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Inter-leg Coordination in the Control of

Walking Speed in *Drosophila*

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Abstract

Legged locomotion is the most common behavior of terrestrial animals and it is assumed to have become highly optimized during evolution. Quadrupeds, for instance, use distinct gaits which are optimal with regard to metabolic cost and have characteristic kinematic features and patterns of inter-leg coordination. In insects, the situation is not as clear. In general, insects are able to alter inter-leg coordination systematically with locomotion speed, producing a continuum of movement patterns. This notion, however, is based on the study of several insect species, which differ greatly in size and weight. Each of these species tends to walk at a rather narrow range of speeds.

We have addressed these issues and examined four strains of *Drosophila*, which are similar in size and weight, but tend to walk at different speed ranges. Our data suggest that *Drosophila* controls its walking speed almost exclusively via step frequency. At high walking speeds we invariably found tripod coordination patterns the quality of which increased with speed as indicated by a simple measure of tripod coordination strength (TCS). At low speeds we also observed tetrapod coordination and wave gait-like walking patterns. These findings suggest not only a systematic speed dependence of inter-leg movement patterns, but imply that inter-leg coordination is flexible. This was further supported by amputation experiments in which we examined walking behavior in animals after the removal of a hind leg. These animals show immediate adaptations in body posture, leg kinematics, and inter-leg coordination thereby maintaining their ability to walk.

Introduction

In terrestrial animals, legged locomotion is a behavior that is highly optimized (Alexander, 1989). It is also flexible and can be adapted to the external environment and to specific behavioral goals. The locomotor apparatus often has to be used on a variety of substrates such as level surfaces, twigs in a bush, or ragged cliffs. Furthermore, the locomotor output can change from slow explorative walking to swift running when it becomes necessary to escape a predator or cross terrain without cover.

Frequently, changes in locomotor output are not restricted to the movements of single legs but also entail changes in the temporal coordination between several or all legs. Many quadrupeds, like cats, dogs or horses, for instance, use specific gaits depending on their movement speed (Alexander, 1989). In these animals, leg coordination changes from walking and pace gaits at slow speeds to trotting gaits at intermediate speeds and, eventually, to gallop at high speeds. The coordination of the front- and hind legs changes from anti-phase in walking to nearly in-phase during gallop (Orlovsky et al., 1999). The transition from one gait to another is discontinuous and it can be shown that quadrupeds select the energetically optimal gait at a given speed (Hoyt and Taylor, 1981).

In hexapods, i.e. insects, the situation appears, at first glance, to be comparable. However, different patterns of leg coordination can occur. These patterns are typically characterized by the number of legs that are on the substrate during stance. Very slow walking insects, for example, generate a metachronal wave of leg movements along each side of the body subsequently from back to front while at least five legs are always in stance phase, a coordination pattern called *wave gait* (Hughes, 1952). For faster walking speed coordination is modified accompanied by an apparent reduction in the number of legs that are on the ground simultaneously. At medium speeds the number of legs is reduced to four, called *tetrapod coordination* (Burns, 1973; Graham, 1972; Hughes, 1952; Spirito and Mushrush, 1979; Wendler, 1964; Wendler 1966), and at high speeds to three, called *tripod coordination* (Bender et al., 2011; Delcomyn, 1971; Graham, 1985). Interestingly, bipedal anti-phase coordination of insect hind legs has been reported for the cockroach, *Periplaneta americana*, during top speed running (Full and Tu, 1991). In this situation the anterior part of the animal is lifted and the front and middle legs no longer touch the ground.

While in quadrupeds the switch between two patterns of inter-leg coordination, or gaits, is distinct and dependent on speed, studies in invertebrates indicate that specific patterns of

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coordination are part of a larger and speed-dependent continuum and that intermediate forms of coordination exist. In the same speed range, insects can use either tetrapod or tripod coordination, seamlessly transitioning from one to the other by modifying stance duration (Cruse, 1990; Graham, 1985; Wendler, 1966). Several genera of ants (*Cataglyphis, Formica, Lasius*, and *Myrmica*), cockroaches (*Periplaneta americana*), fruit flies (*Drosophila melanogaster*), and stick insects (*Carausius morosus*) are known to use tripod coordination during fast locomotion, while at lower speeds leg coordination becomes much more variable, approaching tetrapod coordination (Wendler, 1964; Graham 1972; Bender et al., 2011; Strauss and Heisenberg, 1990; Zollikofer, 1994).

How is inter-leg coordination achieved? Behavioral studies on four-, six-, and eight-legged animals have suggested that sensory signals which reflect the movements of individual legs contribute to the coordination between legs, thereby generating an emergent set of coordination rules (Cruse, 1990; Dürr et al., 2004). Furthermore, the importance of intersegmental neural pathways can also be shown based on studies that reduce or eliminate mechanical interaction between legs (Graham and Cruse, 1981; Cruse and Epstein 1982; Gruhn 2006). In normal walking situations the coordination rules arise from the interplay of mechanical and neural coupling between individual legs during walking. While it is clear that both mechanical and neural influences play important roles, their specific contribution for the generation of leg coordination patterns is not clear, yet. On the one hand, for example, there is evidence confirming the importance of central inter-segmental neural pathways for the coordination of local networks controlling leg movements in insect walking. This has been shown for the cockroach, *Periplaneta americana* (Pearson and Iles, 1973), the locust, *Schistocerca americana* (Ryckebusch and Laurent, 1993), and the hawk moth, *Manduca sexta* (Johnston and Levine, 2002). On the other hand, studies have shown the role of local sensory feedback in establishing inter-leg coordination, e.g. in the hawk moth (Johnston and Levine, 1996; Johnston and Levine, 2002) and the stick insect, *Carausius morosus* (Borgmann et al., 2009; Büschges et al., 1995).

One aspect that has so far hindered further elucidation of the neural mechanisms underlying inter-leg coordination is that insect species at given developmental stages (Graham, 1985) often show a rather narrow range of preferred walking speeds. For example, while it is known that cockroaches can use the full range of inter-leg coordination from metachronal wave gait, in which only one leg is in swing phase at any given time, to tripod coordination (Hughes, 1952), under natural conditions they mostly use tripod coordination (Bender et al., 2011).

115   Adult stick insects also show a preference for a particular coordination pattern. They almost

116   exclusively use tetrapod coordination during level walking, while at high speeds also use tri-

117   pod coordination (Graham, 1972). In adult stick insects tripod coordination is less frequent

118   though; larval stages tend to use tripod coordination much more frequently (Graham, 1972),

119   but are also much smaller. As a consequence, in the insect groups studied so far only a rather

120   limited continuum of walking speeds could be investigated reliably. This is all the more unsa-

121   tisfactory as the specifics of inter-leg coordination are often used as important indicators as to

122   how the neural mechanisms generating walking behavior are structured (Zollikofer, 1994). It

123   is therefore critical to determine the full possible range of walking speeds with regard to inter-

124   leg coordination.

125   In the present study, we used four different *Drosophila* strains in order to address this issue

126   and capture as large a range of walking speeds as possible in a single species. The two wild-

127   type strains *Canton-S* (wt^{CS}) and *Berlin* (wt^{Berlin}) thereby represented the wild-typical beha-

128   vior. These two strains have previously been used in studies on inter-leg coordination (Strauss

129   and Heisenberg, 1990; Strauss and Heisenberg, 1993) and global parameters of locomotor

130   activities (Martin, 2004; Martin et al., 1999). In addition, we selected two mutant *Drosophila*

131   strains, *white^{1118}* (w^{1118}) and w^{1118}, Tbh^{nM18}, to extend the range of observable walking speeds

132   to lower values. w^{1118} flies have reduced levels of octopamine (Sitaraman et al., 2008), while

133   w^{1118}, Tbh^{nM18} lacks this biogenic amine altogether (Monastirioti et al., 1996). Octopamine is

134   implicated in the high-level control of locomotor activity (Brembs et al., 2007; Gal and

135   Libersat, 2008; Gal and Libersat, 2010) and, as we show here, a reduced level or absence of

136   octopamine seems to induce lower walking speeds in *Drosophila*. Furthermore, the results we

137   present here for w^{1118} flies can also serve as a control for future studies in *Drosophila*, since

138   an extensive amount of transgenic flies have a w^{1118} background. As we show, there are im-

139   portant differences between wild-type flies and w^{1118}, and this might be important for the in-

140   terpretation of behavioral studies based on transgenic strains.

141   We can show that under relatively unconstrained conditions individuals of different *Drosophi-*

142   *la* strains cover a broad range of speeds during walking. We find that leg coordination pat-

143   terns change gradually and systematically with walking speed. This suggests that the neural

144   controllers responsible for inter-leg coordination are able to generate a marked flexibility with

145   respect to walking behavior. Furthermore, removing one of the hind legs reveals that *Droso-*

146   *phila* is capable of adapting its leg coordination immediately, thereby maintaining the ability

147   to propel itself forward even after major biomechanical changes in its walking apparatus.

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Materials and Methods

Fly strains and breeding

Flies were raised at 25°C and 60% humidity on a 12/12 h light and dark cycle and maintained on standard medium containing cornmeal, molasses, yeast, and agar. For the experiments presented here, we used the following *Drosophila melanogaster* strains: wild-type *Canton-S* (wt^{CS}), wild-type *Berlin* (wt^{Berlin}), w^{1118}, and w^{1118}, Tbh^{nM18} (Monastirioti et al., 1996). Flies were kindly provided by Dr. M. Leptin (wt^{CS}), Dr. R. Strauss (wt^{Berlin}), and Dr. H. Scholz (w^{1118} and w^{1118}, Tbh^{nM18}).

Experimental procedure

For all experiments, five days old males were used. At least two hours prior to an experiment, flies were cold anesthetized and put into isolation tubes without food but with water. One fly at a time was then transferred from its isolation tube into the experimental setup were it walked spontaneously back and forth on a 5 mm wide transparent walkway (Fig. 1A). Wings were left intact; therefore, to prevent escape by flight, the walkway was enclosed on all sides with acrylic glass. Furthermore, the inner walls of the enclosure were covered with a layer of Fluon (AGC Chemicals Europe) which prevented the flies from scaling the walls. To allow for video recordings a small area (20 mm) on one side of the walkway was kept free of Fluon. Beneath this area, we attached a glass prism providing a ventral view of the walkway. Thus, using a single camera we were able to simultaneously record a lateral (Fig. 1B) and a ventral view (Fig. 1C) of the walking fly. Video recordings were taken with a high-speed digital camera (AOS S-PRI High Speed Color 5.2, AOS Technologies AG, Baden Daettwil, Switzerland) at 500 frames s^{-1}, with a shutter time of 200 µs. The setup was illuminated with infrared LEDs ($\lambda = 880$ nm). The LEDs were externally synchronized to the shutter of the camera in order to provide maximum illumination during the time the shutter of the camera was open. The camera was controlled via AOS Imaging Studio v3 (AOS Technologies AG, Baden Daettwil, Switzerland). After each set of experiments a 10mm wide marker was recorded with the same settings. This marker was then used to calibrate the analyzed videos.

For the amputation experiments, flies were cold anaesthetized followed by the removal of one of the hind legs. This was done by cutting off the leg at the midpoint of the femur, leaving only a stump consisting of coxa, trochanter, and part of the femur. Flies were then moved to isolation tubes and subsequently treated as described above for the intact animals.

180 To determine the average weight of the flies, between 9 and 35 flies (three to seven days old)

181 of each sex and strain were collected into separate 1.5 ml plastic tubes (Table 1). The tubes

182 including the flies were then weighed, the weight of the empty tube was subtracted, and the

183 weight of one single fly was calculated. In addition, the body length of each fly recorded dur-

184 ing the behavioral experiments was determined by marking the base of the antennae and the

185 tip of the abdomen in the ventral view of the fly, using the same software as for the video

186 analysis (ProAnalyst, XCitex, Inc., Cambridge, MA).

187 *Data analysis*

188 During experiments, flies walked spontaneously back and forth on the walkway. We recorded

189 straight walks containing 5 to 12 complete step cycles per leg. The recorded videos were then

190 evaluated frame-by-frame in a semi-automatic fashion. Body position and axes were deter-

191 mined automatically with ProAnalyst (XCitex, Inc, Cambridge, MA). The exact times of tar-

192 sal liftoff and touchdown events were visually determined in the lateral view of the fly, while

193 the associated tarsus positions were visually determined in the ventral view. Data obtained in

194 this manner were then further processed in MATLAB (MathWorks, Inc., Natick, MA).

195 Durations of swing and stance phases were calculated as the difference between the time of

196 liftoff and subsequent touchdown of the same leg (swing) or vice versa (stance). One cycle

197 period was defined as the time difference between two consecutive liftoff events of the same

198 leg. Onset of swing was used as reference time for the analysis of temporal coordination of all

199 legs. In trials with intact animals, the reference leg was always the front leg that completed

200 the most cycles during a given trial. In trials with animals lacking one hind leg, the reference

201 leg was always the front leg contralateral to the lesioned side. Results from the phase analysis

202 of trials in which the right front leg was the reference leg were then flipped in order to com-

203 bine the results with those in which the left front leg was the reference leg. Phase analyses and

204 the corresponding plots were done using the CircStat Toolbox for MATLAB (Berens, 2009).

205 All positional information with regard to tarsal touchdown and liftoff was transformed into

206 the body-centered XY-coordinate system (see also Fig. 1C). Furthermore, in order to compen-

207 sate for small variations in body size, these body-centered data were then normalized to the

208 respective body length of the fly. Based on these data we calculated stance trajectories in the

209 body-centered XY-coordinate system. Step amplitude of a particular step was determined as

210 the distance between the posterior extreme position (PEP) of the tarsus at liftoff and the sub-

211 sequent touchdown at the anterior extreme position (AEP) in body-centered coordinates. It

212 has to be noted that we use step amplitude instead of stride length, which is defined as the

213 distance between two consecutive touchdown positions in floor-fixed coordinates. Stride

214 length is not independent of movement speed and might change even without active changes

215 in the walking motor pattern. This is not true for step amplitude. A change in this measure

216 always necessitates a change in the motor output. Although the two measures are closely re-

217 lated, step amplitude is much more informative, when one is interested in kinematic changes

218 the animal has to make actively.

219 Based on the ventral view, walking speed was calculated for each frame in a trial as the

220 change in position of the fly's body relative to the ground. The resulting speed profile was

221 smoothed with a gliding average of 5 frames width. Based on this complete speed profile, the

222 walking speed associated with a particular swing phase, as used in Fig. 3B and 3D, for in-

223 stance, was calculated as follows: we first determined the time interval between onset and

224 offset of the swing phase and found the section of the complete speed profile associated with

225 this interval. We averaged the speed profile within the interval to obtain a single average

226 speed value. This average speed value was then used as the walking speed associated with a

227 particular swing phase.

228 *Coordination patterns*

229 In hexapod walking, the literature typically distinguishes between three different coordination

230 patterns: tripod coordination, tetrapod coordination and wave gait. The mere existence of

231 these categories implies three distinct gaits, and, as a matter of fact, these coordination pat-

232 terns have often been used synonymous with gaits. The literature, however, also implies that

233 there is a speed-dependent continuum between these prominent patterns (Wendler, 1964;

234 Graham 1972). Therefore, because they are established, we use these terms; however, we do

235 so in a purely descriptive manner and refer to *coordination patterns* rather than *gaits*.

236 In order to describe the walking patterns that occurred during the recorded trials we classify

237 these as either tripod, tetrapod, or undefined coordination according to the following consid-

238 erations: *Tripod coordination* is described as the alternating movement of two distinct groups

239 of legs (Hughes, 1952; Wilson, 1966). These *tripod groups* consist of an ipsilateral front and

240 hind leg, and a contralateral middle leg (L1, L3, R2, and R1, R3, L2, respectively). Tripod

241 coordination is typically found in fast moving animals and therefore constitutes the extreme

242 case at the highest end of the aforementioned speed-dependent continuum. In its ideal form,

243 tripod coordination is characterized as the simultaneous liftoff and touchdown of all legs in

244   one tripod group, while the legs associated with the other tripod group are on the ground.

245   However, using this strict definition of tripod coordination is problematic, mainly because of

246   two reasons. First of all, in this strict sense, tripod coordination occurs only very rarely; even

247   during highly coordinated walking random fluctuations or small systematic shifts in the phase

248   relations between legs of one tripod group tend to persist (Bender et al., 2011); in addition, it

249   is known that in most insects the legs of one tripod group are not completely in phase

250   (Hughes, 1952). Secondly, concentrating on this narrow aspect of inter-leg coordination po-

251   tentially diverts attention from other interesting coordination patterns which do not happen to

252   fall under the tripod definition but might nevertheless be highly regular. In order to address

253   this conceptual problem, we used a more flexible description of tripod coordination: we de-

254   fined a particular walking pattern as tripod coordination when, during one step, the swing

255   phases of all legs associated with a tripod group concurrently overlapped for at least one

256   frame of recorded video. Here, this is equivalent to 2 ms; for comparison, typical swing dura-

257   tions observed during experiments were in the range of 20 to 40 ms. Note, that this is different

258   from the strict definition of tripod coordination, which is defined as *simultaneous* liftoff and

259   touchdown of the legs in a tripod group. In addition, once a walking pattern was defined as

260   tripod, we determined the *tripod coordination strength* (TCS) which we obtained as follows.

261   First, we calculated the time from the earliest swing onset to the latest swing termination. This

262   gave us time t_1, during which at least one of the three legs was in swing phase. Then we de-

263   termined time t_2, during which all three legs were in swing phase at the same time. The ratio

264   t_2/t_1 then described the TCS. A TCS of 1 indicated perfect tripod coordination; it approached

265   0 when the temporal relation of swing phases shifted to other coordination patterns (e.g. see

266   Fig. 7G). *Tetrapod coordination* was defined accordingly: it can be defined as a walking pat-

267   tern in which exactly two legs are lifted off the ground at a particular time (Graham, 1985;

268   Hughes, 1952). Therefore, a walking pattern was defined as tetrapod when, during one step,

269   the swing phases of exactly two legs overlapped for at least one frame of recorded video. Te-

270   trapod coordination constitutes a further special case within the continuum of coordination

271   and is generally associated with intermediate walking speeds. Finally, when a step was neither

272   tripod nor tetrapod we classified it as *undefined*. This category is largely identical with what is

273   usually called *wave gait*, although this was not explicitly tested. It has to be noted, that we

274   used this classification schema on a step-by-step basis; each step was evaluated separately and

275   can either be classified as tripod, tetrapod, or undefined, never as two of the above. Although

276   tripod coordination was predominantly found at high speeds, tetrapod coordination was most

277   frequently found at intermediate speeds, and undefined coordination was most common at low

278 speeds, the classification was completely agnostic with regard to the walking speed during a

279 particular step. Each coordination class could have occurred at any speed.

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Results

The four different strains of *Drosophila* studied here were similar in size and weight (Table 1). The body lengths of males ranged from 2.06 to 2.12 mm, their weight ranged from 0.70 to 0.86 mg. In general, the weight of females was higher, ranging from 1.05 to 1.32 mg. Males of the strains wt^{CS}, w^{1118}, and w^{1118}, Tbh^{nM18} were almost identical in size and weight, while wt^{Berlin} males were slightly larger (5%) and on average 20% heavier. The same was true for females of wt^{Berlin}. In order to minimize potential age- or sex-related influences on walking behavior we selected five day old males for the present study.

Wild-type strain *Canton-S* (wt^{CS})

In a first set of experiments, we studied leg kinematics and inter-leg coordination in wt^{CS} during spontaneous walking. Generally, animals generated walking sequences that were straight and the features of which were in accordance with previously published findings (Strauss and Heisenberg, 1990; Strauss and Heisenberg, 1993). Legs were coordinated in tripod fashion, as exemplified in the trial displayed in Figure 2Ai (highlighted area). The features of all further recorded trials of wt^{CS} were qualitatively similar to the one shown in Fig. 2Ai. Movement speed was always relatively constant during each trial; in the sequence shown in Fig. 2Ai, for instance, movement speed was approx. 13 body lengths per second (BL s^{-1}) on average. However, over all trials, average walking speed ranged from 5 to 16 BL s^{-1}. This was equivalent to absolute values of 11 to 32 mm/s (6 individuals; 555 steps). Average stance phase trajectories of all six legs were relatively straight and almost parallel to the longitudinal body axis (Fig. 2B). The length of stance trajectories was similar for all legs and in the range of half the body length. With regard to temporal coordination, each of the three leg pairs showed anti-phase swing activity on average (Fig. 2C). Legs were generally coordinated in tripod fashion; however, the front leg of a tripod group tended to initiate its swing phase first, followed by the middle leg with a phase shift of approx. 15°. The middle leg was in turn followed by the hind leg with a further phase shift of 15° (Fig 2C).

Only during particularly slow walking sequences tripod coordination was more variable. An example for this is shown in Fig. 2Aii. Here, a section of 0.5s from one of the slower trials in wt^{CS} is shown (approx. 7 BL s^{-1} on average). However, even during these slowest walking sequences coordination was still tripod, according to our conservative definition (see highlighted area in Fig. 2Aii), and phase relations were similar to those of the faster trials (Fig. 2C, green points). In contrast to a tripod group, in which the temporal succession of swing

313 onset was directed posteriorly, the order of swing onsets on each body side was always di-

314 rected anteriorly, beginning with the hind leg, followed by the middle leg and finally the front

315 leg, after which the next series starts again with the hind leg.

316 As it is known that insects walking in tripod coordination adapt swing duration depending on

317 step cycle period (Graham, 1985), we examined this relationship for wt^{CS}. We found that

318 swing duration indeed moderately correlated with cycle period (Fig. 3A); this was true for the

319 complete data set (Fig. 3A, black regression line, coefficient of determination $R^2 = 0.37$), as

320 well as for individual trials (Fig. 3A, gray regression lines). Another parameter that more

321 strongly depended on cycle period was walking speed; we modeled this dependence as a

322 hyperbolic relationship over the complete range of cycle periods (Fig. 3B, black line, pseudo

323 $R^2 = 0.76$). At the same time, cycle period did not correlate with step amplitude (Fig. 3C,

324 black regression line, $R^2 = 0.03$). Although step amplitude contributes weakly to walking

325 speed when we examine the complete range of step amplitudes (Fig. 3D, black regression

326 line, $R^2 = 0.16$), this relation cannot be shown reliably for individual trials (Fig. 3D, gray re-

327 gression lines).

328 Wild-type strain *Berlin* (wt^{Berlin})

329 We then collected data for the wt^{Berlin} (Fig. 4). Similar to wt^{CS} flies, wt^{Berlin} almost exclusively

330 used tripod coordination during all recorded trials. As an example for comparatively strict

331 tripod leg coordination in this strain, Figure 4Ai shows a 0.5s long section of a fast walking

332 trial. Overall, average walking speed ranged from 5 to 15 BL s^{-1}, which was equivalent to

333 absolute speeds of 11 to 34 mm s^{-1} (3 individuals; 403 steps). Stance trajectories in the wt^{Berlin}

334 were on average straight and almost parallel to the longitudinal body axis (Fig. 4B). Each of

335 the three leg pairs showed clear anti-phase swing activity during tripod coordination (Fig.

336 4Ai; highlighted area; Fig. 4C; magenta points for the sequence shown in Fig. 4Ai). In analo-

337 gy to wt^{CS}, we found that the front legs of a tripod group initiated swing first, followed by the

338 middle legs, which in turn were followed by the hind legs (Fig. 4C, blue data points). Only

339 during very slow walking sequences, tripod coordination became more variable and we also

340 found intermittent tetrapod coordination (Fig. 4Aii; highlighted area); this was also reflected

341 in the phase relations which started to deviate more pronouncedly from the typical tripod pat-

342 tern (Fig. 4C, green data points). These shifts to tetrapod coordination were rare though (see

343 also Fig. 7A). The succession of swing onset on each body side was always directed anterior-

344 ly. In analogy to wt^{CS}, walking speed in wt^{Berlin} was clearly correlated with cycle period (Fig.

345 4D), while it did not depend on step amplitude (Fig. 4E).

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346   Mutant strain *white^{1118}* (*w^{1118}*)

347   In the third set of experiments, we analyzed walking in *w^{1118}* flies (Fig. 5). The total range of

348   walking speeds in this strain was similar to those of *wt^{CS}* and *wt^{Berlin}* flies, with values ranging

349   from 2 to 15 BL s^{-1}, i.e. from 4 to 31 mm/s (5 individuals; 695 steps), as exemplified for a

350   single trial in Fig. 5Ai. However, *w^{1118}* flies walked at lower speeds more frequently. In gen-

351   eral, speed appeared to be somewhat more variable within single walking sequences as com-

352   pared to *wt^{CS}* and *wt^{Berlin}* (cf. Figs. 2A and 4A to 5A). Stance trajectories were parallel to the

353   longitudinal body axis for all three pairs of legs. On average, step amplitudes were slightly

354   shorter than 0.5 body lengths and thus shorter than for the other two strains (Fig. 5B). Indi-

355   viduals of *w^{1118}* often used tripod coordination (e.g. Fig. 5Ai; see highlighted area), although

356   the variability of inter-leg coordination seemed to be relatively high (blue points in Fig. 5C; 5

357   individuals; 713 steps). Nevertheless, according to our conservative definition inter-leg coor-

358   dination was still tripod on average (black lines in Fig. 5C). This variability can partially be

359   attributed to the fact that at lower speeds animals no longer used tripod but instead used tetra-

360   pod coordination (Fig. 5Aii; highlighted area with one asterisk) or even wave gait-like coor-

361   dination (Fig. 5Aii; highlighted area with two asterisks). Similar to *wt^{CS}* and *wt^{Berlin}* flies aver-

362   age swing phase onset of posterior legs in a tripod group trailed front legs (Fig. 5C, magenta

363   points for the trial in Fig. 5Ai; blue points for all data). Still, even in the slowest trial the suc-

364   cession of swing phase onsets on a body side was directed anteriorly. The walking speed of

365   *w^{1118}* flies strongly correlated with cycle period (Fig. 5D). We found only a weak correlation

366   between walking speed and step amplitude (Fig. 5E, R^2 = 0.17).

367   Mutant strain *w^{1118}*, *Tbh^{nM18}*

368   The octopaminergic neurotransmitter system has been implicated in the regulation of walking

369   in stick insects, cockroaches, and crabs. *w^{1118}*, *Tbh^{nM18}* mutants lacking the enzyme tyramine-

370   b-hydroxylase enzyme necessary for the conversion of tyramine into octopamine have defi-

371   ciencies in locomotor performance as compared to wild-type flies (Brembs et al., 2007;

372   Scholz, 2005). We found that this offered the chance to extend the range of movement speeds

373   studied here to even lower values. For *w^{1118}*, *Tbh^{nM18}* flies movement speed ranged from 3

374   mm/s to 14 mm/s (5 individuals; 681 steps), i.e. from 1.5 to 7 BL s^{-1}. *w^{1118}*, *Tbh^{nM18}* flies only

375   rarely walked at higher speeds as exemplified for a single trial in Fig. 6Ai (see highlighted

376   area for an instance of tripod coordination). Again, average stance trajectories were parallel to

377   the longitudinal body axis and were slightly shorter than those in the strain *w^{1118}* (Fig. 6B).

378   However, average phase relations of swing onset were no longer typical for tripod coordina-

379   tion: for example, phase values for R1, L2 and R3 relative to L1 were 175°, 120° and 140°,

380   respectively. Phase plots show a substantial variability of inter-leg coordination (Fig. 6C; ma-

381   genta points for the sequence shown in Fig. 6Ai; blue points for all steps; 5 individuals; 713

382   steps). At low speeds (< 5 BL s^{-1}) w^{1118}, Tbh^{nM18} flies often used tetrapod coordination; during

383   the slowest trials (2 to 3 BL s^{-1}) coordination resembled wave gait (Fig. 6Aii: see highlighted

384   areas: * tetrapod; ** wave gait-like coordination; green points of this sequence in Fig. 6C). In

385   analogy to the other strains examined here, the succession of swing onset on each body side is

386   directed anteriorly. Only small deviations from this pattern could be observed during very

387   slow trials (cf. third swing of R3 in Fig. 6Aii). Again, walking speed in w^{1118}, Tbh^{nM18} was

388   strongly correlated to cycle period (Fig. 6D). The correlation between walking speed and step

389   amplitude was weak (Fig. 6E, $R^2 = 0.15$).

390   *Inter-leg coordination depends on movement speed*

391   While all strains used tripod coordination during fast walking, at lower speeds inter-leg coor-

392   dination became more variable or changed to other patterns such as tetrapod coordination.

393   Based on this observation, we wanted to know whether inter-leg coordination depends syste-

394   matically on walking speed. Therefore, we first determined the relative frequency of occur-

395   rence of tripod, tetrapod, and undefined coordination in all four fly strains. We found that wt^{CS}

396   and wt^{Berlin} flies almost exclusively used tripod coordination, while in w^{1118} and w^{1118}, Tbh^{nM18}

397   flies tetrapod and undefined coordination patterns represented almost one third of all patterns

398   (Fig. 7A). When we pooled the data of all strains and plotted the relative frequency of occur-

399   rence of coordination types in three different speed ranges we found that tetrapod and unde-

400   fined coordination patterns occur almost exclusively at speeds below 5 BL s^{-1} (Fig. 7B). Be-

401   cause we chose a rather conservative tripod definition, we frequently found this coordination

402   type in all four strains. To further flesh out the relation between tripod coordination and walk-

403   ing speed we examined the tripod coordination strength (TCS) as a function of speed in all

404   four strains (Fig. 7C: wt^{CS}; Fig. 7D: wt^{Berlin}; Fig. 7E: w^{1118}; Fig. 7F: w^{1118}, Tbh^{nM18}). Figure 7G

405   shows five exemplary footfall patterns illustrating TCS ranging from 0.8 to 0.1. Generally, in

406   all four strains TCS was variable, but depended systematically on movement speed. While we

407   did not expect TCS to reach 1.0 due to the aforementioned phase lags within a tripod group, at

408   speeds higher than 10 BL s^{-1} it reached maximal values of up to 0.85 (cf. Fig. 7G). Below 10

409   BL s^{-1} TCS ranged from values 0.02 to 0.8. In general, at speeds higher than 10 BL s^{-1} inter-

410   leg coordination is tripod. Its variability increases noticeably towards lower speeds, as indi-

411   cated by lower TCS values. In the range of low walking speeds (<10 BL s^{-1}) *Drosophila*

412   seems to be able to also use tetrapod coordination or even wave gait.

413   *Inter-leg coordination changes after loss of one hind leg*

414   The results presented here suggest that *Drosophil*a's walking system does not generate a fixed

415   motor output. Instead, it seems to be able to flexibly produce inter-leg coordination patterns

416   which change in a systematic and gradual fashion with walking speed. At very slow walking

417   speeds, *Drosophila* uses wave gait; with an increase in speed, inter-leg coordination then tran-

418   sitions to tetrapod and finally becomes tripod at the highest speeds. In order to further study

419   the basis of this apparent flexibility, in a final set of experiments we examined walking in wt^{CS}

420   flies shortly after the removal of one hind leg (Fig. 8). The loss of a leg drastically changes

421   the body geometry and if the animal wants to continue walking successfully it has to adapt its

422   movement pattern to this new geometry. One necessary prerequisite for such an adaptation is

423   that sensory information originating in the legs is taken into account by the neural system that

424   generates walking behavior.

425   We observed five changes in the walking behavior of flies after the loss of one hind leg: (i)

426   wt^{CS} flies with a missing hind leg walked on average slower than intact animals of the same

427   strain (Fig. 8 A; cf. Fig. 2 A). Average walking speeds ranged from 1 to 13 BL s^{-1}, which is

428   equivalent to approx. 2 to 26 mm/s (5 individuals; 664 steps), as compared to a range of 4 to

429   18 BL s^{-1} in intact animals. (ii) The shape of stance trajectories changed after amputation of

430   one hind leg and showed distinct curvatures. (iii) In all legs, AEPs and PEPs changed within

431   the body coordinate system (Fig. 8B). Generally, we found an outward shift of AEPs and

432   PEPs. In addition, especially in the remaining middle and hind legs these positions were also

433   shifted caudally. (iv) The average stance trajectories of the remaining hind leg and of both

434   middle legs became noticeably longer. Stance trajectory length increased in the remaining

435   hind leg from 0.43 to 0.47 BL, and in middle leg contralateral to the lesion from 0.50 to 0.53

436   BL. The most noticeable increase was found in the middle leg ipsilateral to the lesion. Here,

437   average stance trajectory length increased from 0.50 to 0.60 BL. (v) Phase relations of both

438   the contralateral middle and the remaining hind leg were altered. The hind leg contralaterally

439   to the lesion (leg I3) was, on average, no longer in phase with the ipsilateral middle leg (C2);

440   it increased its phase with regard to I3 to 0.85 rad (Fig. 8C) as compared to the intact animal

441   in which the phase of C2 with regard to I3 was 0.16 on average (Fig. 2C). Furthermore, the

442   contralateral middle leg showed an increase in phase with regard to the contralateral front leg

443   (Fig. 8 C, cf. with Fig. 2C). As a consequence, generally three to four legs were simultaneous-

444   ly on the ground. Slow walking individuals used either tetrapod or wave gait coordination

445   (Fig. 8Aii). The correlation between walking speed and cycle period was still present though,

446   and step amplitude was not correlated with speed (Figs. 8D and E).

447

Discussion

We have shown that the walking system of *Drosophila* is able to generate a broad range of locomotion speeds and different strains walked at preferred parts of this complete range. wt^{CS} flies tended to walk faster than wt^{Berlin} and w^{1118} individuals, respectively. Mutant w^{1118}, Tbh^{nM18} individuals walked at the lowest speeds. At high speeds, all individuals walked in tripod coordination. With decreasing walking speed, TCS decreased as well (Fig. 7C to F) and animals also used tetrapod coordination more frequently (Fig. 7B). Finally, at very low speeds, walking was often accomplished by simultaneous stance phases of five legs while only a single leg is in swing phase at a time. These findings imply that *Drosophila's* walking behavior is more flexible than previously thought (Strauss and Heisenberg, 1990): there are no clearly separable gaits and, more specifically, the neural controller producing inter-leg coordination is not restricted to a fixed tripod pattern.

This notion is substantiated by amputation experiments, in which we examined the walking behavior of animals after the loss of one hind leg. These experiments were carried out with individuals of wt^{CS}, which is the strain that showed the most robust tripod coordination when intact. Removal of a hind leg in these flies resulted in an immediate reorganization of overall posture, single leg kinematics and inter-leg coordination: the legs of the animals were positioned in a broader frame, the stance trajectories of the remaining middle and hind legs were elongated while the phase of these legs was increased.

Changes in inter-leg coordination related to walking speed

In the first part of the present study we have analyzed walking in the *Drosophila* strains wt^{CS} and wt^{Berlin} as well as the mutant strains w^{1118} and w^{1118}, Tbh^{nM18} with respect to single leg kinematics and inter-leg coordination. Walking speed differed noticeably between strains, with wt^{CS} and wt^{Berlin} ranging from 5 to 16 BL s^{-1} (11 to 32 mm/s), w^{1118} ranging from 2 to 15 BL s^{-1} (3.5 to 31 mm/s), and w^{1118}, Tbh^{nM18} ranging from 1.5 to 7 BL s^{-1} (3 to 14 mm/s). For the strains wt^{CS} and wt^{Berlin}, the reported average walking speeds in the literature range from 2.2 and 2 to 3 mm/s (Serway et al., 2009) to 15 and 21 mm/s (Poeck et al., 2008; Strauss and Heisenberg, 1993), respectively. Average walking speed for w^{1118} was reported to be approx. 2 mm/s and for w^{1118}, Tbh^{nM18} 4 mm/s (Scholz, 2005). More detailed data concerning the range of walking speeds are only available for the strain wt^{Berlin}, for which speeds of 12 to 40 mm/s were found (Strauss and Heisenberg, 1990). These values correspond with our data in which we find only slightly lower speeds for wt^{Berlin} (11 - 34 mm/s). It has to be noted though

480 that we used a different behavioral paradigm than previous studies. Some of these used Buri-

481 dan's paradigm (Bülthoff et al., 1982; Götz, 1980) to elicit straight walks on level ground

482 (Poeck et al., 2008; Serway et al., 2009; Strauss and Heisenberg, 1990; Strauss and

483 Heisenberg, 1993), while others studied walking in *Drosophila* under ambient light conditions

484 without the presentation of visual cues (Scholz, 2005; Wolf et al., 2002).

485 For all strains examined here we found that walking speed is controlled via changes in step

486 cycle period and stance duration. Over the complete range of walking speeds we found only

487 moderate changes with regard to swing duration, and no systematic modification of step am-

488 plitude could be detected. This complements and extends a previous study in which *Drosophi-*

489 *la* altered not only its cycle period but also its stride length over the range of walking speeds

490 (Strauss and Heisenberg, 1990). These authors, however, examined stride length, while the

491 present study focused on step amplitude (see also Materials and Methods section). The find-

492 ings presented here do not contradict the previous ones; here, however, we wanted to disso-

493 ciate the effect body translation during swing phases has on stride length from actual adapta-

494 tions in leg kinematics during a step cycle. As a consequence, our findings indicate that *Dro-*

495 *sophila* controls walking speed solely by adjusting step cycle period while it keeps step ampli-

496 tude mostly constant.

497 Strauss and Heisenberg (1990) reported that *Drosophila* uses tripod coordination for a large

498 part of the observed speed range. They found tetrapod coordination only during "... decelera-

499 tion episodes prior to turns or to a complete stop,...". In general, we can confirm these find-

500 ings. However, in the present study *Berlin* flies also spontaneously generated relatively slow

501 walking bouts. In these trials we found that inter-leg coordination deviated from a strong tri-

502 pod pattern, as indicated by low TCS values. Comparing this result with the data for wt^{CS} and

503 w^{1118} revealed that this change in coordination is indeed systematically found when *Drosophi-*

504 *la* walks more slowly. At walking speeds higher than 10 BL s^{-1} inter-leg coordination was

505 always tripod. At lower speeds, TCS decreased and within this speed domain we also ob-

506 served tetrapod coordination. This analysis suggests that the kinematics of the movement pat-

507 tern generally change systematically and continuously with walking speed.

508 It is important to emphasize what a decrease in TCS means with regard to inter-leg coordina-

509 tion: a TCS of 0.5 means that the swing phases of the legs associated with a tripod group

510 overlap 50% of the time during which any of these legs move. For a TCS of 0.4 this decreases

511 to 40%; however, this also means that during 60% of the time these legs are not in swing

512 phase simultaneously. In other words, during this time four or five legs are on the ground.

513 This time only increases with a further decrease in TCS. Consequently, although low TCS

514 levels still indicate tripod coordination (according to our conservative definition), swing phase

515 overlap in these cases might be more consistent with coordination patterns that conventionally

516 have rather been associated with tetrapod coordination. In addition, examining the two mutant

517 strains w^{1118} and w^{1118}, Tbh^{nM18} shows that at very low walking speeds *Drosophila* no longer

518 uses tetrapod and instead coordinates its legs in a pattern that resembles wave gait, a pattern

519 first described for larger insects (Hughes, 1952; Wilson, 1966).

520 Interestingly, it appears that inter-leg coordination in *Drosophila* reflects all possible coordi-

521 nation patterns known in insects. Studies on inter-leg coordination in other, much larger in-

522 sects, including cockroaches and beetles (Hughes, 1952), or grasshoppers (Burns, 1973),

523 showed that inter-leg coordination is tripod only at high walking speeds and short cycle pe-

524 riods. At lower speeds inter-leg coordination becomes increasingly variable, including tetra-

525 pod walking patterns. However, in these studies the examined species often differed noticea-

526 bly in size and weight. Burns (1973), for instance, studied two orthopteran species, locusts

527 and grasshoppers, which differed in size by a factor of two. With respect to a systematic anal-

528 ysis of inter-leg coordination and walking speed previous insights derive from studies on ants

529 (Zollikofer, 1994), cockroaches (Delcomyn, 1971), and stick insects (Graham, 1985;

530 Wendler, 1966). Freely walking ants predominantly use tripod coordination in a speed range

531 between 5 and 32 BL s^{-1}; no data, however, is available for slower walking speeds. Unre-

532 strained cockroaches walk at speeds in the range of 1 to 20 BL s^{-1} (Bender et al., 2011;

533 Delcomyn, 1971) and it has been reported that tripod coordination is present across a broad

534 range of speeds, i.e. above 1.2 BL s^{-1}. However, inter-leg coordination in cockroaches be-

535 comes more variable with slower speeds. Delcomyn (1971) used the term "uncoupled alter-

536 nating triangle" for the increasing variability in tripod coordination occurring at slow speeds

537 (Kozacik, 1981). Bender and coworkers (2011) also reported clear changes in inter-leg coor-

538 dination related to walking speed. They proposed the term *ambling gait* for inter-leg coordina-

539 tion that is found during slow walking. It is important to note that although cockroaches tend

540 to move the legs of a tripod group simultaneously at low speeds the coordination pattern be-

541 comes much more variable and there does not seem to be a fixed coupling anymore. When

542 adult stick insects walk on a level surface they mostly do so at speeds well below 1 BL s^{-1}; in

543 this situation their preferred inter-leg coordination is tetrapod (Cruse et al., 2009; Graham,

544 1972). At higher speeds sequences of tripod coordination can also be observed (Graham,

545 1972). Our results on *Drosophila* show two things: firstly, as it has been found in the stick

546 insect, inter-leg coordination in *Drosophila* is not fixed, but changes systematically and grad-

547 ually as a function of walking speed over a broad speed range. Secondly, below walking

548 speeds of 5 to 6 BL s^{-1}, *Drosophila* seems to be able to choose which coordination type it uses

549 and can walk in tripod, tetrapod, or even wave gait-like inter-leg coordination. Importantly,

550 we found that swing duration was uncorrelated with walking speed. These findings have im-

551 plications for the organization of the neural structure controlling walking in *Drosophila*: there

552 is no justification to hypothesize a specific neural tripod generator in *Drosophila*.

553 This conclusion is corroborated by the changes observed in inter-leg coordination following

554 the loss of one hind leg in *wt*^{CS}, which is the strain that had the most robust tripod coordina-

555 tion pattern. We found that inter-leg coordination as well as stance kinematics changed after

556 the loss of one hind leg (Fig. 8). In the present study, compensatory changes were observed on

557 two different levels: the temporal and the kinematic level. With regard to temporal coordina-

558 tion, the stepping activity of the remaining legs, specifically the contralateral middle and hind

559 legs, was modified such that the now absent support of the missing hind leg was compensated.

560 Swing phase activity in the contralateral hind and middle legs was delayed as compared to an

561 intact animal. Kinematic changes entail an extended stance trajectory in the ipsilateral middle

562 leg and a general outward shift of AEPs and PEPs, i.e. overall, the animal adopts a broader

563 posture. Especially, this outward shift suggests an overall compensatory modification of body

564 posture. In addition, the extended stance trajectory of the ipsilateral middle leg nicely corres-

565 ponds to Cruse's coordination rule 1 (Cruse et al., 1998; Dürr et al., 2004). This rule ensures

566 that a leg in swing phase inhibits the transition to swing phase in an anterior neighbor. Since

567 the amputated leg in the present study can be interpreted as locked in swing phase this would

568 explain the extended stance phase in the ipsilateral middle leg. These findings are interesting

569 as it provides evidence for cooperative interactions (neural and mechanical) between the legs

570 in the generation of propulsion and posture. Similar changes in inter-leg coordination after the

571 loss of one leg have been reported for stick insects (Bässler, 1972; Graham, 1977) and cock-

572 roaches (Delcomyn, 1991; Hughes, 1957). Hughes (1957), for instance, found in cockroaches

573 that upon removal of one hind leg other legs have extended stance trajectories and that stance

574 trajectories are shifted outward. Our results also parallel findings reported by Delcomyn

575 (1991), who showed that inter-leg coordination during walking became more variable after

576 the loss of one hind leg (cf. Fig. 2C with 8C). We have to note, however, that the postural

577 adaptations we observed, especially the broader placement of the tarsi, might at least in part

578 be due to a relative increase in load, a consequence of the loss of muscle force available to the

579 animal.

580   Based on the changes in inter-leg coordination with regard to walking speed and upon remov-

581   al of one leg, we conclude that the neural control system for walking in *Drosophila* allows for

582   a modular control of single-leg stepping in which individual legs are largely independent of

583   each other and are only loosely coupled. We hypothesize that the neural control system for

584   walking in *Drosophila* is similar to that in fast walking insects, like ants and cockroaches, as

585   well as to that found in insects like the stick insect. The behavior of *Drosophila* agrees well

586   with that found in stick insects (see Introduction). Behavioral studies in stick insects suggest

587   that inter-leg coordination is the result of the interplay of individual leg controllers based on

588   specific rules (Cruse, 1990; Dürr et al., 2004). Although not (yet) studied in *Drosophila*, it is

589   quite conceivable that the 'coordination rules 1-3', as proposed by Cruse (Dürr et al., 1990),

590   would suffice to generate the walking behavior observed here. However, it also needs to be

591   taken into account that the output of any locomotor system is shaped by the complex interac-

592   tion between neural as well as mechanical influences. In order to further substantiate how

593   *Drosophila's* walking system compares to that of other insects it will be necessary to distin-

594   guish between the level of neural control and the level of mechanical coupling. Experimental

595   paradigms for insect locomotion are available that allow for this dissection, e.g. slippery sur-

596   faces that reduce or even remove mechanical coupling between the legs (Graham and Cruse,

597   1981; Gruhn et al., 2006).

598   Another interesting aspect of the present study are the results for the two mutant strains w^{1118}

599   and w^{1118}, Tbh^{nM18}. Both of these strains exhibited walking speeds that were lower than in the

600   two wild-type strains, a fact that allowed us to extend the range of speeds that we investi-

601   gated. Walking speed in w^{1118}, Tbh^{nM18} was lower than in w^{1118}. It is quite conceivable that

602   w^{1118} flies walk slower because of visual impairment (Kalmus, 1943). The even lower speed

603   range used by w^{1118}, Tbh^{nM18} can likely be attributed to the fact that w^{1118}, Tbh^{nM18} lacks octo-

604   pamine (Monastirioti et al., 1996), a biogenic amine that plays an important role during vari-

605   ous locomotor behaviors in invertebrates. It is known to influence the initiation and mainten-

606   ance of flight (Brembs et al., 2007) and pre-flight jumps in *Drosophila* (Zumstein et al., 2004)

607   and is also implicated as a modulator of walking behavior in cockroaches, for instance (Gal

608   and Libersat, 2008; Gal and Libersat, 2010). Interestingly, in all of these studies octopamine

609   appears to selectively influence high-level aspects of locomotion, while more low-level as-

610   pects, such as leg kinematics, for instance, remain unaffected. Although the present study did

611   not focus specifically on the effects of octopamine, we can support the findings of these pre-

612   vious works. Individuals of the w^{1118}, Tbh^{nM18} strain walked noticeably slower and less fre-

613   quently, while inter-leg coordination and kinematics seemed to be very similar to w^{1118}. It is

614 important to note that these low octopamine levels might only explain reduced walking speed
615 in w^{1118}, Tbh^{nM18}. While w^{1118} also has reduced levels of other biogenic amines like dopamine
616 and serotonin (Sitaraman, 2008), its octopamine levels are similar or only very slightly re-
617 duced (Sitaraman et al., 2008; Yarali et al., 2009). Modifying the octopamine levels might be
618 useful in future studies in order to specifically modulate the walking behavior in *Drosophila*
619 mainly with regard to movement speed.

620

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755

Figure Legends:

Figure 1: A: Schematic of the experimental setup. Flies walked spontaneously back and forth on a walkway along the direction of the red arrow. Walks were recorded through a 20mm wide window simultaneously from one side and from below (a: acrylic glass, inside coated with a layer of Fluon to prevent the flies from scaling the glass; b: 5 mm wide transparent walkway; c: camera viewpoint; d: camera field of view, free of Fluon; e: glass prism, providing a ventral view of the walkway). B: Exemplary lateral view of a male *Drosophila*, wt^{CS}, during one of the recorded walks. C: Ventral view of the same fly in the same video frame. Tips of the tarsi are marked with colored circles. (R1, R2, R3: right front, middle, and hind leg; L1, L2, L3: left front, middle, and hind leg). Red and green arrows indicate origin and orientation of the body coordinate system.

Figure 2: Walking parameters of wt^{CS}. A: Footfall pattern of all six legs during (i) 0.5 s of one faster trial, (ii) 0.5 s of one slower trial and (iii) walking speed of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (BL = body lengths; R1, R2, R3: right front, middle, and hind leg; L1, L2, L3: left front, middle, and hind leg). Black bars indicate swing phase, white bars indicate stance phase, magenta lines indicate onset and end of complete step cycles in the faster trial, green bars in the slower trial, respectively. Shaded areas highlight coordination patterns of interest (see main text). B: Average stance trajectories of all legs of all trials in relative body coordinates. C: Phase plots of swing onset of all legs with respect to the left front leg (blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector, length of mean vector indicates variance).

Figure 3: Evaluation of leg stepping parameters of wt^{CS}. A: Swing duration as a function of cycle period (black: data from all trials; magenta: data from trial in 2Ai; green: data from trial in 2Aii). B: Walking speed as a function of cycle period (same color coding as in A). C: Step amplitude as a function of cycle period (same color coding as in A). D: Walking speed as a function of step amplitude (same color coding as in A). Each panel contains a regression line for the complete data set (black) as well as several further regression lines (gray) each of which is associated with one trial (n = 15).

787 **Figure 4:** Walking parameters of wt^{Berlin}. A: Footfall pattern of all six legs during (i) 0.5 s of
788 one faster trial, (ii) 0.5s of one slower trial and (iii) walking speed of the body during the 0.5 s
789 of the trials shown in Ai (magenta graph) and 2Aii (green graph) (BL = body lengths; R1, R2,
790 R3: right front, middle, and hind leg; L1, L2, L3: left front, middle, and hind leg). Black bars
791 indicate swing phase, white bars indicate stance phase, magenta lines indicate onset and end
792 of complete step cycles in the faster trial, green bars in the slower trial, respectively. Shaded
793 areas highlight coordination patterns of interest (see main text). B: Average stance trajectories
794 of all legs of all trials in relative body coordinates. C: Phase plots of swing onset of all legs
795 with respect to the left front leg (blue: data from all trials; magenta: data from the Ai; green:
796 data from Aii; black line: mean vector, length of mean vector indicates variance). D: Cycle
797 period as a function of walking speed (black: data from all trials; magenta: data from Ai;
798 green: data from Aii). E: Step amplitude as a function of walking speed (black: data from all
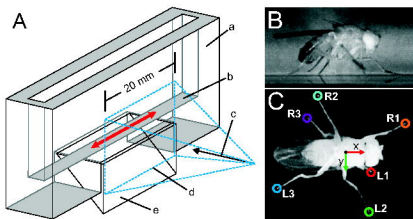799 trials; magenta: data from Ai; green: data from Aii).

800

801 **Figure 5:** Walking parameters of w^{1118}. A: Footfall pattern of all six legs during (i) 0.5 s of
802 one faster trial, (ii) 0.5 s of one slower trial and (iii) walking speed of the body during the 0.5
803 s of the trials shown in Ai (magenta graph) and Aii (green graph) (BL = body lengths; R1, R2,
804 R3: right front, middle, and hind leg; L1, L2, L3: left front, middle, and hind leg). Black bars
805 indicate swing phase, white bars indicate stance phase, magenta lines indicate onset and end
806 of complete step cycles in the faster trial, green bars in the slower trial, respectively. Shaded
807 areas highlight coordination patterns of interest (see main text). B: Average stance trajectories
808 of all legs of all trials in relative body coordinates. C: Phase plots of swing onset of all legs
809 with respect to the left front leg (blue: data from all trials; magenta: data from Ai; green: data
810 from Aii; black line: mean vector, length of mean vector indicates variance). D: Cycle period
811 as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data
812 from Aii). E: Step amplitude as a function of walking speed (black: data from all trials; ma-
813 genta: data from Ai; green: data from Aii).

814

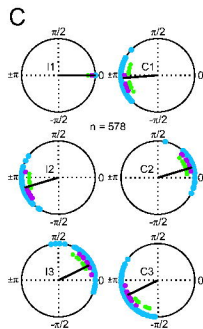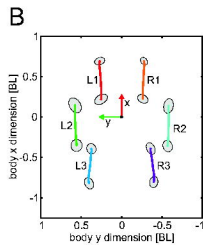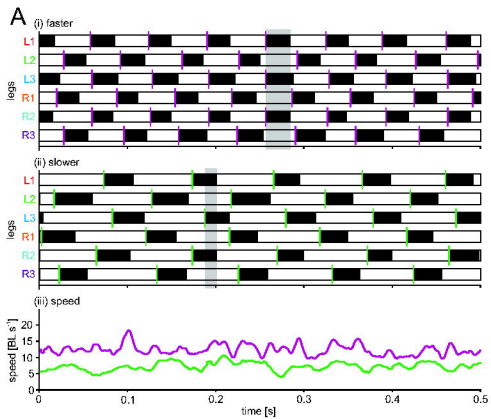815 **Figure 6:** Walking parameters of w^{1118}, Tbh^{nM18}. A: Footfall pattern of all six legs during (i)
816 0.5 s of one faster trial, (ii) 0.5 s of one slower trial and (iii) walking speed of the body during
817 the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (BL = body lengths;
818 R1, R2, R3: right front, middle, and hind leg; L1, L2, L3: left front, middle, and hind leg).

819   Black bars indicate swing phase, white bars indicate stance phase, magenta lines indicate on-

820   set and end of complete step cycles in the faster trial, green bars in the slower trial, respective-

821   ly. Shaded areas highlight coordination patterns of interest (see main text). B: Average stance

822   trajectories of all legs of all trials in relative body coordinates. C: Phase plots of swing onset

823   of all legs with respect to the left front leg (blue: data from all trials; magenta: data from Ai;

824   green: data from Aii; black line: mean vector, length of mean vector indicates variance). D:

825   Cycle period as a function of walking speed (black: data from all trials; magenta: data from

826   Ai; green: data from Aii). E: Step amplitude as a function of walking speed (black: data from

827   all trials; magenta: data from Ai; green: data from Aii).

828

829   **Figure 7:** Analysis of inter-leg coordination. A: Relative frequency of tripod, tetrapod and

830   undefined coordination in the four different strains (BL = body length, for definition of coor-

831   dination types see main text). B: Relative frequency of tripod, tetrapod and undefined coordi-

832   nation at slow walking speeds (< 5 BL s^{-1}), medium speeds (5 to 10 BL s^{-1}) and high speeds

833   (> 10 BL s^{-1}). C to F: Tripod coordination strength (TCS, for definition see main text) as a

834   function of walking speed for the different strains. C: wt^{CS}, D: wt^{Berlin}, E: w^{1118}, and F: w^{1118},

835   Tbh^{nM18}. G: Five exemplary footfall patterns with TCS of 0.8, 0.6, 0.4, 0.2, and 0.1 taken from

836   footfall patterns of five different flies. Shaded areas highlight the concurrent overlap of swing

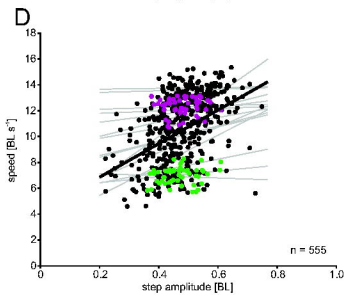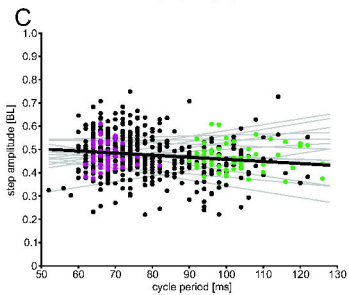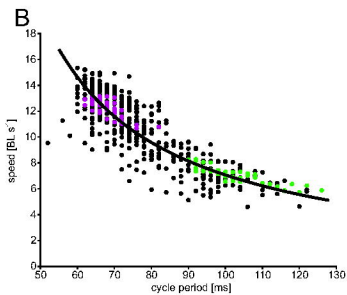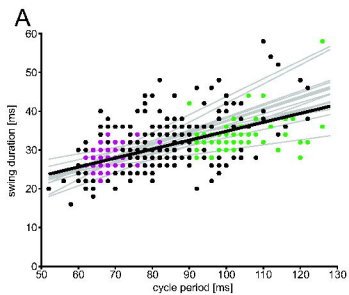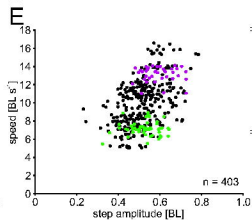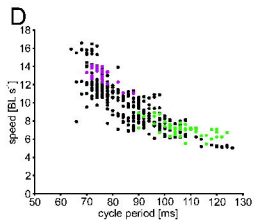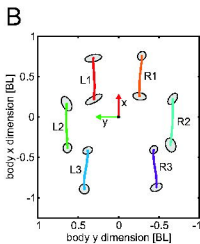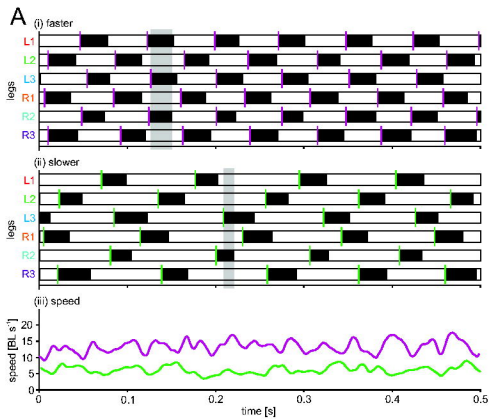837   phases in the legs of one tripod group.

838

839   **Figure 8:** Walking parameters of wt^{CS} after removal of one hind leg. A: Footfall pattern of all

840   six legs during (i) 0.5 s of one faster trial, (ii) 0.5 s of one slower trial and (iii) walking speed

841   of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph)

842   (BL = body lengths; R1, R2, R3: right front, middle, and hind leg; L1, L2, L3: left front, mid-

843   dle, and hind leg). Black bars indicate swing phase, white bars indicate stance phase, magenta

844   lines indicate onset and end of complete step cycles in the faster trial, green bars in the slower

845   trial, respectively. B: Average stance trajectories of all legs of all trials in relative body coor-

846   dinates. Black arrows indicate the shifts of AEP and PEP (cf. Fig. 2B). C: Phase plots of

847   swing onset of all legs with respect to the left front leg (blue: data from all trials; magenta:

848   data from Aii; green: data from Aii; black line: mean vector, length of mean vector indicates

849   variance). D: Cycle period as a function of walking speed (black: data from all trials; magen-

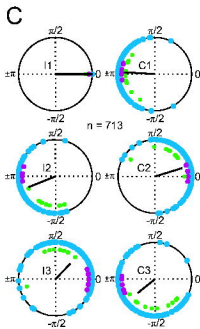850   ta: data from Ai; green: data from Aii). For comparison, gray inset shows rescaled data from
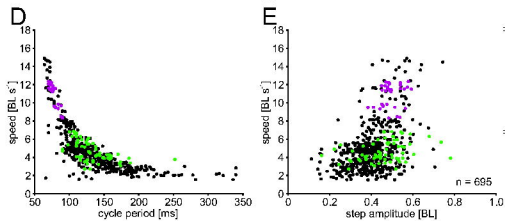
851   Fig. 3B. E: Step amplitude as a function of walking speed (black: data from all trials; magen-

852   ta: data from Ai; green: data from Aii).

A

20 mm

a

b

c

d

e

B

C

R2

R3

R1

x

y

L1

L3

L2

A

(i) faster

L1
L2
L3
R1
R2
R3

(ii) slower

L1
L2
L3
R1
R2
R3

(iii) speed

B

C

D

E

A

(i) faster

L1	
L2	
L3	
R1	
R2	
R3	

legs

(ii) slower

	*	**
L1		
L2		
L3		
R1		
R2		
R3		

legs

(iii) speed

time [s]

B

C

D

E

A

(i) faster

legs

L1
L2
L3
R1
R2
R3

(ii) slower

legs

L1
L2
L3
R1
R2
R3

    *   **

(iii) speed

speed [BL s⁻¹]

10
6
2

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7   0.8   0.9
                        time [s]

B

body x dimension [BL]

1
0.5
0
-0.5
-1

1   0.5   0   -0.5   -1
        body y dimension [BL]

L1   R1

L2   R2

L3   R3

C

I1   C1

n = 713

I2   C2

I3   C3

D

speed [BL s⁻¹]

10
8
6
4
2

50   100 150 200 250 300 350
        cycle period [ms]

E

speed [BL s⁻¹]

10
8
6
4
2

0.2   0.4   0.6   0.8   1.0
        step amplitude [BL]

n = 681

A
y-axis: rel. freq. for resp. coordination (0 to 1.0)
Legend: undefined, tetrapod, tripod
x-axis: fly strain
Bars labeled: wt^{cs}, wt^{berlin}, wt^{rria}, wt^{rria}; Tbh^{nM18}

B
y-axis: rel. freq. for resp. coordination (0 to 1.0)
Legend: undefined, tetrapod, tripod
x-axis: speed range [BL s⁻¹]
Bars: <5 (n=1099), 5-10 (n=755), >10 (n=636)

C
wt^{cs}
y-axis: TCS [a.u.]
x-axis: speed [BL s⁻¹]
n = 202

D
wt^{berlin}
y-axis: TCS [a.u.]
x-axis: speed [BL s⁻¹]
n = 140

E
w^{rria}
y-axis: TCS [a.u.]
x-axis: speed [BL s⁻¹]
n = 163

F
w^{rria}; Tbh^{nM18}
y-axis: TCS [a.u.]
x-axis: speed [BL s⁻¹]
n = 137

G
legs: L1, L2, L3, R1, R2, R3
TCS [a.u.]: 0.8, 0.6, 0.4, 0.2, 0.1

Table 1

	Male				Female	
	weight [mg]	N	size [mm]	N	weight [mg]	N
wt^{CS}	0.70	29	2.06 ± 0.08	6	1.17	29
wt^{Berlin}	0.86	22	2.12 ± 0.01	3	1.32	22
w^{1118}	0.70	27	2.09 ± 0.08	5	1.05	35
w^{1118}, TbH^{nM18}	0.71	12	2.07 ± 0.03	5	1.21	9