
208 touchdown (hind leg) positions, either parallel (Fig. 3A) or perpendicular to the body axis
209 (Fig. 3B) were significantly different from one another. Significant differences between the
210 three groups of data are one prerequisite for linear correlation. We then looked for linear
211 correlation between the data groups. For 180 pairs of positions each, we performed a pair wise
212 analysis of data from middle leg standing positions that only differed along one of the two
213 axes.

214 On average, the x-coordinate of the touchdown position of the hind leg increased with
215 increasing x-coordinate of the standing middle leg (Table 1A). Although the distribution of
216 the hind leg touchdown positions for the three middle leg positions along the body axis (two,
217 five, and eight) was relatively big, they were nevertheless all significantly different from one
218 another (Fig. 3A; p-values in Table 1B). We used these data pairs to identify a linear

219 correlation parallel to the body axis. With a coefficient of determination of $r^2_x = 0.28$, such a
220 correlation can indeed be assumed. To test for a possible correlation perpendicular to the body
221 axis, we used middle leg positions four, five, and six (Fig. 3B). Although the mean values of
222 these three data groups did not differ much (Table 1A) they were still significantly different
223 from each other (Table 1B) as a result of their small variability. The linear correlation along
224 this axis was smaller but still present ($r^2_y = 0.14$).

225 We also calculated the distances between the standing position of the middle leg and the
226 touchdown position of the hind leg parallel (Fig. 3C) and perpendicular to the body axis (Fig.
227 3D). These values were plotted against the standing position of the middle leg. We calculated
228 their mean values, tested for significant differences between the groups and for linear
229 correlation. This comparison helps to estimate the targeting accuracy of the hind leg. The
230 same or no significant difference in the distance between middle and hind leg for the differing
231 middle leg standing positions would suggest targeting by the hind leg. A systematic increase
232 in the distance between the two positions with a more anteriorly or distally standing middle
233 leg would instead indicate weak or no targeting by the hind leg. On average, the distances
234 parallel to the body axis between middle leg standing position two and five and the resp. hind
235 leg touchdown positions did not increase significantly, while the distance at position eight
236 was significantly bigger than those at positions two and five (Table 1A, B). There was almost
237 no correlation between the standing positions of the middle leg and the distances to the touch
238 down position of the hind leg along the body axis ($r^2_x = 0.07$), again supporting targeting of
239 the hind leg towards the standing middle leg parallel to the body axis. On the other hand, the
240 average distances between hind leg touchdown and the standing middle leg at the three
241 positions perpendicular to the body axis increased significantly from one standing position to
242 the next by about five millimeters each (Fig. 3D; Table 1A, B). Because of the small
243 variability within the groups, and the big systematic increase of the mean values, the linear
244 correlation between these standing positions and the distances was strong ($r^2_y = 0.82$),
245 suggesting no or only minor targeting of the hind leg towards the standing position of the
246 middle leg perpendicular to the body axis. We repeated the series of experiments with the
247 middle legs of the same animals glued to the standing platform. This did not change the
248 distribution of touch down positions, and the targeting accuracy in both directions was largely
249 unchanged (data not shown, see supplementary figure 1).

250

251 *Targeting accuracy of the middle leg towards the standing front leg*

252 To our knowledge, targeting of the middle towards the front leg has not been studied
253 quantitatively. To test the targeting accuracy of the middle leg, we therefore performed the
254 same experiments as above with the standing front and stepping middle leg. A plot of the
255 investigated seven different standing positions of the front, and the respective touchdown
256 positions of the stepping ipsilateral middle leg (Fig. 4) shows that the touchdown of the
257 middle leg usually occurred close to its maximum reach (dotted semi circle). The middle leg
258 only rarely had its touchdown anterior of the front leg coxa (vertical dotted line). The front leg
259 positions six, eight, and nine were even out of reach for the middle leg. To identify a potential
260 systematic dependence between the touchdown position of the middle and the standing
261 position of the front leg, we plotted these two positions against each other and tested for linear
262 correlation parallel (Fig. 5A) and perpendicular to the body axis (Fig. 5B). Again, we used
263 180 pairs of data from front leg positions that only differed along one of the two axes.

264 To identify a potential correlation parallel to the body axis we used positions two, five, and
265 eight of the standing front leg (Fig. 5A). Although the scatter of touchdown positions along
266 the body axis was relatively large, the average x-coordinate of the touchdown position
267 increased significantly parallel to the body axis with increasing x-coordinate of the standing
268 front leg (Table 1A, B), but they were only weakly correlated ($r^2_x = 0.13$). To test for a
269 correlation perpendicular to the body axis, we used positions four, five, and six (Fig. 5B).
270 Here the mean values of the three data groups did not change significantly (Table 1A and 1B).
271 Consequently, no linear correlation along this axis was detected ($r^2_y = 0.08$). For easier
272 comparison of all coefficients of determination the r^2 -values of all evaluations are also listed
273 in Table 2, lines a and b.

274 We then calculated the distances between the position of the standing front and the
275 touchdown position of the middle leg parallel (Fig. 5C) and perpendicular to the body axis
276 (Fig. 5D). Although the touchdown positions of the middle leg were on average more anterior
277 when the front leg was standing on a more anterior position (Fig. 5A), the distance between
278 middle leg and front leg tarsus parallel to the body axis also increased significantly from
279 positions two through eight (Table 1A, B). With 7.3 mm and 13.0 mm the difference between
280 the distances at positions five and eight, respectively is particularly big. This might be caused
281 by the fact that the middle leg was still anatomically able to reach position five, while this was
282 not possible for position eight. We found a linear correlation between the position of the
283 standing front leg and the distances to the middle leg touchdown parallel to the body axis (r^2_x
284 = 0.35), which is again indicative of only weak targeting of the middle leg towards the

285 standing position of the front leg in this direction. The average distances increased from one
286 standing position to the next significantly by about five millimeters (Fig. 5D, Table 1A and
287 1B), resulting in a strong linear correlation between the standing positions of the front leg and
288 the distances to the middle leg touchdown position perpendicular to the body axis ($r^2_y = 0.74$).
289 This is again means no or only weak targeting of the middle leg towards the front leg in this
290 axis. We again repeated the series of experiments with the front legs of three of the animals
291 glued to the platform. This had only minor effects on the distribution of touch down positions
292 or the targeting accuracy in both directions (data not shown, see supplementary figure 2).

293 *Targeting accuracy in the tethered walking animal*

294 The experimental situation with a standing anterior leg corresponds to a situation where the
295 animal starts locomotion after standing still, but this is a special case that may have limited
296 relevance for the freely locomoting animal. Therefore, we also analyzed the targeting
297 precision of the hind and middle legs onto their anterior neighbor during tethered stationary
298 walking. The animal was again tethered above the slippery surface as before, but this time the
299 middle or front legs were not placed on one of the defined positions but moved freely. This
300 approach differs from that of Dean and Wendler (1983), who looked at targeting in stick
301 insects walking on a treadwheel, and was chosen to remove mechanical influences between
302 the legs and investigate especially the neuronal basis of targeting. The position of the
303 posterior leg used for the analysis, was again its touchdown position. However, since it is not
304 known at what time during the step cycle of the posterior leg its touchdown position is
305 determined, we tested if we could see a correlation of this touchdown position with the
306 position of the anterior leg at three different time points during its step cycle: 1. The position
307 of the anterior leg at the time when the posterior leg finished its swing phase and touches the
308 ground (comparable to the control with a standing anterior leg, only without pre-defined
309 positions). 2. The position of the anterior leg at the time when the posterior leg was lifted off
310 the ground and began its swing phase. 3. The next posterior extreme position that the anterior
311 leg takes up after liftoff in the posterior leg (this point can be identical to the situation 1, but
312 need not).

313 We calculated the coefficients of determination for each of these three combinations, and, to
314 ensure that the results were not caused by noise, we also calculated the coefficients of
315 determination between the touchdown positions of the posterior leg and a set of random
316 variables. The random variables had the same distribution as the real data (front leg: X
317 between -10.2 and 28.7 mm; Y between 0.5 and 31.2 mm; middle leg: X between -11.2 and

318 15.4 mm; Y between 2.0 and 28.3 mm). Table 2 (lines c-f) lists the numbers of data pairs and
319 the corresponding r^2 -values of the linear regressions. All linear regressions of the real data are
320 significantly different from zero ($P < 0.001$), while the linear regressions with the random
321 variables are not ($P > 0.05$). In addition, all coefficients of determination of the real data are
322 bigger than the values for the used random variables. The strongest linear correlation in both
323 directions for middle and hind leg as posterior legs was found between the touchdown
324 position of the posterior leg and the position of the anterior leg at the time of liftoff of the
325 posterior leg (scenario 2). For all further evaluations of targeting during walking, we
326 therefore used this position. We determined all liftoff and touchdown events of the posterior
327 leg, and identified the position of the anterior leg for all liftoff events of the posterior leg. If
328 the anterior leg was performing a swing phase at that time point, the corresponding
329 touchdown position of the posterior leg was removed from the data set.

330 All data pairs from the hind and middle leg are plotted in figure 6. Most of the time, the
331 touchdown positions of the hind leg were posterior of the middle leg coxa (dotted vertical
332 line), but occasional stepping to more anteriorly located positions occurred. The mean values
333 and the overall scatter of the touchdown positions of the hind leg perpendicular to the body
334 axis were similar to those of the hind leg touchdown positions in all experiments with
335 predefined middle leg standing positions (Fig.6; $Y = 20.0$ mm, s.d. 4.3; see for comparison
336 Fig. 2), but were slightly shifted caudally ($X = -13.1$ mm, s.d. 6.4; see for comparison Fig. 2).
337 Since the reference positions of the middle leg were taken at the time of liftoff in the hind leg,
338 the middle leg had not completed its stance phase and thus had not reached its liftoff position,
339 yet. Therefore the middle leg positions are comparably far rostral, and distances to the hind
340 leg touchdown positions were larger than for the standing middle leg. Under tethered walking
341 conditions, the touchdown positions of the hind legs were on average 16.1 mm (s.d. = 5.7)
342 posterior (X-dist) of the middle leg positions, while the lateral distribution of the two data
343 groups was similar (average Y-dist = -4.4 mm, s.d. = 3.3). Most of the middle leg positions
344 were within the reach of the hind leg. We tested for linear correlation of the hind and middle
345 leg positions and distances parallel and perpendicular to the body axis (Fig. 7). The
346 coefficient of determination for positions parallel to the body axis (Fig. 7A) was similar to the
347 results with standing middle leg and targeting hind leg ($r^2_x = 0.30$, cf. Fig. 3A). A much
348 stronger linear correlation was now found for the positions perpendicular to the body axis r^2_y
349 = 0.51 (Fig. 7B). Distances between the two positions either parallel (Fig. 7C; $r^2_x = 0.09$) or
350 perpendicular (Fig. 7D; $r^2_y = 0.15$) to the body axis showed only very weak linear
351 correlations. Altogether it appears that the state of activity of the middle leg positively

352 influences the targeting accuracy of the hind leg perpendicular to the body axis when the
353 animal locomotes steadily, while no additional improvement was found for the aiming
354 precision along the body axis.

355 To find out if there were also state-dependent changes in the aiming precision of the middle
356 onto the front leg, we repeated this analysis for these two legs under tethered walking
357 conditions. We again determined all liftoff and touchdown events of the middle leg and their
358 positions and also identified the position of the front leg for all liftoff events in the middle leg.
359 If the front leg was performing a swing phase at the time, the corresponding touchdown
360 position of the middle leg was again not included in the analysis. The majority of touchdown
361 positions of the middle leg was close to the legs maximum reach, with no touchdown
362 positions anterior of the coxa of the front leg (figure 8, vertical dotted line). The overall
363 distribution (mean values: $X = -9.3$ mm, s.d. 4.4; $Y = 18.2$ mm, s.d. 2.5) was similar to that of
364 the touchdown positions with standing front leg (cf. Fig. 4). Interestingly, the spread among
365 touchdown positions of the middle leg was much smaller than that among the touchdown
366 positions of the hind leg (cf. Fig. 6). Similar to middle and hind leg, the reference positions of
367 the front leg were taken at the time of the liftoff of the middle leg. As a result, the front leg
368 positions are all relatively far anterior and in most cases even out of reach for the middle leg
369 (dotted semi circle). There was only a very small overlap in the spread of the middle and front
370 leg positions parallel to the body axis. On average the touchdown positions of the middle leg
371 were 22.7 mm, s.d. 6.3 (X-dist) posterior of the front leg positions while the lateral
372 distribution of the two data groups was similar (Y-dist = -1.4 mm, s.d. 4.3). Despite the large
373 distance, with $r^2_x = 0.27$, one can assume linear correlation between the positions of middle
374 and front leg along the body axis (Fig. 9A). This value was in the same range as that for the
375 walking middle and targeting hind leg (cf. Fig 7A, table 2d) and about twice as high as the
376 coefficient of determination of the standing front and targeting middle leg (cf. Fig 5A, table
377 2a). Perpendicular to the body axis, there was only a slight linear correlation between the
378 positions of the middle and front leg (Fig. 9B; $r^2_y = 0.18$), but this was still more than twice as
379 large than that between standing front and targeting middle leg (cf. Fig 5B, table 2a). The
380 distances between the touchdown position of the middle leg and the position of the front leg at
381 middle leg liftoff also showed a strong linear correlation parallel (Fig. 9C; $r^2_x = 0.639$) as well
382 as perpendicular to the body axis (Fig. 9D; $r^2_y = 0.717$). Overall these results indicate
383 targeting of the middle leg to the position of the moving front leg along the body axis and at
384 least a slight targeting perpendicular to the body axis. Similar to the findings for the hind to

385 middle leg, the targeting accuracy of the middle to the front leg appears to improve in a state-
386 dependent manner, once the animal locomotes steadily.

387 One can summarize that, markedly extending earlier assumptions, the middle leg is less
388 precise than the hind leg in finding its anterior neighbor in the standing and the walking
389 animal. In addition, we could demonstrate that movement of the respective anterior leg seems
390 to be of importance for targeting accuracy suggesting not only a segment-specific but also a
391 state-dependent effect.

392 **Discussion**

393 We have investigated the aiming accuracy of middle and hind legs of stick insects on a
394 slippery surface. With our analyses we could demonstrate that targeted leg movements
395 towards their rostral neighboring leg can occur under certain conditions, even without
396 mechanical coupling through the ground, but that this ability is not equally strong between the
397 hind and the middle legs, and not equally strong between standing and walking animals.

398

399 *Targeted leg movements without mechanical coupling*

400 In earlier investigations it had been shown that stick insects can perform targeted movements
401 with their hind legs and that the touchdown position of the hind leg depends on the position of
402 the middle leg that was standing on a separate platform when the rest of the legs are walking
403 on the same treadmill (Cruse, 1979). This constitutes a setup where the first step of the hind
404 leg is virtually mechanically uncoupled from standing middle leg. A different approach to
405 study the neuronal control of stepping uses animals tethered above a slippery surface and
406 could show that stick insects are able to perform normal walking movements under this
407 condition (Graham and Cruse, 1981; Cruse and Epstein, 1982; Epstein and Graham, 1983;
408 Graham and Epstein, 1985; Gruhn et al., 2006; Gruhn et al., 2009a). However, information
409 about targeting movements of the legs on the slippery surface has been relatively scarce and
410 inconclusive. While Graham and Cruse (1981) as well as Cruse et al. (1995) reported
411 targeting of the legs based on the distribution of touchdown and liftoff positions of
412 ipsilaterally neighboring legs, Epstein and Graham (1983) claimed that they could not observe
413 targeting behavior during their experiments with walking stick insects. By specifically
414 analyzing the linear correlation of corresponding pairs of leg positions of stick insects tethered
415 above a slippery surface setup, we could now confirm that stick insects actually can perform
416 targeted leg movements towards their anteriorly neighboring leg even in the absence of
417 mechanical coupling through the ground. However, targeting precision is different between
418 thoracic segments. This targeting in the absence of mechanical coupling provides evidence for
419 a neuronal mechanism that must be involved in spatial coordination of leg movements.

420

421 *Targeting accuracy of hind and middle legs is different*

422 By comparing the targeting accuracy of the hind towards the middle legs with the targeting
423 accuracy of the middle towards the front legs we could show that the precision has a segment-
424 specific quality, and that targeting of the hind leg was distinctly more accurate than targeting
425 of the middle leg. In fact, when the front leg was standing and the middle leg performed its

426 first step of the walking sequence, this step forwards can hardly be called targeted at all (see
427 results above). This is a novel result because none of the previous studies investigating
428 targeting behavior of stick insects (e.g. Cruse, 1979; Cruse et al., 1984; Dean, 1984; Dean and
429 Wendler, 1983) measured the accuracy of the middle leg foot placement towards its ipsilateral
430 front leg to compare it with the targeting accuracy of the hind leg, although middle leg
431 targeting was reported by Cruse et al. (1995) as unpublished observations. In earlier studies, it
432 was assumed from comparing distances between average touchdown and liftoff positions of
433 neighboring legs (Cruse, 1976) that the hind legs showed better targeting than the middle legs
434 (Cruse, 1979). With our results we could now confirm this assumption. It is, however,
435 interesting that targeting perpendicular to the body axis in both legs was virtually non-existent
436 in our study, unlike in earlier studies. However, in these previous studies, the targeting hind
437 leg was either standing (Cruse, 1979; Cruse et al., 1984) or moving (Dean and Wendler, 1983;
438 Dean, 1984) along a treadwheel. It cannot be excluded that under these conditions the
439 treadwheel may have a predefining influence on the leg movement perpendicular to the body
440 axis. In addition, the position analyses were performed between the touchdown position of the
441 hind leg and the position of the middle leg at the same time which, as will be discussed below,
442 may not be the best choice for the moving animal.

443 It remains unclear why the targeting of the hind leg is more accurate. The induction of the
444 first step by a light touch to the abdomen was the same between activating either leg and thus
445 seems unlikely to be the reason for the difference. One explanation for the distinctly better
446 targeting accuracy of the hind legs compared the middle legs could be based on simple
447 anatomical constraints for the middle legs. The middle leg is the shortest leg of the stick insect
448 (Cruse, 1976) and is anatomically not capable of reaching all posterior extreme positions of
449 the front leg, while the distinctly longer hind leg (Cruse, 1976) is anatomically capable of
450 reaching almost every posterior position of the middle leg. This could also lead to better
451 targeting accuracy of the hind leg by simply bumping into the middle leg. Such an effect,
452 however, may only be relevant at the beginning of a movement when the body is not
453 simultaneously displaced forwards by the movement of several legs at the same time.

454 The reason for the better targeting performance by the hind legs may be that the center of
455 mass of the stick insect is located close to and posterior of the coxae of the hind legs (Cruse,
456 1976). It might therefore be of greater importance for the stability of the animal to reliably
457 find foothold with the hind than with the middle legs. As a consequence, processing of
458 sensory information on the target leg's location in the resting animal may be different
459 between meso- and the metathoracic segment. So far, no direct evidence exists to support this

460 hypothesis for the case of targeting. However, Hellekes and colleagues (2012) have shown
461 that there is segment specificity in the processing of sensory information from the femoral
462 chordotonal organ (fCO), which signals the femur-tibia joint angle, and which could also be
463 integrated with other known sensory signals to yield distance information to a neighboring
464 leg. Further implications of this differential processing will be discussed below.

465

466 *Targeting accuracy changes between standing and moving target leg*

467 Interestingly, targeting performance improved when the animal was moving as compared to
468 when the animal was stationary. We found this to be true for the middle leg targeting the front
469 leg parallel to the body axis, as well as for hind and middle legs targeting perpendicular to the
470 body axis. This suggests that targeting precision is in fact dependent on the state of the
471 animal, i.e. movement of the legs.

472 It is currently unknown, at what time or at what position of the target leg the targeting
473 information is read out in order to produce aimed movements by the targeting leg. For exact
474 targeting, the animal would have to know the position of the target leg at the targeting legs
475 touchdown, which, during walking, is not trivial, because the target position has to be read out
476 and extrapolated while the target leg is still moving towards this position. However, the time
477 of readout can be assumed to be within a time frame that allows the nervous system to process
478 the information and for the targeting leg to actually produce a targeted movement that is not
479 made obsolete by the forward movement of the animal.

480 One can get a rough estimate for the minimal time span necessary for this information transfer
481 by calculating conduction times. First, the position information from the sense organs of the
482 targeted leg has to be transmitted to the local thoracic ganglion. Spikes take 12ms to travel
483 from the stick insect tarsus to the ganglion, and from data of stick insect and the locust it can
484 be assumed that it takes about 2 ms for the first spikes to travel from coxal sense organs to
485 interneurons within its own hemiganglion (Fisch, 2007, Höltje and Hustert, 2003). The
486 information then has to travel to the neighboring segment. Hardly any direct connections from
487 sensory neurons into neighboring segments have been demonstrated, yet (Hustert 1978), but
488 with connective lengths averaging about 17 mm between pro- and mesothorax and 10 mm
489 between meso- and methathorax (Cruse, 1976), and with conductance velocities within the
490 connective of about 2-2.8 mm/s (Brunner et al., 1990) one can assume at least another 4-9 ms
491 until the first spikes reach the neighboring ganglion. Depending on how far distal in the leg
492 the innervated muscle is, it takes additional 1-5 ms for the motoneuron spikes to travel to the
493 neuromuscular end plate (Höltje and Hustert, 2003). Finally, the muscle needs a minimum of

494 20-40 ms to build up the muscle tension needed for the movement of the leg (Guschlbauer et
495 al. 2007; Hooper et al., 2009; Blümel et al. 2012). It is unclear how many synapses and
496 interneurons have to be crossed before the information reaches the motoneurons of the
497 targeting leg, but both intersegmental as well as local interneurons have been described that
498 could take part in the targeting process (Brunn and Dean, 1994). Altogether, in the most
499 conservative estimate, and without considering synaptic transmission, it would take at least 27
500 ms to process and target a measured leg position, most likely more. This estimate seems to
501 match the finding by Schütz and Dürr (2011), who could show that re-targeting of an ongoing
502 swing movement by the front leg occurs with a delay of about 40 ms after antennal contact
503 with an object. Therefore the position information has to be collected and read out during the
504 swing phase of the targeting leg.

505

506 Taking the above considerations into account, the position of the target leg at the time when
507 the targeting leg finishes its swing phase and touches the ground, or even the posterior
508 extreme position of the target leg, do not leave enough time for processing. They could only
509 have a good correlation with the touchdown position if one assumes a perfect prediction of
510 this position by the animal. Indeed, the r^2 -values were very small (see Table 2, c and e). Since
511 we did not know the exact point in time that is used by the animal, we chose the position of
512 the target leg at the time when the targeting leg lifted off the ground and began its swing
513 phase. This is well above the range reported by Schütz and Dürr (2011), and hence leaves
514 enough time (on average 141 ms s.d. 57; A. Wosnitza, unpublished) for the neuromuscular
515 system to transmit and process the information. However, we cannot exclude that the time
516 point at which the placement of the foot is actually decided lies further back similar to Schütz
517 and Dürr (2011), or even further in the future, as has been reported for vertebrates that use
518 visual and mechanosensory information to guide leg trajectories during walking (cat: McVea
519 and Pearson, 2007; McVea et al., 2009; Wilkinson and Sherk, 2005; human: Mohagheghi et
520 al., 2004; Patla and Vickers, 2003). In the case of humans wanting to place their foot at a
521 specific target position, it has been reported that they fixate on this position on average two
522 steps ahead, and at least 800–1,000 ms before the limb is placed on the target area (Patla and
523 Vickers, 2003).

524

525 The questions that arise now are why targeting of the hind and the middle leg generally
526 improved during walking, why this is not the case for the hind leg in parallel to the body axis,
527 and what the underlying neuronal mechanisms could be. It is known that sensory information

528 signaling leg angles is integrated by intersegmental and local interneurons and could therefore
529 also be used to provide the targeting information for the hind leg (Brunn and Dean, 1994).
530 Primarily responsible for the targeting accuracy perpendicular to the body axis is the fCO
531 which measures the angle between femur and tibia (Bässler, 1977; Cruse et al., 1984).
532 Processing of fCO activity changes between standing and walking animals (Bässler, 1974;
533 Bässler, 1976; Bässler, 1988; Stein et al., 2006; Hellekes et al., 2012). In addition, it is also
534 known that fCO signals from an anterior leg in the actively stepping animal affect the next
535 posterior leg (Ludwar et al., 2005; Stein et al., 2006). So far, however, no interneurons have
536 been described that solely receive position information from the fCO. Most of the
537 interneurons receive a combination of movement velocity and acceleration information from
538 the femoral chordotonal organ (Büschges, 1989; Brunn and Dean, 1994). Altogether, these
539 findings make it very plausible that fCO signals from the anterior leg may help targeting the
540 posterior leg to its anterior neighbor perpendicular to the body axis, but that they are only
541 processed to do so in a state-dependent manner, that is, if the animal is actually walking.
542 Targeting of the hind leg in parallel to the body axis, seems to be primarily controlled by
543 coxal hair rows and hair fields which measure the position of the coxa and pro- and retraction
544 movements of the leg (Bässler, 1977; Dean and Wandler, 1983; Cruse et al., 1984). So far, no
545 data exist on state-dependent or thoracic-segment-dependent processing of this type of
546 sensory information, however, it is again known from the fCO, that its signals are processed
547 differently in the different thoracic segments (Hellekes et al., 2012). Therefore, in addition to
548 the state-dependence of sensory processing, a different segment-specific processing in the
549 metathorax may be responsible for a lack of improvement in hind to middle leg targeting
550 when the animal switches from standing to walking. In other words, since the hind legs could
551 be more important for the animal's stability, their targeting is already almost at its best in the
552 standing animal.
553 Interestingly, this state-dependent influence of sensory input on the spatial coordination
554 between the legs also matches the description of movement-induced temporal coordination in
555 the stepping stick insect by Borgmann et al. (2009), and its improvement with acceleration
556 (Gruhn et al., 2009b). It also bears similarities with the changes in the coordinating influences
557 between straight and curve walking described by Dürr (2005). The fact that these influences
558 may not be equally strong between different thoracic segments also matches earlier
559 descriptions of stick insect walking, in which the front legs have been described to act as
560 "feelers" (Cruse, 1976), and is also in accordance with Dürr (2005) and Grabowska et al.
561 (2012), who could show that temporal coupling between middle and hind legs during walking

562 is much stronger than that of either leg to the front legs. The functional significance of this
563 could be that the front legs may, in addition to their function in locomotion, also be used for
564 exploratory purposes, while the middle and hind legs serve as entity mostly for locomotion. In
565 this context, it will be interesting to see if targeting accuracy changes with ground properties
566 such as solid planar ground or even irregular profiles such as stair-like structures or even
567 grids, which more resemble the natural habitat of a stick insect.

568

569 In conclusion, our data, together with findings of previous studies, support a notion in which
570 stick insect middle and hind legs can aim at their anterior neighbor either when performing a
571 first step or during steady walking. However, the correlations are not always very strong,
572 especially for the first step in the standing animal. This suggests that processing of the
573 relevant sensory information is differently achieved in middle and hind legs as the hind leg is
574 more accurate than the middle leg in finding its anterior neighbor under both conditions. The
575 fact that movement of the animal strongly improves targeting accuracy suggests that
576 processing of information on leg position to produce spatial coordination in the stick insect is
577 not only segment-specific but also state-dependent and supports previous findings of state-
578 dependent and segment-specific processing of sensory information for temporal coordination.

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716 **Figure Legends:**

717

718 **Figure 1:** Schematic drawing of the stationary stick insect with coordinate system of the
719 labeled standing positions of the middle leg. The yellow dots mark the tracked positions on
720 the animal. The red arrow marks the movement direction of the posterior leg, in this case the
721 hind leg. Experiments with the front leg as target leg were done accordingly (see also inset in
722 fig. 4).

723 **Figure 2:** Standing positions of the middle leg (red dots) and touchdown positions of the
724 stepping hind leg (black crosses) on the slippery surface. Each sub-plot shows data from one
725 of the seven standing positions of the middle leg. The vertical dotted line marks the position
726 of the middle leg coxa which is located at zero on the x-axis. The dotted semi-circle depicts
727 the calculated average maximum range of fully stretched hind legs. The inset gives a
728 schematic overview of the standing positions of the middle leg.

729 **Figure 3:** Scatter plot of the middle leg standing positions against the touchdown positions of
730 (**A** and **B**) and distances from (**C** and **D**) the ipsilateral hind leg. Separated into the
731 components parallel (**A** and **C**) and perpendicular (**B** and **D**) to the body axis, and including
732 linear correlation and test for significant differences between the groups of data using the
733 Man-Whitney-U-test. Plotted are pairs of data that belong to middle leg standing positions
734 which only differ in the considered coordinate. In figure **A** and **C**, these are positions two,
735 five, and eight. In **B** and **D**, these are positions four, five and six.

736 **Figure 4:** Standing positions of the front leg (red dots) and touchdown positions of the middle
737 leg (black crosses) on the slippery surface. Each sub-plot shows data from one of the seven
738 positions of the standing front leg. The vertical dotted line marks the level of the front leg
739 coxa which is located at zero on the x-axis. The dotted semi-circle depicts the calculated
740 average maximum reach of fully stretched middle legs. The inset gives a schematic overview
741 of the standing positions of the front leg.

742 **Figure 5:** Scatter plot of the front leg standing positions against the touchdown positions of
743 (**A** and **B**) and distances from (**C** and **D**) the ipsilateral middle leg. Separated into the
744 components parallel (**A** and **C**) and perpendicular (**B** and **D**) to the body axis. Each panel also
745 shows linear correlation and test for significant differences between the groups of data using
746 the Man-Whitney-U-test. Plotted are pairs of data that belong to front leg standing positions

747 which only differ in the considered axis. In figure **A** and **C**, these are positions two, five, and
748 eight. In **B** and **D**, these are positions four, five and six.

749 **Figure 6:** Scatter plot of middle and hind leg positions during walks on the slippery surface.
750 The red dots represent the positions of the middle leg at the time of the liftoff of the hind leg.
751 The black crosses show the subsequent touchdown position of the hind leg. The vertical
752 dotted line marks the zero on the x axis and also the position of the coxa of the middle leg.
753 The dotted semi-circle depicts calculated average maximum range of fully stretched hind legs
754 with its standard deviation (grey area).

755 **Figure 7:** Scatter plot with test for linear correlation of the positions of the middle leg at the
756 time of the liftoff of the hind leg against the subsequent touchdown position of the hind leg (**A**
757 und **B**) and against the distance between middle and hind leg (**C** und **D**), respectively. The
758 plots are separated into the components parallel (**A** und **C**) and perpendicular (**B** und **D**) to the
759 body axis.

760 **Figure 8:** Scatter plot of the positions of the front leg and middle leg during walks on the
761 slippery surface. The red dots represent the positions of the front leg at the time of the liftoff
762 of the middle leg. The black crosses show the subsequent touchdown position of the middle
763 leg. The vertical dotted line marks the zero on the x axis, and also the position of the coxa of
764 the front leg. The dotted semi-circle depicts calculated average maximum range of fully
765 stretched middle legs with its standard deviation (grey area).

766 **Figure 9:** Scatter plot with test for linear correlation of the positions of the front leg at the
767 time of the liftoff of the middle leg against the subsequent touchdown position of the middle
768 leg (**A** und **B**) and against the distance between front and middle leg (**C** und **D**), respectively.
769 Separated into the component parallel (**A** und **C**), and perpendicular (**B** und **D**) to the body
770 axis.

771 **Table 1: A.** Mean and s.d. of the positions and distances of the touchdown positions of hind
772 or middle leg with respect to the targeted position of the middle or front leg. For targeting
773 along the long axis of the animal (X), the values are given with respect to positions P2, P5 and
774 P8, for positions perpendicular to the long axis of the animal (Y), values are given with
775 respect to positions P4, P5 and P6. **B:** P-values from the Mann-Whitney-U-test for significant
776 differences between the data groups listed in **A**.

777 **Table 2:** Coefficients of determination of the linear regressions parallel (r^2_x) and
778 perpendicular (r^2_y) to the body axis and size of the evaluated data groups. The r^2 -values are
779 given for the touchdown positions (a) and the distance (b) of the posterior leg against the
780 standing position of the anterior leg. Additionally the r^2 -values are given for the touchdown
781 positions of the posterior leg against the position of the anterior leg at three different time
782 points (c-e), and against a set of random variables (f). Finally, the distances of the posterior
783 leg against the positions of the anterior leg at the time of the last liftoff of the posterior leg (g).
784 All linear regressions of the real data are significantly different from zero ($P < 0.001$). The
785 linear regressions with the random variables are not significantly different from zero ($P >$
786 0.05). LO means liftoff, TD means touchdown.

787

788 **Table 1A**

[mm]	HL - ML				ML - FL			
	Positions		Distances		Positions		Distances	
	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
X _{P2}	-8.0	± 5.3	0.9	± 5.3	-10.1	± 4.8	3.7	± 4.6
X _{P5}	-1.8	± 6.3	1.6	± 6.3	-7.0	± 5.0	7.3	± 5.0
X _{P8}	0.7	± 5.8	5.0	± 5.9	-5.5	± 5.8	13.0	± 5.8
Y _{P4}	23.8	± 2.3	-14.5	± 2.6	19.8	± 2.9	-8.6	± 2.7
Y _{P5}	24.9	± 2.2	-9.5	± 2.4	20.6	± 3.2	-2.8	± 2.9
Y _{P6}	26.0	± 1.9	-3.8	± 2.3	21.3	± 2.6	3.1	± 2.6

789

790 **Table 1B**

	HL - ML		ML - FL	
	Positions	Distances	Positions	Distances
p _{P2-P5}	< 0.0001	0.6612	0.0002	0.0001
p _{P5-P8}	0.0255	0.0024	0.0280	< 0.0001
p _{P2-P8}	< 0.0001	0.0002	< 0.0001	< 0.0001
p _{P4-P5}	0.0154	< 0.0001	0.1333	< 0.0001
p _{P5-P6}	0.0058	< 0.0001	0.1825	< 0.0001
p _{P4-P6}	< 0.0001	< 0.0001	0.0022	< 0.0001

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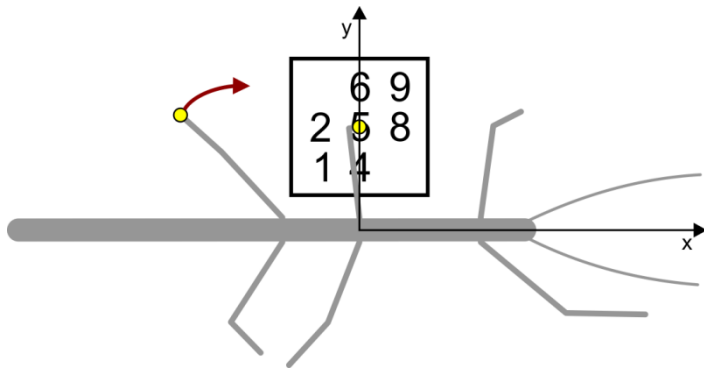
793 **Table 2**

		HL - ML			ML - FL		
		r^2_x	r^2_y	n	r^2_x	r^2_y	n
a	positions - standing anterior Leg	0.28	0.14	180	0.13	0.08	180
b	distances - standing anterior Leg	0.07	0.82	180	0.35	0.74	180
c	TD of the posterior Leg	0.15	0.30	216	0.19	0.15	494
d	last LO of the posterior Leg	0.30	0.51	356	0.27	0.18	501
e	next LO of the anterior Leg	0.07	0.22	216	0.20	0.06	494
f	random variables	-0.04	-0.07	356	-0.01	0.01	501
g	distances - last LO of post. Leg	0.09	0.15	356	0.64	0.72	501

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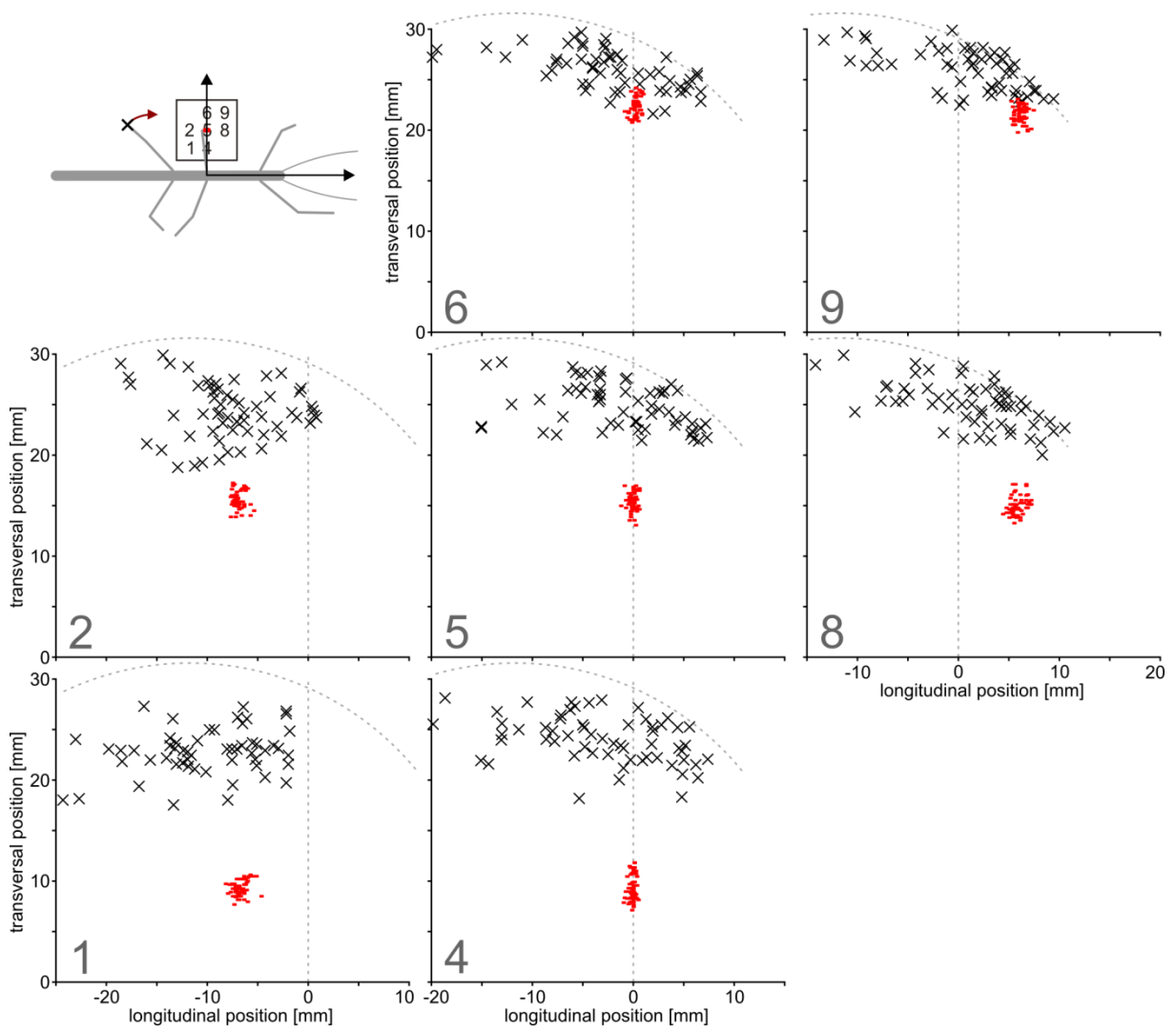
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796 **Figure 1:**



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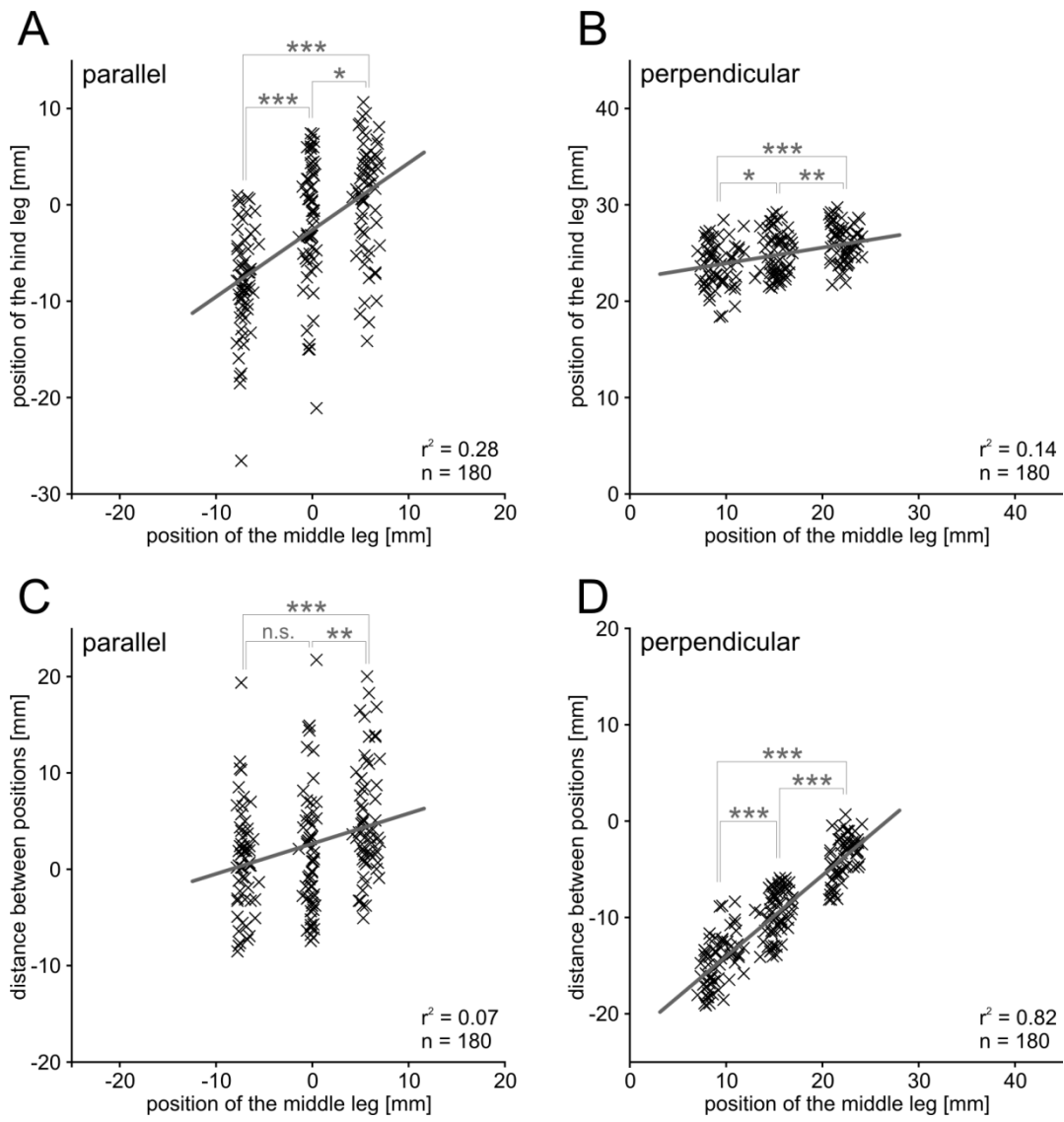
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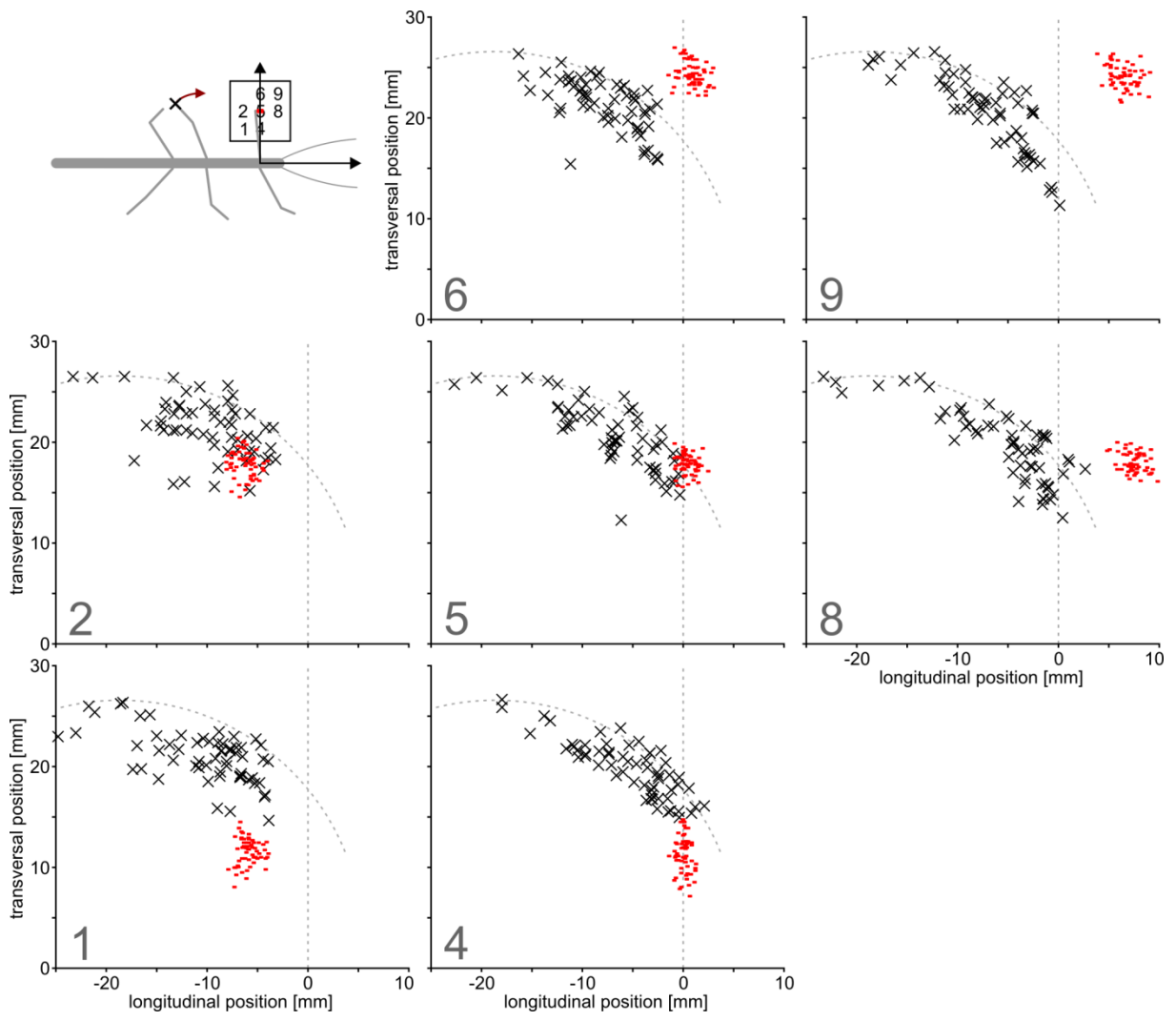
802 **Figure 3:**



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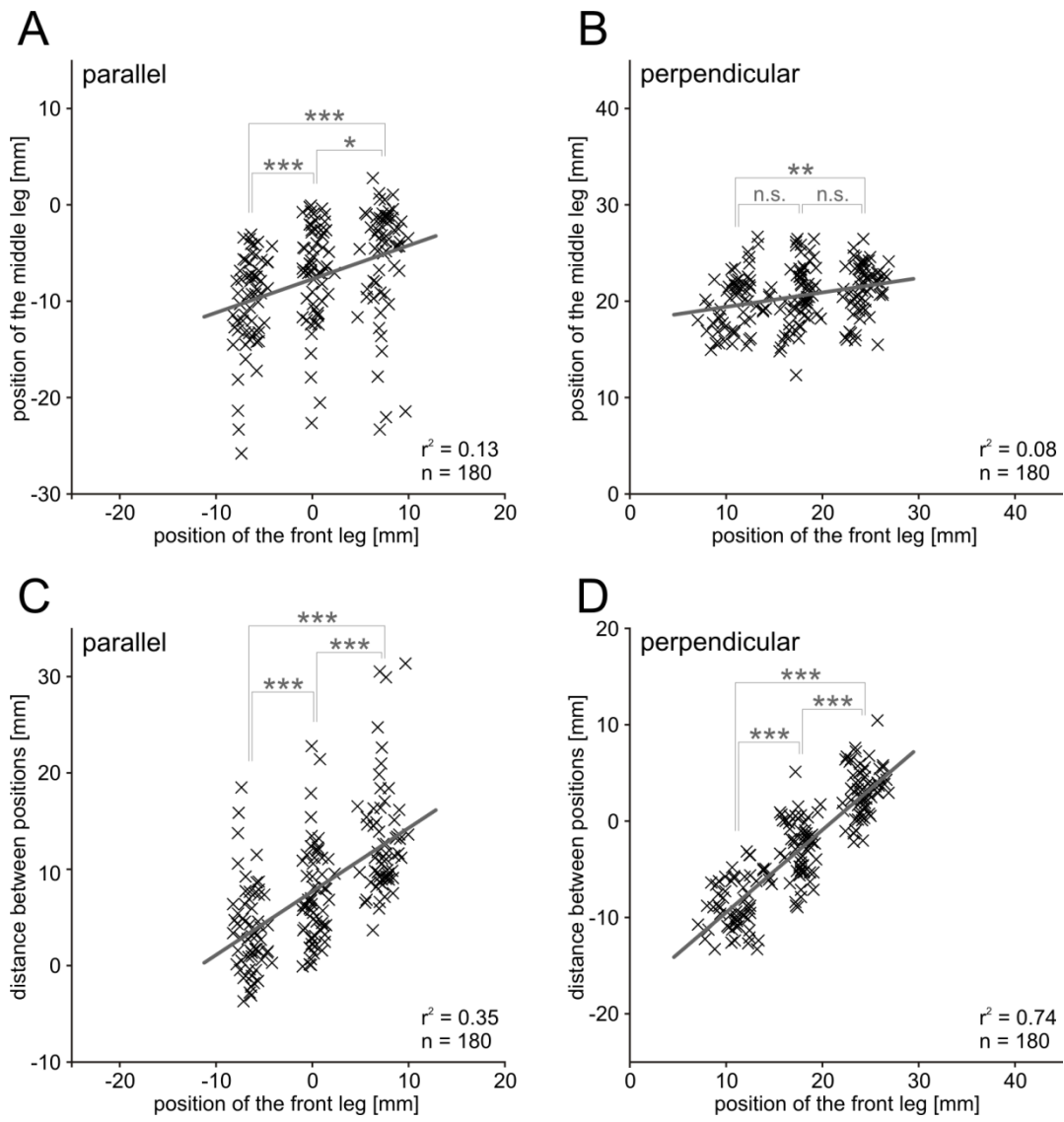
805 **Figure 4:**



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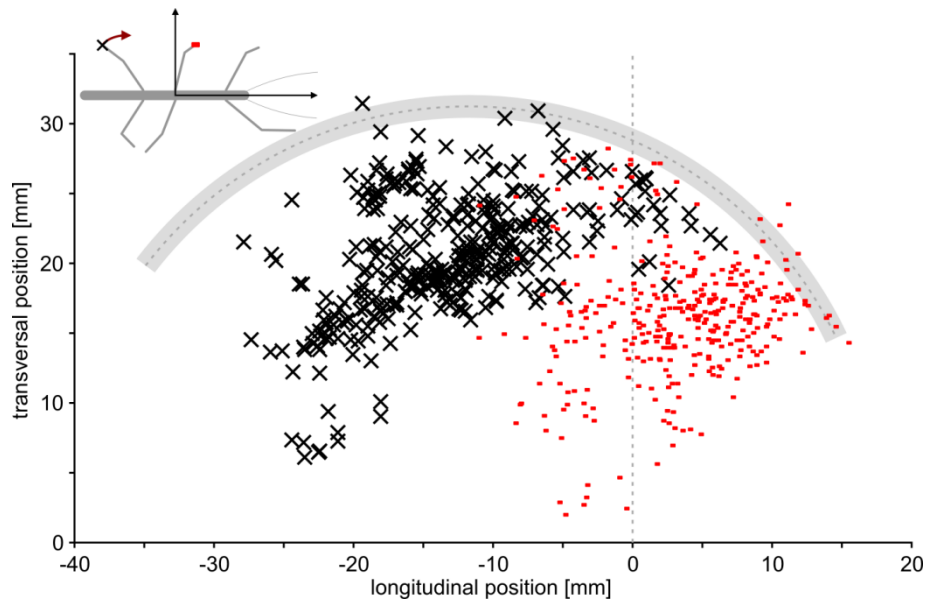
808 **Figure 5:**



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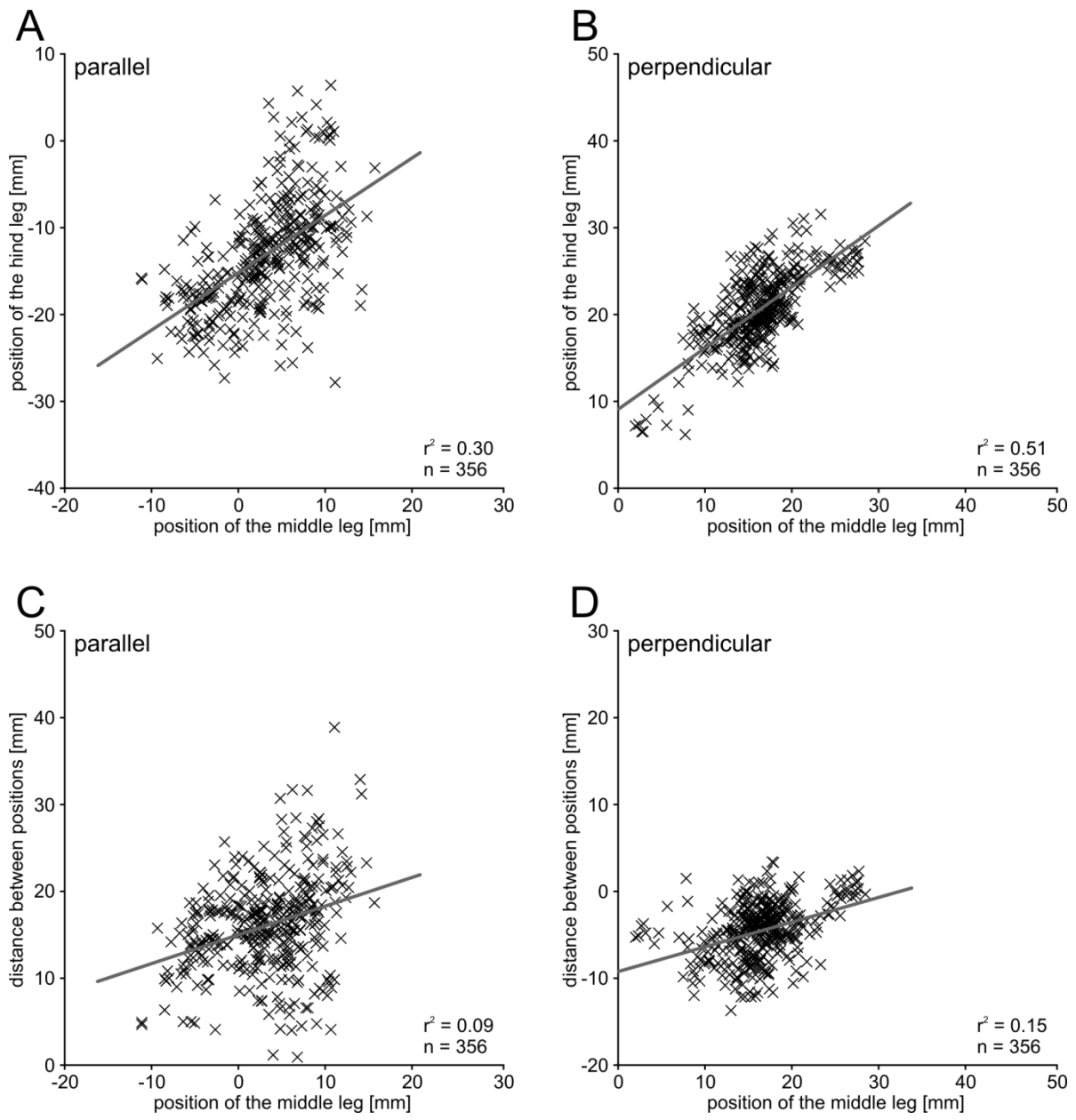
811 **Figure 6:**



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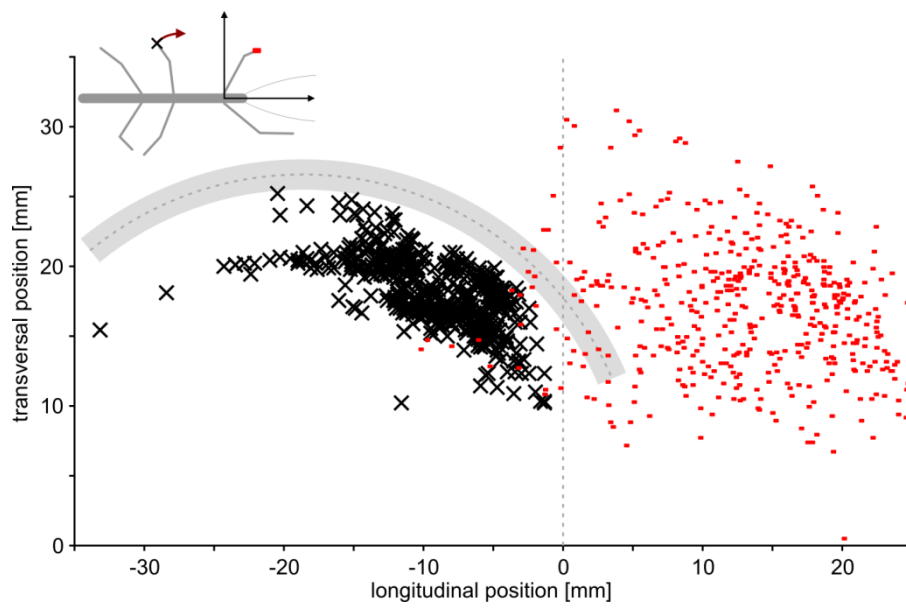
814 **Figure 7:**



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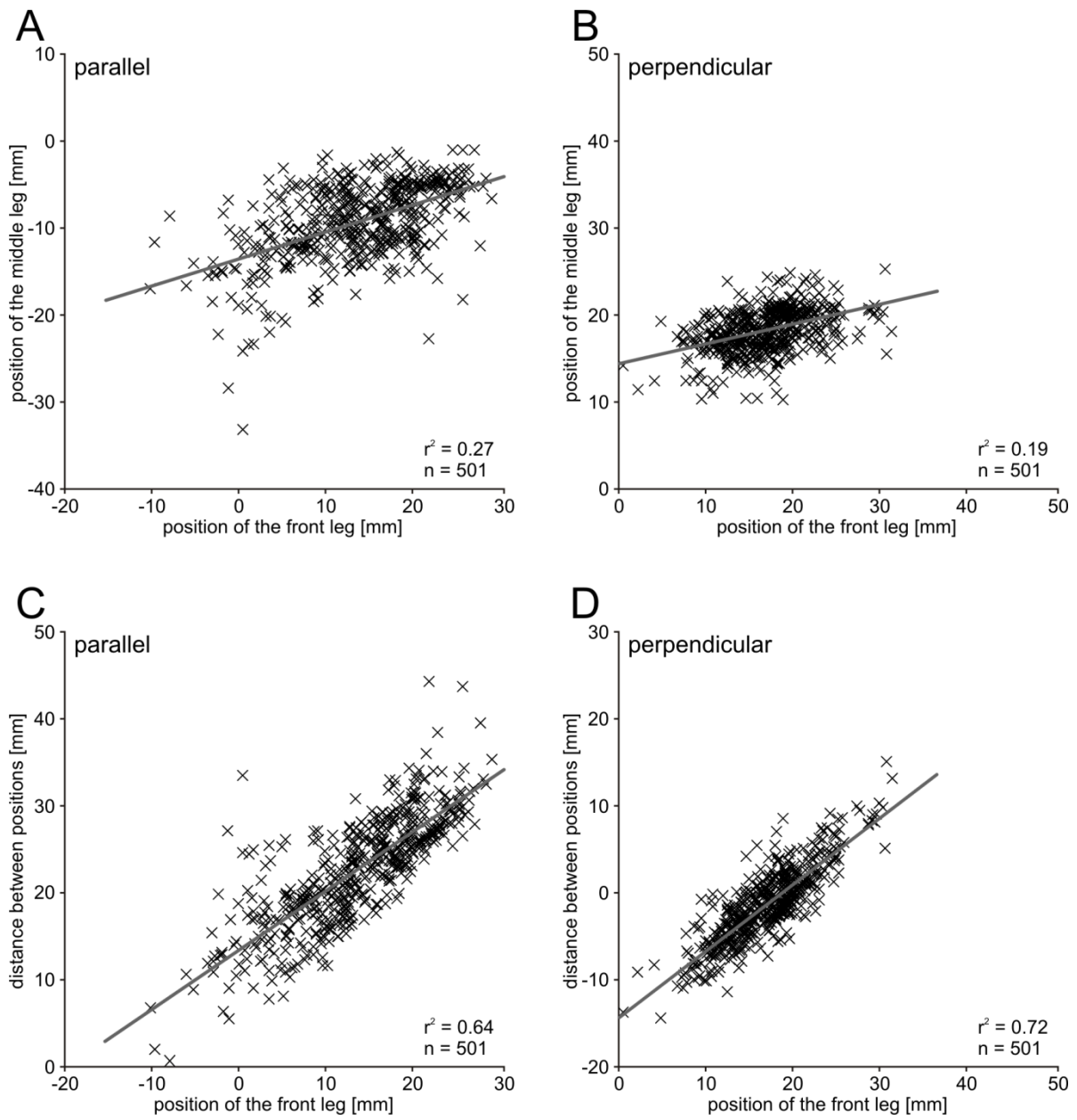
817 **Figure 8:**



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819

820 **Figure 9:**



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