

1 **Miniature linear and split-belt treadmills reveal mechanisms of adaptive motor control in** 2 **walking *Drosophila***

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6 **Abstract**

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9 To navigate complex environments, walking animals must detect and overcome unexpected perturbations. One technical
10 challenge when investigating adaptive locomotion is measuring behavioral responses to precise perturbations during
11 naturalistic walking; another is that manipulating neural activity in sensorimotor circuits often reduces spontaneous
12 locomotion. To overcome these obstacles, we introduce miniature treadmill systems for coercing locomotion and tracking
13 3D kinematics of walking *Drosophila*. By systematically comparing walking in three experimental setups, we show that
14 flies compelled to walk on the linear treadmill have similar stepping kinematics to freely walking flies, while kinematics
15 of tethered walking flies are subtly different. Genetically silencing mechanosensory neurons alters step kinematics of flies
16 walking on the linear treadmill across all speeds, while inter-leg coordination remains intact. We also found that flies can
17 maintain a forward heading on a split-belt treadmill by adapting the step distance of their middle legs. Overall, these new
18 insights demonstrate the utility of miniature treadmills for studying insect locomotion.

19 **Introduction**

20
21
22 Many animals rely on legged locomotion to move through diverse and unpredictable environments. To achieve behavioral
23 goals in the face of this unpredictability, nervous systems have evolved to control the body in an adaptive manner. Animals
24 as diverse as cockroaches (Couzin-Fuchs et al., 2015) and humans (Eng et al., 1994) use similar strategies to recover from
25 unexpected motor outcomes (e.g., tripping), by rapidly adjusting coordination within and between legs. Understanding
26 how sensorimotor neural circuits detect perturbations and generate adaptive motor responses remains a fundamental
27 question in neuroscience (Tuthill and Wilson, 2016).

28 A common method to investigate the neural control of movement is to perturb neurons within candidate circuits and
29 measure the effect on an animal's behavior. For example, past efforts to identify sensorimotor circuits have relied on
30 anatomical lesions (Andersson and Grillner, 1983; Dietz, 2002). While these methods revealed regions of the nervous
31 system that are important for proprioceptive sensing and motor control, they lack cell-type specificity and produce wide-
32 ranging behavioral effects. More recently, genetic methods have enabled targeted manipulation of specific cell-types that
33 sense or control the body. However, these experimental manipulations often decrease the probability and vigor of
34 spontaneous behavior. For example, the loss of feedback from mechanosensory neurons in both mammals (Chesler et al.,
35 2016) and insects (Mendes et al., 2013) reduces walking speed and probability. This confound has made it challenging to
36 dissect the relative roles of mechanosensory feedback vs. feedforward motor commands across different walking speeds
37 (Bidaye et al., 2018).

38 One strategy to overcome this reduction in spontaneous behavior is to compel animals to walk, for example by placing
39 them on an actuated treadmill. Treadmills have been historically used to study the neural basis of motor control and
40 adaptive locomotion in both vertebrates (Belanger et al., 1996; Hasan and Stuart, 1988; Wetzel and Stuart, 1976) and
41 invertebrates (Dean and Wendler, 1983; Foth and Bässler, 1985; Foth and Graham, 1983; Herreid and Full, 1984; Herreid

42 et al., 1981; Watson and Ritzmann, 1998, 1997). For instance, treadmills have been used to drive walking in cats (Whelan,
43 1996) and rodents (Fujiki et al., 2018), leading to important insights into spinal circuits for adaptive locomotor control.

44 Because treadmills are externally controlled, they can also deliver calibrated mechanical perturbations to walking animals.
45 Previous work showed that cats walking on a treadmill learn to increase the height of their steps to avoid being smacked
46 by a paddle (McVea and Pearson, 2007). Split-belt treadmills, which consist of two independently controlled belts, are
47 another classic paradigm to investigate walking coordination and motor adaptation. Both humans (Kambic et al., 2023;
48 Reisman et al., 2007, 2005) and mice (Darmohray et al., 2019) learn new inter-leg coordination patterns when their left
49 and right legs are driven at different speeds on a split-belt treadmill. This phenomenon of split-belt adaptation has been
50 used to investigate behavioral and neural mechanisms of adaptive locomotion (Torres-Oviedo et al., 2011). A final
51 advantage of treadmills is that they enable the study of locomotion within a confined space, which is important for
52 capturing body kinematics or physiological signals from neurons and muscles.

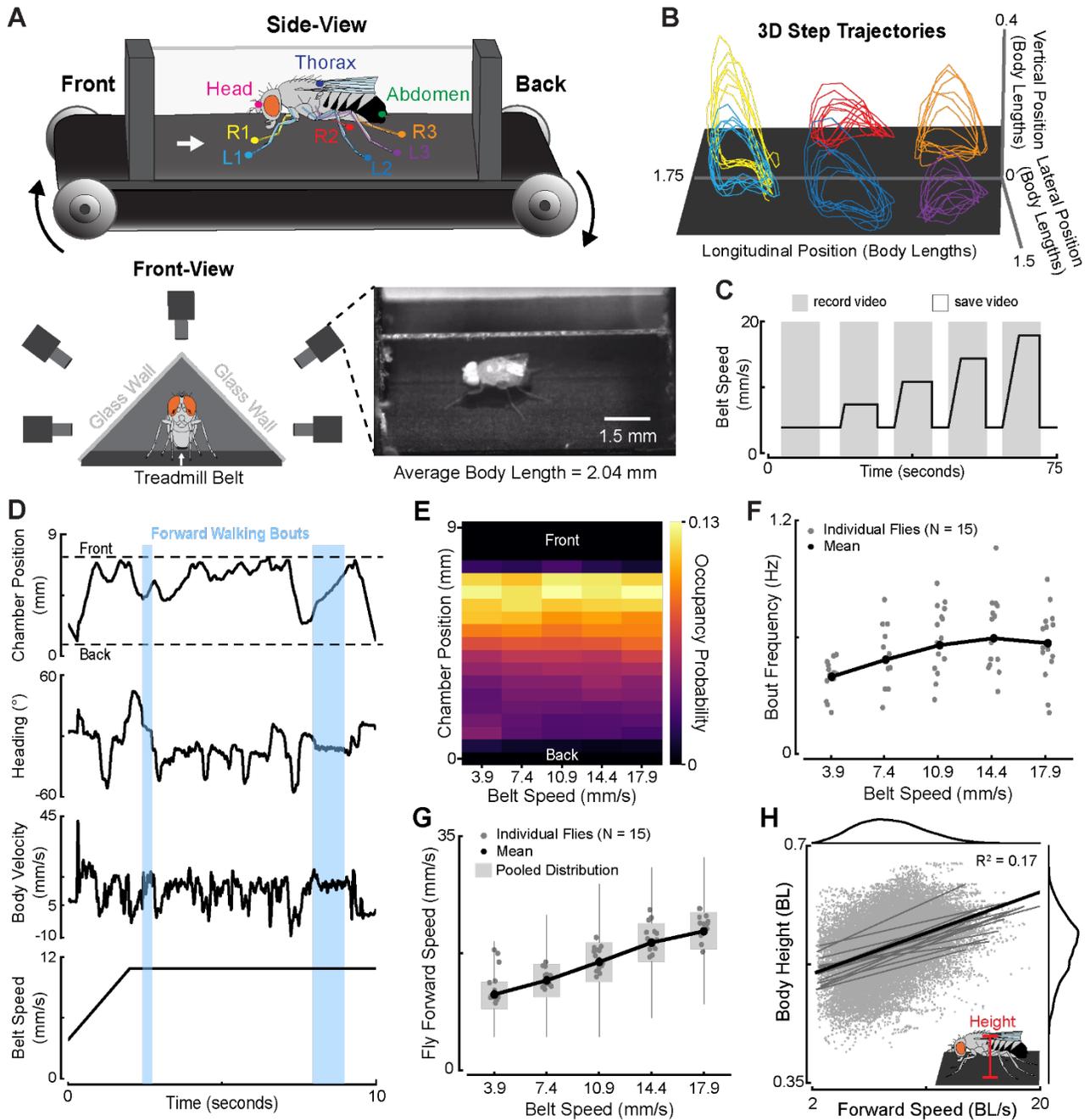
53 In recent years, the fruit fly, *Drosophila melanogaster*, has emerged as an important model system for studying
54 proprioceptive sensing and adaptive locomotion (Agrawal et al., 2020; Chen et al., 2021; Chockley et al., 2022; Isakov et
55 al., 2016; Mamiya et al., 2023, 2018; Mendes et al., 2013). The key advantages of the fly are a compact, fully-mapped
56 nervous system (Anthony Azevedo et al., 2022; Shin-ya Takemura et al., 2023; Sven Dorkenwald et al., 2023) and cell-
57 type specific tools for targeted genetic manipulations. Fly locomotion has been previously studied in tethered animals
58 walking on a floating sphere (Berendes et al., 2016; Buchner, 1976; Creamer et al., 2018; Götz and Wenking, 1973), or
59 in freely walking animals constrained to a behavioral arena (DeAngelis et al., 2019; Fujiwara et al., 2022; Mendes et al.,
60 2013; Simon and Dickinson, 2010; Strauss and Heisenberg, 1990; York et al., 2022). One advantage of the tethered
61 preparation is that it enables 3D tracking of the fly's body and legs (Günel et al., 2019; Karashchuk et al., 2021), which
62 has not previously been possible in freely walking flies. It is also possible to record neural signals of tethered flies using
63 optical imaging (Seelig et al., 2010) or electrophysiology (Fujiwara et al., 2017; Turner-Evans et al., 2017). However, one
64 disadvantage of studying locomotion in tethered flies is that their posture is constrained, and normal ground reaction
65 forces may be disrupted, which could affect walking kinematics.

66 To bridge these established methodologies, we introduce a new linear treadmill system that enables long-term 3D tracking
67 of walking *Drosophila*. We systematically compare walking kinematics on the linear treadmill to those of freely walking
68 and tethered flies. We then use the linear treadmill to investigate step kinematics and inter-leg coordination following
69 genetic silencing of mechanosensory neurons. Last, we introduce a novel split-belt treadmill for fruit flies, which we use
70 to uncover behavioral mechanisms of adaptive motor control. We provide open-source software and hardware designs for
71 these treadmill systems as resources for the community.

72 **Results**

73 74 **A linear treadmill for tracking 3D walking behavior in *Drosophila***

75
76 We engineered a miniature linear treadmill system to measure 3D walking kinematics in flies (**Figure 1A**). A fly was
77 constrained to walk on the treadmill within a transparent chamber. Its wings were trimmed to discourage flight initiation.
78 We used 5 high-speed video cameras (180 fps) to record fly walking behavior. To test the treadmill system, we used wild-
79 type Berlin flies that had a body length of 2.04 ± 0.10 mm. We used DeepLabCut (Mathis et al., 2018) and Anipose
80 (Karashchuk et al., 2021) to track and extract 3D kinematics of the fly leg tips and body (**Figure 1B**).



82

83 **Figure 1. The linear treadmill controls locomotor speed and enables tracking of 3D kinematics in walking *Drosophila*.** (A) Schematic of the
 84 linear treadmill setup. Key points associated with the head, thorax, abdomen, and each leg tip were tracked in 3D. Flies were recorded with 5 high-
 85 speed cameras as they were driven to walk on the treadmill within an attic-shaped chamber. Flies had an average body length of 2.04 ± 0.10 mm.
 86 Note that the schematic flies are not to scale. (B) 3D leg tip (tarsi) trajectories during forward walking. Colors correspond to the labels in (A). (C)
 87 Flies were compelled to walk at different speeds by moving the treadmill belt at one of 5 steady-state speeds. The belt gradually increased in speed
 88 to reach steady-state speeds greater than 5 mm/s. Each speed was presented to a fly 10 times, with speeds randomly interleaved. Each trial was
 89 composed of a 10 s recording period (gray) followed by a 5s saving period (white) in which the belt speed returned to the baseline speed. (D) A
 90 representative trial of a fly walking on the treadmill with a belt speed of 10.9 mm/s. The position of the fly along the chamber (measured at the
 91 thorax), the fly's heading angle and body velocity, and the belt speed profile are plotted from top to bottom, respectively. Forward walking bouts are
 92 highlighted by the light blue shaded regions. Forward walking bouts were classified as periods lasting at least 200 ms where the fly walked in the
 93 middle of the chamber (see Methods), had a heading angle between -15 to 15 degrees with respect to the front of the chamber, and had a body
 94 velocity greater than 5 mm/s. (E) Flies walked between the middle and front of the chamber across all belt speeds. (F) Flies increased their walking
 95 bout frequency as the belt speed increased. Gray dots are the mean frequency of individual flies, while the black line denotes the mean across all
 96 flies. (G) Flies increased their forward walking speed as the belt speed increased. Box plots show the distribution of pooled data. Black line connects

97 the median forward speed across all flies and belt speeds. (H) Flies increased their body height (vertical distance between the thorax and ground) as
98 they walked faster (in body lengths per second, or BL/s). Black: population fit; Gray line: individual fit. See also Video 1.

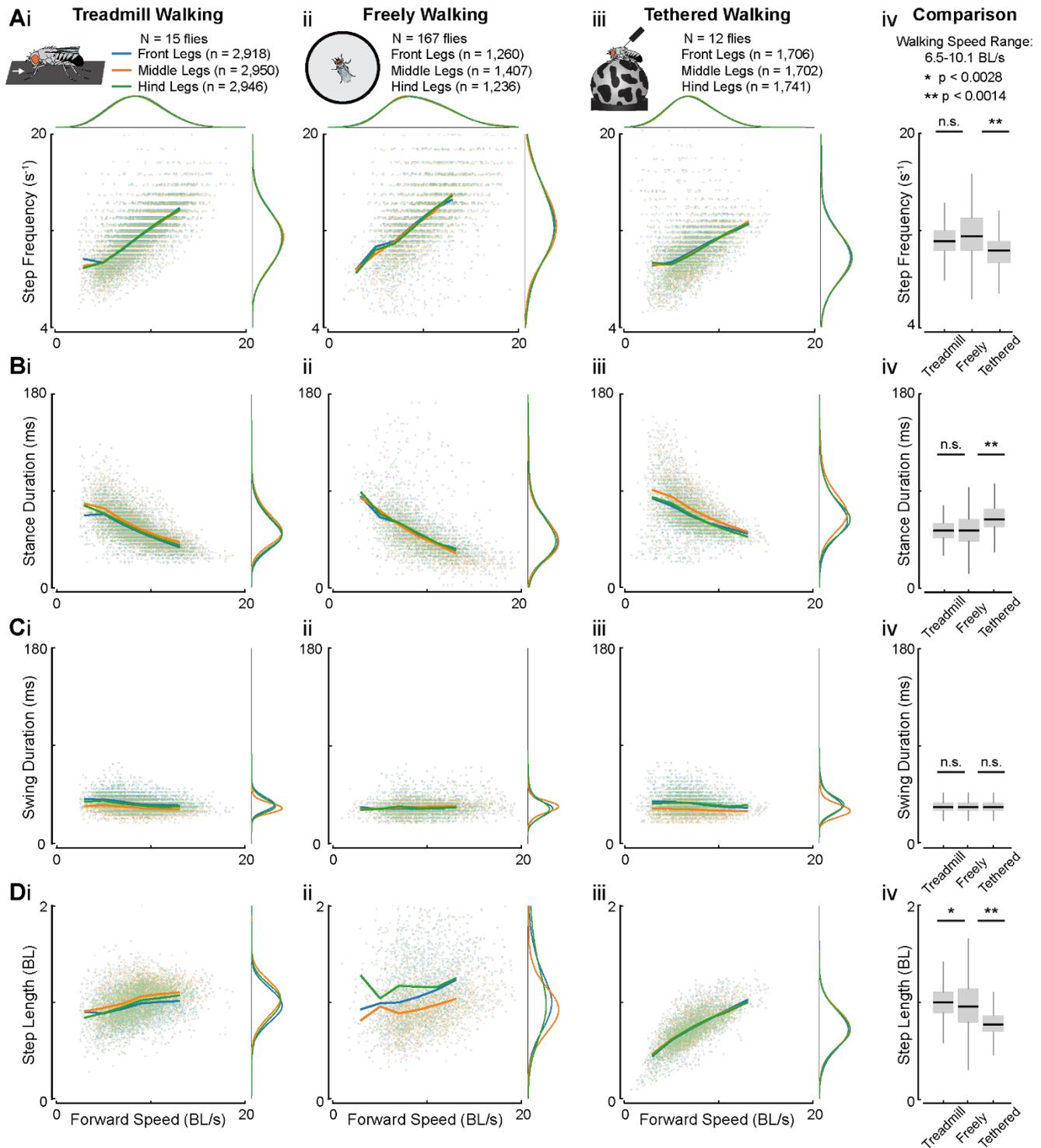
99
100 We first measured the 3D kinematics of flies as they walked across a range of belt driving speeds (**Figure 1C**). As
101 illustrated by a representative fly walking at an intermediate belt speed, flies on the treadmill displayed forward walking
102 bouts, standing, and other lateral movements (**Figure 1D, Video 1**). Flies traversed the entirety of the chamber but spent
103 most of their time toward the front (**Figure 1E**). They typically walked in short bursts, accelerating toward the front of
104 the chamber, where they would walk for a short period, after which they would ride the belt to the back of the chamber;
105 contact with the back of the chamber would then initiate another walking bout. Flies increased their walking bout
106 frequency as the belt speed increased, as measured by the frequency at which they crossed the middle of the chamber from
107 the rear (**Figure 1F**). The sporadic structure of fly treadmill walking is similar to that previously reported for freely
108 walking flies (Sorribes et al., 2011). Flies on the treadmill also consistently increased their walking speed to keep up with
109 the treadmill's belt driving speed (**Figure 1G**). At the extremes, flies on the treadmill were able to sustain walking at a
110 max belt speed of 40 mm/s (**Video 2**) and surpassed an instantaneous walking velocity of 50 mm/s (**Video 3**), which is
111 the fastest walking speed ever recorded for *Drosophila melanogaster*. By driving flies to walk across a range of speeds
112 while recording their 3D kinematics, we found that flies increased their body height as they walked faster by 0.007 BL
113 per BL/s (**Figure 1H**).

114 In summary, our engineered treadmill makes it possible to force individual flies to walk for long periods (up to 1 hour)
115 while tracking 3D body and leg kinematics. Flies remained upright 97% of the time on the treadmill and spent an average
116 of 54% of the time walking, enabling collection of large amounts of useful kinematic data from each animal. Using 3D
117 tracking, we discovered that flies elevate their body as they walked faster, a relationship that has been shown in other
118 walking animals, from cockroaches (Full and Tu, 1991) to humans (Struzik et al., 2021), but had not been previously
119 described in *Drosophila*.

120 **Comparison of walking kinematics between treadmill, freely, and tethered walking flies**

121
122 Multiple previous studies have quantified step kinematics of freely walking flies (Chun et al., 2021; DeAngelis et al.,
123 2019; Fujiwara et al., 2022; Mendes et al., 2013; Strauss and Heisenberg, 1990; Szczecinski et al., 2018; Wosnitza et al.,
124 2013). To compare treadmill walking to freely walking kinematics, we tracked and analyzed a new dataset of wild-type
125 flies walking in a circular arena. We focused our kinematic analyses on forward walking bouts, which were identified
126 from fly heading direction and body velocity. Note that more detailed definitions of all kinematic parameters are included
127 in the Methods.

128 We found that the key relationships between stepping kinematics and forward walking speed were similar between flies
129 walking on the treadmill (**Figure 2i**) and freely walking flies (**Figure 2ii, Video 4**). Flies in both setups increased step
130 frequency as they walked faster (**Figure 2Ai-ii**). Correspondingly, stance duration was inversely related to walking speed
131 for flies in both setups (**Figure 2Bi-ii**). However, swing duration remained fairly constant across speeds and was of a
132 similar magnitude for treadmill and freely walking flies (**Figure 2Ci-ii**). Step length, the distance between the footfalls of
133 each leg, was also comparable between treadmill and freely walking flies (**Figure 2Di-ii**). Flies in both setups had similar
134 increases in step length with increasing walking speed. The largest difference between treadmill and freely walking flies
135 was that the step kinematics of freely walking flies were more variable (e.g., step frequency within the walking speed
136 range of 6.5-10.1 BL/s: freely walking $\sigma^2 = 5.18 \text{ s}^{-1}$; treadmill walking $\sigma^2 = 1.98 \text{ s}^{-1}$). This could be because the treadmill
137 has a single driving axis, which may result in straighter walking bouts. The step kinematics of flies in our freely walking
138 dataset were also consistent with prior work (DeAngelis et al., 2019; Mendes et al., 2013; Strauss and Heisenberg, 1990;
139 Szczecinski et al., 2018; Wosnitza et al., 2013), even though fly strain and sex were sometimes different in those studies
140 (**Table S1**). Overall, the step kinematics of flies walking on the treadmill were similar to freely walking flies.



141

142 **Figure 2. Step kinematics are similar across treadmill and freely walking flies, but different from tethered flies.** (A) Step frequency of the
 143 front (blue), middle (orange), and hind (green) legs as a function of forward walking speed for treadmill (i), freely walking (ii), and tethered flies
 144 (iii). Distributions (iv; gray box plots) that combine step frequency across leg pairs for treadmill, freely, and tethered walking flies over an overlapping
 145 and dense range of walking speeds (6.5-10.1 BL/s). (B) Stance duration as a function of forward walking speed for flies in the different setups. (C)
 146 Swing duration as a function of forward walking speed. (D) Step length as a function of forward walking speed. In A-D, lines are speed-binned
 147 averages (2 BL/s bins between 3 and 13 BL/s) for each kinematic parameter and leg pair. Step frequency, stance duration, and swing duration (A-C)
 148 were computed on interpolated data for treadmill (i; 180 to 300 fps) and freely walking flies (ii; 150 to 300 fps) to enable the comparison to tethered
 149 flies. Distributions associated with each leg were visually offset from each other for presentation in A-C. In A-C iv, Chi-squared test for goodness
 150 of fit with a Bonferroni correction of 18 was used to statistically compare the distributions across the different walking setups. In D, t-test with a

151 Bonferroni correction of 18 was used to compare the distributions. N: number of flies; n: number of steps. See also Figure S1 for comparisons of the
152 similarity between the setups with respect to step kinematics, leg pairs, and walking speeds.

153

154 Having developed a framework for comparing walking kinematics across experimental setups, we took the opportunity
155 to extend our analysis to a third setup: tethered flies walking on a floating sphere (**Figure 2iii, Video 5**). The “fly-on-a-
156 ball” setup is commonly used in our lab (Agrawal et al., 2020; Azevedo et al., 2020; Karashchuk et al., 2021) and many
157 others (e.g., Berendes et al., 2016; Creamer et al., 2018; Seelig et al., 2010), but leg kinematics of tethered and freely
158 walking flies have not been systematically compared.

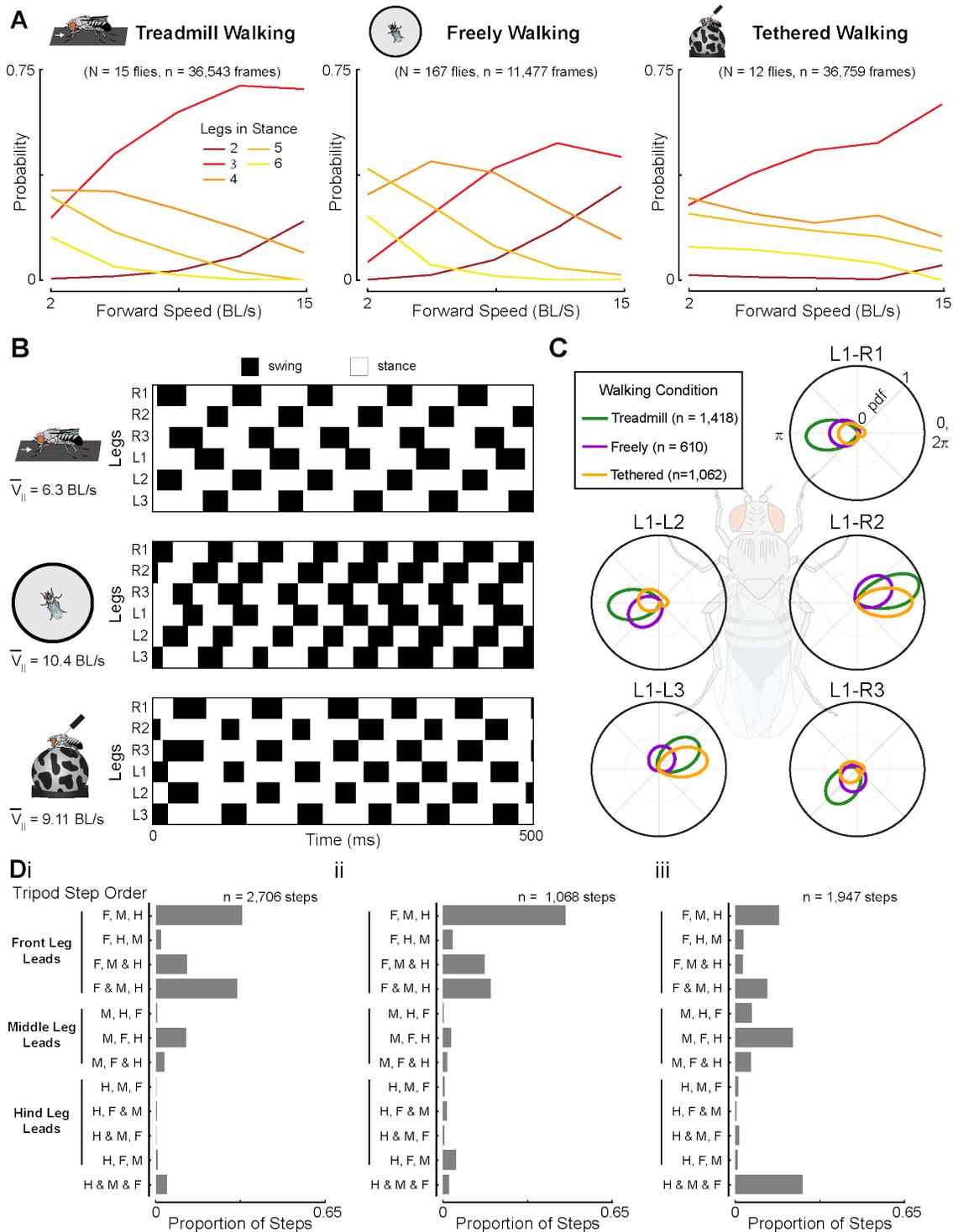
159 In general, we found that the relationships between stepping kinematics and walking speed were similar across all three
160 setups. However, tethered flies differed from untethered ones (i.e. treadmill and freely walking flies) in several key
161 aspects. First, tethered flies didn’t reach the faster walking speeds displayed by untethered flies. Step frequency of tethered
162 flies was also significantly lower than that of untethered flies (**Figure 2Aiv**), whereas stance duration was significantly
163 longer (**Figure 2Biv**). There was no significant difference in swing duration across all setups (**Figure 2Civ**). Therefore,
164 the reduced step frequency in tethered flies resulted from longer stance durations across walking speeds. Tethered flies
165 also had a significantly lower step length than untethered flies (**Figure 2Div**). However, the step length of tethered flies
166 was more correlated with walking speed (tethered: $r = 0.41$; freely: $r = 0.05$; treadmill: $r = 0.22$). We also compared step
167 kinematics between the different setups across leg pairs and walking speeds (**Table S1**). We found that there was a greater
168 similarity in the step kinematics between treadmill and freely walking flies, especially at fast walking speeds. These
169 differences in step kinematics and walking speed ranges between tethered and untethered flies may be because the tethered
170 flies are walking on a spherical surface and/or because their body weight is supported by a rigid tether.

171 We next compared inter-leg coordination across the three setups. We quantified the number of legs that were in the stance
172 phase of the step cycle at each point in time, which remains constant for idealized coordination patterns, such as the tripod
173 coordination pattern where 3 legs are in stance (DeAngelis et al., 2019). We found that the probability of having a specific
174 number of legs in stance was similar between tethered and untethered flies. Specifically, flies in all three setups showed
175 an increased probability of having 3 legs in stance as walking speed increased (**Figure 3A**). This is consistent with prior
176 work showing that freely walking flies are more likely to use a canonical tripod coordination pattern at higher speeds
177 (DeAngelis et al., 2019; Mendes et al., 2013; Pereira et al., 2019; Wosnitza et al., 2013).

178 Given that flies across all three setups had similar speed-dependent changes in inter-leg coordination, we next asked if the
179 stepping pattern underlying inter-leg coordination was different between tethered and untethered flies. We therefore
180 examined the relative swing and stance relationships across legs. We observed that the order in which legs entered stance
181 conformed to the so-called *Cruse Control* rules for both tethered and untethered flies (Bidaye et al., 2018; Cruse, 1990,
182 1985). This is illustrated by the anterior progression of ipsilateral leg stepping (diagonal black stripes in **Figure 3B**). We
183 also examined the relative phase relationships between the left front leg and all other legs across the different setups. we
184 computed phase by determining when a leg entered stance within the left front leg’s step cycle. We found that the relative
185 phase relationships across all legs were similar for treadmill, freely, and tethered walking flies (**Figure 3C**). However, the
186 phase relationships of the ipsilateral front and hind legs and contralateral middle leg that make up the canonical tripod
187 were more coupled for tethered flies.

188 Finally, we looked at the order in which legs within a tripod entered stance with respect to the left front leg’s step cycle.
189 We found differences in the stance onset order between tethered and untethered flies. For example, the front leg within a
190 tripod was usually the first leg to contact the ground for treadmill (**Figure 3Di**) and freely walking flies (**Figure 3Dii**),
191 whereas the stance order was more variable for tethered flies, having more instances of the middle leg entering stance first
192 (**Figure 3Diii**). Tethered flies also had more instances where all legs within a tripod entered stance at the same time, called
193 an “ideal tripod”. Therefore, the inter-leg phase coupling of tethered flies was stronger than that of untethered flies. One
194 explanation could be that the added stability from the tether induces a more tightly coupled inter-leg coordination pattern.

195 In summary, we found that although step kinematics were subtly different between tethered and untethered flies (**Figure**
 196 **2**), inter-leg coordination was broadly similar (**Figure 3**).



197
 198

199 **Figure 3. Subtle differences in inter-leg coordination between tethered and untethered flies.** (A) Probability of treadmill, freely walking, and
 200 tethered flies exhibiting 2-6 legs in stance across forward walking speeds. Lines are speed-binned averages of the probability of a certain number of
 201 legs in stance (3.2 BL/s bins between 2 and 15 BL/s). N: number of flies; n: number of camera frames. (B) Representative plots of the swing and
 202 stance phases of each leg across all setups. Black: swing; White: stance. (C) Polar plots of the relative phase between the left front leg and each other
 203 leg for flies in each setup. Kernel density estimations were used to determine the probability density functions. n: number of phase comparisons. (D)

204 Bar plot of the proportion of steps attributed to each step order combination of the legs with a tripod for treadmill (i), freely (ii), and tethered (iii)
205 walking flies. The leading leg is indicated on the left of the graph. ‘&’ denotes legs contacting the ground at the same time. n: number of steps.

206

207 **Silencing mechanosensory feedback alters step kinematics across walking speeds but not inter-leg coordination**

208

209 One of our motivations to develop a linear treadmill was to investigate the role of mechanosensory feedback in fly
210 locomotion. This has historically been challenging, because silencing mechanosensory neurons typically leads to a
211 reduction in locomotor probability and speed in flies (Mendes et al., 2013) and other animals (Chesler et al., 2016; Dietz,
212 2002). To test whether flies lacking mechanosensory feedback will walk on the linear treadmill, we genetically silenced
213 chordotonal neurons (*iav-GAL4 > UAS-kir2.1*), which are found at multiple joints throughout the fly’s body, including
214 in the femur and tibia (**Figure 4A**). As expected, silencing chordotonal neurons drastically reduced locomotion in freely
215 walking flies (**Figure 4B**). However, flies lacking chordotonal feedback walked a greater proportion of the time and across
216 a wider range of speeds when driven to walk on the linear treadmill (**Figure 4C**). Silencing chordotonal neurons altered
217 the structure of fly locomotion on the treadmill compared to genetically-matched controls. In particular, flies with silenced
218 chordotonal neurons spent more time towards the back of the chamber at faster belt speeds (**Figure 4D**) and exhibited a
219 lower bout frequency (**Figure 4E**).

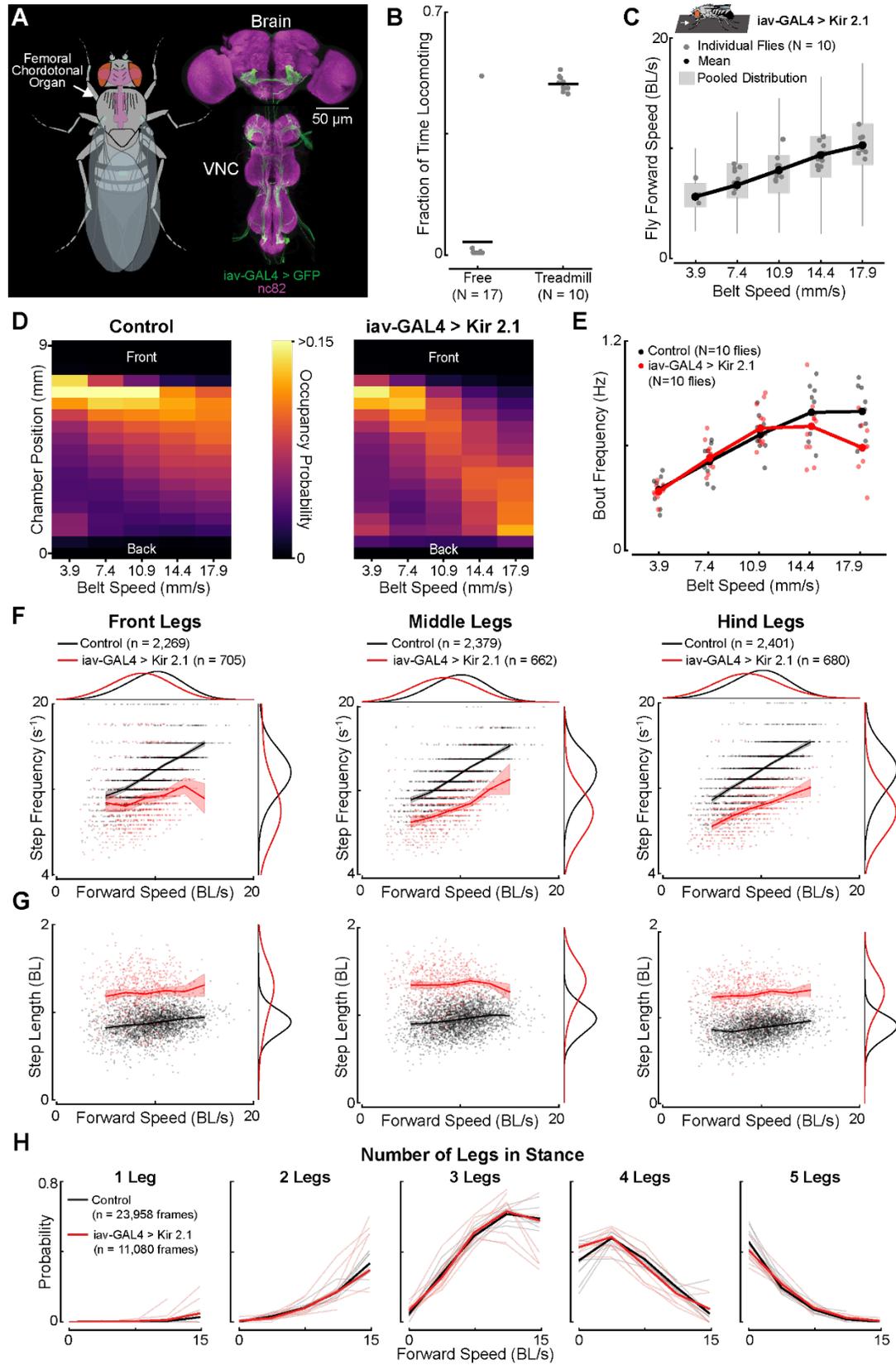
220 The leg movements of flies with silenced chordotonal neurons were noticeably different. Qualitatively, the legs appeared
221 less rigid and moved with less precision (**Video 6**). Therefore, we next analyzed the impact of silencing chordotonal
222 neurons on step kinematics and inter-leg coordination. We found that flies lacking chordotonal feedback had a lower step
223 frequency across legs and speeds compared to control flies (**Figure 4F**). In addition, flies with silenced chordotonal
224 neurons had greater step lengths across speeds, suggesting that they increased the size of their steps to compensate for
225 taking fewer of them (**Figure 4G**). However, inter-leg coordination was not significantly different between controls and
226 flies lacking chordotonal feedback (**Figure 4H**).

227 In summary, we found that silencing mechanosensory feedback from chordotonal neurons altered step kinematics across
228 all walking speeds but did not have a significant effect on inter-leg coordination. We conjecture that other proprioceptor
229 classes, such as hair plates and campaniform sensilla (Tuthill and Azim, 2018), may play a more important role than
230 chordotonal neurons in inter-leg coordination. Overall, these results also demonstrate the advantage of the linear treadmill
231 to investigate the role of sensory feedback in locomotion.

232 **A split-belt treadmill reveals that middle legs correct for rotational perturbations**

233

234 We next engineered a split-belt treadmill to investigate behavioral mechanisms of adaptive motor control in walking flies
235 (**Figure 5A**). We compared the leg kinematics of walking flies while the belts moved at the same speed (tied) vs. when
236 the belts moved at different speeds (split: slow and fast), focusing our analysis on periods of forward, straight walking
237 (see Methods, **Figure 5B left, Video 7**). We tested splits in both directions and pooled symmetric conditions for
238 subsequent analyses.



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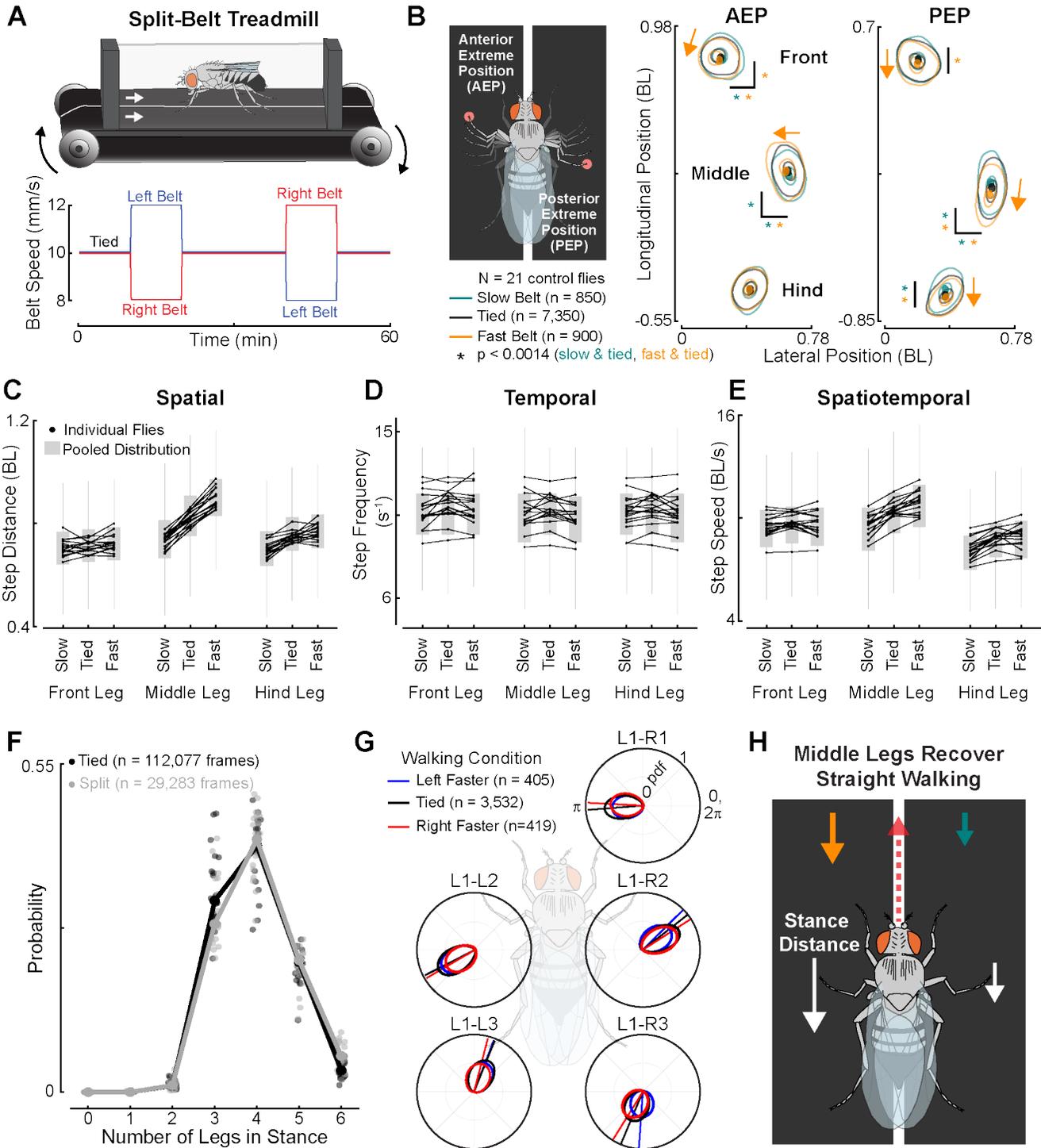
240 **Figure 4. Silencing mechanosensory chordotonal neurons alters step kinematics but not inter-leg coordination across walking speeds.** (A) A
 241 schematic of the locations of chordotonal neurons (green), including the femoral chordotonal organ in each leg (white arrow for left front leg), labeled
 242 by the *iav-GAL4* driver line. The axons of chordotonal neurons (green) are shown in the max intensity projection of a confocal stack of the fly brain

243 and VNC (magenta). **(B)** Flies with silenced chordotonal neurons (*iav-GAL4*> Kir 2.1) spent more walking on the treadmill compared to the arena.
244 Gray dots: individual flies; Black lines are means. **(C)** The flies in **(B)** increased their forward walking speed as the belt speed increased. N: number
245 of flies. Box plots show the distribution of pooled data. Black line connects the median forward speed for all flies for each belt speed. Gray dots are
246 the median forward speed of each fly. **(D)** Heatmap of the occupancy probability along the chamber of genetically-matched controls (*R52A01 GAL4*-
247 *DBD* > Kir2.1; left panel) and flies lacking chordotonal feedback (right) shows that the latter has a greater probability of being located towards the
248 back of the chamber at fast belt speeds. **(E)** Bout frequency was lower at fast belt speeds for flies lacking chordotonal feedback (red) compared to
249 controls (black). Dots are the mean bout frequency for each fly. N: number of flies. **(F)** The step frequency of the front (left), middle (center), and
250 hind (right) legs was lower across walking speeds for flies lacking chordotonal feedback (red) compared to genetically-matched controls (black).
251 Lines are speed binned averages (2 BL/s bins from 5-15 BL/s) for each kinematic parameter and the 95% confidence interval is shown by the shaded
252 region. Distributions between control and experimental flies were offset from each other so that they could be visualized. n: number of steps. **(G)**
253 Step length was greater across walking speeds for flies lacking chordotonal feedback (red) compared to controls (black). **(H)** The probability of a
254 certain number of legs in stance (1-5: left-right) was not different across walking speeds between flies with silenced chordotonal neurons (red) and
255 controls (black). Solid lines: pooled data; Thin lines: individual flies. n: number of camera frames.

256

257 We found that flies achieved straight walking by modifying spatial rather than temporal step kinematics. For instance,
258 flies significantly altered where the legs contacted or lifted off of the ground (i.e., the anterior and posterior extreme
259 positions (**Figure 5B**). Specifically, the mean anterior extreme position (AEP) shifted posteriorly and medially for the
260 front leg on the fast belt, and laterally for the front leg on the slow belt. The front leg's mean posterior extreme position
261 (PEP) also shifted posteriorly when on the fast belt. The middle leg's AEP shifted medially when on the fast belt but
262 translated posteriorly and laterally on the slow belt. Meanwhile, the PEP of the middle legs shifted in the opposite
263 direction. The hind leg shifted its mean PEP in a similar manner along the longitudinal axis. These changes in leg
264 placement produced changes in the total distance that the legs, particularly the middle legs, traveled during a step cycle
265 with respect to the body (i.e., step distance, **Figure 5C**). Step frequency, the timing component of the step cycle, did not
266 change (**Figure 5D**), which suggests that changes in step distance alone dictated the speed at which legs moved through
267 their step cycle (**Figure 5E**). For example, the increased step distance of the middle leg on the fast belt resulted in a faster
268 step speed. Although changes in step speed could in principle have been achieved by altering temporal kinematics, spatial
269 kinematics, or a combination of both, our results suggest that flies overcome the belt asymmetries to maintain forward
270 locomotion by specifically adjusting the step size of the middle legs.

271 Split-belt walking had minimal effects on inter-leg coordination. For instance, there was only a small difference in the
272 probability of 3 legs being in stance during asymmetric belt movement compared to when the belts were tied in speed
273 (**Figure 5F**). Moreover, the mean phase offsets between legs were either not altered or shifted slightly when the belts were
274 driven at different speeds compared to when they were tied (**Figure 5G**). These subtle changes in inter-leg coordination
275 may help correct for the rotational perturbation induced by the treadmill. However, the more substantial changes in the
276 spatial positioning of the middle legs appear to be the primary mechanism that flies use to achieve straight walking during
277 split-belt walking (**Figure 5H**).



278

279 **Figure 5. A split-belt treadmill reveals that flies adjust spatial kinematics to correct for rotational perturbations.** (A) A schematic of the split-
 280 belt treadmill (top) and the belt speed protocol (bottom). The split-belt treadmill consists of two independently controlled belts, which were initially
 281 driven at the same speed (tied). The right (red) and left (blue) belts then differed in speed by 40%, and the direction of the speed change reversed on
 282 the subsequent split period. (B) We focused our analysis on forward walking bouts where the left and right legs moved on their respective belts (left).
 283 The distributions of where the legs first contact the ground, the anterior extreme positions (AEP), and where they takeoff from the ground, the
 284 posterior extreme positions (PEP), are shown for when legs walked on belts during the tied condition (black) or on the slow (teal) and fast (orange)
 285 belt during the split condition. Distributions are shown by kernel density estimations and means are denoted by dots. Arrows indicate the direction
 286 the distributions shifted with respect to the tied distribution. Bootstrapping with a Bonferroni correction of 36 was used to statistically compare the
 287 means of the tied and split distributions. An absence of a “*” indicates no significant difference. N: number of flies; n = number of steps. (C) Step

288 distance increased for the middle leg as belt speed increased. The hind leg had a lower step distance when walking on the slow belt. Box plots show
289 the distribution of pooled data. Black lines and dots are the median step distances of individual flies. (D) Step frequency did not change across
290 conditions for any leg. (E) Step speed increased for the middle leg as belt speed increased and was lower for the hind leg on the slow belt. (F)
291 Probability of 0-6 legs being stance when the belts were tied (black) or split (gray) in speed. Dots connected by lines are the global probabilities.
292 Individual dots are the probability that a given fly shows a certain number of legs in stance. (G) Polar plots of the relative phase between the left
293 front leg and the other legs when the belts were tied (black) or split (left faster: blue; right faster: red) in speed. n: number of phase comparisons. (H)
294 Summary schematic showing that the middle legs adjust their step distance to recover straight walking in the presence of asymmetric belt speeds.

295

296 Discussion

297

298 In this study, we engineered miniature linear and split-belt treadmills for walking *Drosophila*. Flies walking on the
299 treadmill exhibited similar walking behavior, step kinematics, and inter-leg coordination to freely walking flies. The
300 treadmill allowed us to achieve the first 3D tracking of untethered fly walking, which revealed that flies elevate their body
301 height as they walk faster. We also used the linear treadmill to show that flies lacking mechanosensory feedback from
302 chordotonal neurons are able to walk at higher speeds if compelled to do so. Across all walking speeds, silencing
303 chordotonal neurons altered motor control of individual legs, but not coordination between legs. Finally, we found that
304 flies can maintain a forward heading on a split-belt treadmill by adjusting the step size of their middle legs. These insights
305 illustrate how treadmills fill an important gap between freely walking and tethered preparations for investigating neural
306 and behavioral mechanisms of fly locomotion.

307 Although our primary goal was to compare treadmill and freely walking flies, we also took the opportunity to examine
308 walking kinematics of tethered flies. The advantage of tethered walking is that it enables full 3D joint tracking (Günel et
309 al., 2019; Karashchuk et al., 2021), spatially targeted optogenetic stimulation (Agrawal et al., 2020), and recordings of
310 neural activity with calcium imaging (Seelig et al., 2010) or electrophysiology (Fujiwara et al., 2017; Turner-Evans et al.,
311 2017). However, our results suggest that studies of tethered walking should be interpreted with caution, because walking
312 kinematics of tethered flies differ in subtle but important ways from untethered flies (Figures 2-3; Figure S1). Although
313 speed-dependent changes in walking kinematics and coordination were consistent between tethered and untethered flies,
314 the magnitude of step kinematics and the coupling strength between legs were different. One reason for these differences
315 may be that tethered flies walk on a sphere (i.e., a foam ball), whereas treadmill and freely walking flies walk on a flat
316 surface. Tethered flies also do not support their own body weight, but instead use their legs to rotate the floating sphere,
317 a configuration that is unlikely to mimic normal ground reaction forces. Indeed, prior work showed that changing the load
318 on the body alters walking kinematics in freely walking flies (Mendes et al., 2014). On the other hand, it is remarkable
319 that flies and other animals walk at all while tethered on a floating sphere. Given the major mechanical differences between
320 tethered and untethered conditions, the kinematic differences we found are relatively subtle.

321 One of our primary motivations for developing a treadmill system for flies was to investigate the role of mechanosensory
322 feedback across walking speeds. Prior work has suggested that proprioception is most important at slower walking speeds,
323 and that flies use a more feedforward motor program when walking faster (Bidaye et al., 2018). However, testing this
324 hypothesis has been challenging, because silencing mechanosensory neurons causes flies to walk less and at lower
325 velocities (Mendes et al., 2013). The linear treadmill makes it possible to drive fly locomotion across a wide range of
326 speeds, including after genetic manipulations to mechanosensory neurons. We found that silencing mechanosensory
327 feedback alters step kinematics across all walking speeds. Indeed, the greatest deviation in step kinematics between control
328 and experimental flies occurred at faster walking speeds. However, the lack of mechanosensory feedback did not impact
329 inter-leg coordination at any walking speed. We chose to use a blunt manipulation with a broad driver line (*iav-Gal4*) to
330 illustrate the utility of the treadmill for driving walking even when mechanosensory feedback is profoundly altered. One
331 important caveat is that we expressed Kir 2.1 in chordotonal neurons throughout development, so the nervous system
332 could have compensated to maintain normal inter-leg coordination. In the future, it will be interesting to silence
333 mechanosensory feedback of flies walking on the treadmill with more temporal and genetic specificity, for example using

334 optogenetic manipulation of proprioceptor subtypes in the femoral chordotonal organ (Chen et al., 2021; Chockley et al.,
335 2022). Our results are consistent with the hypothesis that proprioceptors in the femoral chordotonal organ contribute to
336 individual leg kinematics, whereas descending commands from the brain (Bidaye et al., 2018) or feedback signals from
337 other proprioceptor classes may have more influence on inter-leg coordination (Tuthill and Azim, 2018).

338 In hexapod insects, each pair of legs plays a specialized role in controlling locomotion. In freely walking insects, the front
339 legs are typically used for steering (Isakov et al., 2016), while the hind legs contribute to propulsion and jumping
340 (Burrows, 2013, 2007; Card and Dickinson, 2008). Using the split-belt treadmill, we found that the middle legs play a
341 unique role in correcting for perturbations that displace flies from a forward walking trajectory. The middle legs are ideally
342 positioned to stably pivot the body of the fly about its center of mass, like rowing a boat from its center. In larger insects,
343 the middle legs have been shown to play a role in executing tight turns (Cruse et al., 2009). Although the split-belt treadmill
344 puts the fly in artificial circumstances, it mimics many situations in the wild when flies may need to perform rotational
345 body corrections. For example, heterogeneous terrain, meddlesome conspecifics, or unilateral wind gusts could
346 asymmetrically act on the movement of the left and right legs to induce a rotation of the body. In the future, the split-belt
347 treadmill may also provide a useful method to study the neural mechanisms that underlie adaptive heading stabilization
348 in walking flies (Haber Kern et al., 2022).

349 In addition to their utility for investigating sensorimotor control of fly walking, we anticipate several additional
350 applications of miniature treadmill systems. One will be to investigate motor adaptation during split-belt walking, a
351 phenomenon which has been extensively studied in mammals (Hinton et al., 2020). The split-belt treadmill is also used
352 as a clinical tool for diagnosing cerebellar deficits (Hoogkamer et al., 2015) and post-stroke rehabilitation (Reisman et al.,
353 2007) in humans. The linear treadmill may also be useful for the study of insect respiratory physiology, which has
354 previously been studied during flight (Lehmann, 2001) and in running cockroaches (Herreid and Full, 1984). Finally,
355 because our treadmill system is constructed of simple and inexpensive belts, pulleys, and motors, it can be easily
356 customized to study other walking insects, such as ants (Dahmen et al., 2017) and snow flies (Golding et al., 2023).

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369

370 **Author Contributions**

371

372 B.G.P. and J.C.T. conceived of the study. B.G.P. engineered the linear and split-belt treadmills. B.G.P. developed high-
373 speed videography acquisition scripts and code to control the treadmill belts. B.G.P. collected and analyzed linear and
374 split-belt treadmill data. S.J.L. collected and analyzed freely walking data. G.M.C. collected and analyzed tethered
375 walking data. B.P. and G.M.C. performed the statistical analyses. B.G.P. and J.C.T. wrote the manuscript with input from
376 other authors.

377

378 **Data and Code Availability**

379

380 Data is available on Dryad (<https://doi.org/10.5061/dryad.mpg4f4r73>). Code for analyzing and visualizing treadmill,
381 freely, and tethered walking kinematics is located on GitHub (https://github.com/Prattbuw/Treadmill_Paper). The .stl file
382 used for the 3D printed treadmill chamber and Python scripts to acquire high-speed video and control the treadmills are
383 also located there.

384

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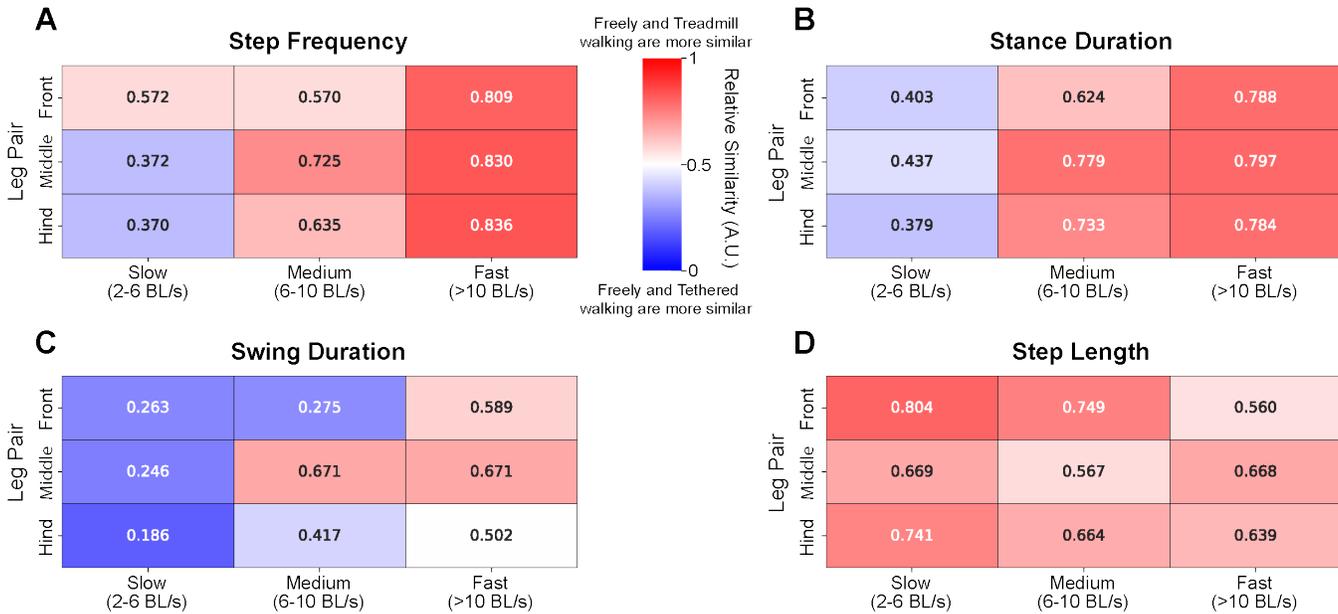
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589

590 **Supplemental information**

591



592

593

594 **Figure S1. Distributions of step kinematics are more similar between freely and treadmill walking flies than between freely walking and**
 595 **tethered flies. (A)** Step frequency was more similar between treadmill and freely walking flies, especially at medium (6-10 BL/s) and fast (>10
 596 BL/s) walking speeds. At slow (2-6 BL/s) walking speeds, freely and tethered walking step frequencies were more similar. Relative similarity was
 597 determined by dividing the KL divergence of freely and treadmill walking kinematic distributions by the sum of the KL divergences between freely
 598 and treadmill walking, and freely and tethered walking kinematic distributions. We then reversed the similarity scale by computing 1 minus these
 599 values. A value close to 1 indicates that freely and treadmill step kinematics are more similar than freely and tethered walking kinematics for a given
 600 walking speed range. The opposite is true for values close to 0. **(B)** Stance duration was more similar between freely and treadmill walking. At slow
 601 walking speeds, freely and tethered walking stance durations were slightly more similar than that of treadmill walking. **(C)** Swing duration was more
 602 similar between freely and treadmill walking flies at fast speeds, whereas the swing duration of freely and tethered walking flies was more similar at
 603 slower speeds. **(D)** Step length was most similar between freely and treadmill walking flies across all speeds and legs.

604

605

Kinematics Parameter	Source	Fly Strain	Sex	Mean Value @ 10 mm/s	Mean Value @ 20 mm/s	Mean Value @ 30 mm/s
Step Frequency (s ⁻¹)	Szczecinski et al., 2018	WT Berlin, Canton S, w ¹¹¹⁸	Male	9	12.5	15
	Present Study – Treadmill Walking	WT Berlin	Male	9	12.5	15
	Present Study – Freely Walking	WT Berlin	Male	10	13	15.5
	Present Study – Tethered Walking	WT Berlin	Male	9	11	N.A.
Stance Duration (ms)	Szczecinski et al., 2018	WT Berlin, Canton S, w ¹¹¹⁸	Male	80	50	45
	DeAngelis et al., 2019	WT flies from Gohl et al., 2011	Female	80	50	40
	Mendes et al., 2013	Oregon R	Female	100	60	45
	Present Study – Treadmill Walking	WT Berlin	Male	75	50	37.5
	Present Study –	WT Berlin	Male	75	50	37.5

	Freely Walking					
	Present Study – Tethered Walking	WT Berlin	Male	80	55	N.A.
Swing Duration (ms)	Szczecinski et al., 2018	WT Berlin, Canton S, w ¹¹¹⁸	Male	35	30	28
	Wosnitza et al., 2013 (data used in above ref.)	WT Canton S	Male	35	30	25
	DeAngelis et al., 2019	WT flies from (Gohl et al., 2011)	Female	30	35	40
	Mendes et al., 2013	Oregon R	Female	35	32.5	30
	Strauß and Heisenberg, 1990	WT Berlin	Female	35	30	28
	Present Study – Treadmill Walking	WT Berlin	Male	37.5	35	32.5
	Present Study – Freely Walking	WT Berlin	Male	35	35	35
	Present Study – Tethered Walking	WT Berlin	Male	37.5	35	N.A.
Step Length (mm)	DeAngelis et al., 2019	WT flies from Gohl et al., 2011	Female	1	1.5	2
	Mendes et al., 2013	Oregon R	Female	1.25	1.75	2.25
	Strauß and Heisenberg, 1990	WT Berlin	Female	1.5	2.25	2.5
	Present Study – Treadmill Walking	WT Berlin	Male	1.8	2.1	2.125
	Present Study – Freely Walking	WT Berlin	Male	1.8	2.05	2.2
	Present Study – Tethered Walking	WT Berlin	Male	1.3	1.75	N.A.

606

607 **Table S1. Summary of previously reported relationships between step kinematics and forward walking speed in *Drosophila melanogaster***
608 **and those reported in this study.** Mean values were determined through visual inspection of fits within relevant plots of previous literature. The
609 mean values for the relationships obtained in this study were approximated through visual inspection of the fits in Figure 2, as we did for the other
610 papers.

611

612

613 Video Legends

614

615 **Video 1. A representative fruit fly walking on the linear treadmill with a steady-state belt speed of ~11 mm/s.** Top-down and side views of a
616 wild-type Berlin fly walking on the treadmill within the chamber are shown by the top left and right videos, respectively. The videos were recorded
617 at 180 fps. Below, in descending order, are the position of the fly within the chamber, its heading angle, its body velocity, and the belt speed
618 throughout the video. The light blue regions indicates forward walking bouts. This data is displayed in **Figure 1D**.

619

620 **Video 2. Fruit flies are capable of walking across a wide range of treadmill belt speeds.** Top-down and side views of a wild-type Berlin fly
621 walking on the linear treadmill within a chamber are shown by the top left and right videos, respectively. The tracked head (red), thorax (green), and
622 abdomen (blue) are displayed for each camera view, as well as the belt speed profile contained within the video. Note that this early chamber design
623 differs from that used in the rest of the paper.

624

625 **Video 3. The linear treadmill forces high-speed walking in flies.** Top-down and side views of a control (R52A01 DBD > tnt) fly walking on the
626 linear treadmill within a chamber are shown by the top left and right videos, respectively. The fly was driven at a belt speed of about 18 mm/s and
627 achieved walking velocities greater than 50 mm/s, which is the fastest walking velocity reported for *Drosophila melanogaster*. Note that flies of this
628 genotype are not used elsewhere in the paper.

629

630 **Video 4. A fruit fly walking freely and forward in an arena.** The top-down view shows a wild-type Berlin fly walking in an arena (i.e. the fly
631 bowl). The forward walking bout is indicated by the appearance of the “Forward Walking” label. The video was recorded at 150 fps and was slowed
632 down 10x. This data is contained in **Figures 2 & 3**.

633

634 **Video 5. A representative tethered fruit fly walking on a sphere suspended by air.** Side and top-down views (left and right, respectively) show
635 a wild-type Berlin fly walking on a floating sphere while tethered. The forward walking bout is indicated by the appearance of the “Forward Walking”
636 label. The video was recorded at 300 fps and was slowed down by 5x. This data is contained in **Figures 2 & 3**.

637

638 **Video 6. Silencing chordotonal neurons alter walking in flies.** Top-down and side views (left and right, respectively) are shown for the same
639 genetically-matched control fly (R52A01 DBD > Kir 2.1; top) and fly with chordotonal neurons broadly silenced (iav-GAL4 > Kir 2.1) as each
640 walked on the linear treadmill. The belt speed was 17.9 mm/s for both flies. Videos were recorded at 180 fps and were slowed down by 2x. This data
641 is contained in **Figure 4**

642

643 **Video 7. Flies use their middle legs to correct for rotational perturbations induced by asymmetric belt speeds on the split-belt treadmill.**
644 Top-down views of the same control (R48A07 AD > Kir 2.1) fly walking on the split-belt treadmill when the left and right belts were tied in speed
645 (10 mm/s) and when the left belt moved faster (12 mm/s) than the right (8 mm/s). The period of asymmetric belt movement is called the “split”
646 period. During the representative split period, a forward walking bout is shown where the left and right legs of the fly walked on the corresponding
647 belts. Videos were recorded at 200 fps and the video showing the forward walking bout was slowed 5x. This data is contained in **Figure 5**.

648

649 **Methods**

650

651 **Fly Husbandry and Genotypes**

652

653 Adult male *Drosophila melanogaster* between 2-7 days post-eclosion were used for experiments (Table 1). Flies were
654 reared in a 25°C incubator with 14:10 light:dark cycle within vials filled with a standard cornmeal and molasses medium.

655

Stock Name	Genotype	Figure(s)	Stock Source
WT Berlin	+, +, +	1-3	Gifted from Heisenberg Lab
R52A01 DBD > Kir 2.1 (Control)	w[1118]/+ DL; +/+ DL; pJFRC49-10XUAS-IVS-eGFP::Kir2.1(attP2)/P{y[+t7.7]w[+mC]=R52A01-GAL4.DBD}attP2	4	Crossed Dickinson Lab Stock U-111 with Bloomington Stock #69141
iav-GAL4 > Kir 2.1	w[*]/+ DL; +/+ DL; pJFRC49-10XUAS-IVS-eGFP::Kir2.1(attP2)/P{w[+mC]=iav-GAL4.K}3	4	Crossed Dickinson Lab Stock U-111 with Bloomington Stock #52273
R48A07 AD > Kir 2.1 (Control)	w[1118]/+ DL; P{+t[7.7]w[=mC]=R48A07-p65.AD}attP40/+ DL; pJFRC49-10XUAS-IVS-eGFP::Kir2.1(attP2)/+	5	Crossed Dickinson Lab Stock U-111 with Bloomington Stock #71070
R39B11 AD > Kir 2.1 (Control)	w[1118]/+ DL; P{+t[7.7]w[=mC]=R39B11-p65.AD}attP40/+ DL; pJFRC49-10XUAS-IVS-eGFP::Kir2.1(attP2)/+	5	Crossed Dickinson Lab Stock U-111 with Bloomington Stock #71040

656

657 **Table 1. *Drosophila melanogaster* genotypes used for experiments.**

658

659 **Miniature Treadmills**

660

661 *Linear treadmill and experiments*

662

663 The key components of the linear treadmill system are a custom 3D-printed chamber, pulleys, a belt, a DC motor
664 controlled programmatically with a PID controller, and 5 high-speed cameras. The chamber was designed using 3D CAD
665 software (Autodesk Fusion 360: *Treadmill_Chamber.stl* in GitHub repository) and printed with black resin using a high
666 spatial resolution 3D printer (Formlabs Form 2; black resin RS-F2-GPBK-04). The region of the chamber where a fly
667 walked had transparent, sloped walls, a length of 8.929 mm, a max width of 6.5 mm, and a max height of 1.5 mm.

668 Coverslips that were 22m x 22m and size #1 were used as the transparent walls of the chamber (Electron Microscopy
669 Sciences: #72200-10). Rain-X was applied to the inner surface of the coverslips to limit flies from walking on the glass.
670 The pullies (B & B Manufacturing: 28MP025M6FA6) were attached to steel bars which rotated with bearings (AST
671 Bearings: SMF126ZZ) that were mounted on custom fabricated brackets. The distance between the brackets was
672 adjustable, which enabled the belt (B & B Manufacturing: 100MXL025UK) held between the pullies to be tensioned. A
673 mounted DC motor (Phidgets: 12V/0.8Kg-cm/46RPM 50:1 DC Gear Motor w/ Encoder ID: 3256E_0) actuated the belt.
674 An infrared ring-light (Olympus Controls: R130-850) was used to illuminate flies on the belt. 5 high-speed cameras
675 (Machine Vision Store; USB 3.0 Basler camera acA800 x 600, Basler AG) with adjustable lenses (Computar: MLM3X-
676 MP) and IR filters (Olympus Controls: FS03-BP850-34) recorded flies walking on the belt and within the chamber at 180
677 fps. The DC motor and high-speed cameras were controlled using a microcontroller (Phidgets: PhidgetMotorControl 1-
678 Motor ID: 1065_1B) and DAQ (National Instruments: BNC-2110), respectively, and a custom Python script
679 (*linear_treadmill_belt_stim_videography.py* in GitHub repository).

680
681 Male *Drosophila* (specific genotypes in Table 1) were driven to walk on the linear treadmill while the belt's steady state
682 speed was either 3.9, 7.4, 10.9, 14.4, or 17.9 mm/s. To smoothly reach steady-state belt speeds above 3.9mm/s, the belt
683 linearly increased in speed with a slope of 3.5 mm/s². Wild-type Berlin and *iav-GAL4 > Kir 2.1* flies were subjected to
684 each belt speed 10 times, whereas *R52A01 DBD > Kir 2.1* flies were presented each belt speed 15 times. Trials in which
685 flies walked at a given belt speed were 10 seconds. There was a 5 second period between trials where the belt moved at
686 3.9 mm/s and the high-speed videos were saved. DeepLabCut and Anipose (Karashchuk et al., 2021; Mathis et al., 2018)
687 were used track the fly's leg tips (i.e. tarsi), head, thorax, abdomen, and key points on the chamber in 3D using 2,250
688 annotated frames as the training dataset. The test prediction error of the tracking was 5.45 pixels and the reprojection error
689 was 2.88 pixels. Walking kinematics were analyzed and visualized using custom Python scripts
690 (*linear_treadmill_walking_analysis.ipynb* & *linear_treadmill_visualization_walking_comparisons.ipynb* in GitHub
691 repository).

692
693 We also tried driving tethered flies to walk on the linear treadmill. Occasionally, the front legs moved with the belt, but
694 the overall movement between legs was uncoordinated. We typically observed legs being dragged along the surface of
695 the belt. It should be noted that we tried many different tether designs, from rigid ones to light-weight, low-resistance
696 ones inspired from a treadmill used for desert ants (Dahmen et al., 2017). Overall, we were unable to drive coordinated
697 walking in tethered flies using the treadmill.

698
699 *Split-belt treadmill and experiments*

700
701 The construction of the split-belt chamber was similar to the linear treadmill. The key difference was the addition of a
702 second independently actuated belt. Therefore, we used smaller belts (B & B Manufacturing: 100MXL012UK) and pullies
703 (B & B Manufacturing: 28MP012M6FA6), while the chamber size remained the same. We also used DC motors (Phidgets:
704 12V/3.0Kg-cm/78RPM 51:1 DC Gear Motor w/ Encoder ID: 3263E_1) that were of a newer model. Finally, the frame
705 rate of the high-speed cameras was increased to 200 fps. A custom Python script controlled the motors and cameras
706 (*splitbelt_treadmill_belt_stim_videography.py* in GitHub repository).

707
708 Only male flies were used in split-belt experiments (specific genotype in Table S1). Flies initially walked on belts that
709 were tied in speed (i.e. 10 mm/s) for 10 minutes. Then, one belt increased in speed by 20% (i.e. 12 mm/s) while the other
710 belt decreased in speed by 20% (i.e. 8 mm/s). This split period also lasted 10 minutes. Following the split period, the belts
711 again moved at 10 mm/s for 10 minutes. At the end of the 10 minutes, this trial structure was repeated, but the belts
712 switched which increased or decreased in speed during the split period. 5 high-speed cameras recorded the movement of
713 the fly during this task and the same key points in the linear treadmill experiments were tracked and reconstructed in 3D.

714 The training dataset consisted of 4,140 annotated frames and the DeepLabCut network achieved a test error of 6.13 pixels.
715 The reprojection error was 1.82 pixels. Custom Python scripts were used to analyze and visualize walking kinematics
716 (*splitbelt_walking_analysis.ipynb* and *splitbelt_walking_visualization.ipynb* in GitHub repository).

717

718 **Freely Walking Experiments**

719

720 Groups of 10, 2-7 day old, male wild-type Berlin flies were placed in a 10 cm circular arena with sloped walls (i.e. fly
721 bowl) and allowed to freely walk (Simon and Dickinson, 2010). A high-speed camera (Machine Vision Store: USB 3.0
722 Basler camera acA1300-200um, Basler AG) recorded a 2.4 cm x 2.7 cm region of the arena from above at 150 fps in 10s
723 bouts. Leg tips, head, thorax, and abdomen of flies were tracked using SLEAP, which is optimized for multi-animal pose
724 estimation (Pereira et al., 2022). Custom Python scripts quantified and visualized walking kinematics
725 (*freely_walking_analysis_visualization.ipynb* in GitHub repository).

726

727 **Tethered Experiments**

728

729 De-winged male wild-type Berlin flies, 2-5 days old, were attached to a thin tungsten tether (0.1 mm) with UV curing
730 glue (KOA 300). Flies were then positioned with a micromanipulator on a spherical foam ball (weight: 0.13 g; diameter:
731 9.08 mm) suspended by a regulated air supply. The 2D trajectory, and forward, rotational, and side-slip velocities of the
732 fly were measured from the movement of the ball with FicTrac (Moore et al., 2014). 6 high-speed cameras (Machine
733 Vision Store: USB 3.0 Basler camera acA800 x 600, Basler AG) recorded flies walking on the ball at 300 fps over 2
734 second bouts. Custom python and MATLAB scripts were used to acquire the high-speed videos. The leg joints, tips, head,
735 and abdomen were tracked and reconstructed in 3D using DeepLabCut and Anipose, respectively. Walking kinematics
736 were analyzed and visualized using Python (*tethered_walking_analysis_visualization.ipynb* in GitHub repository).

737

738 **Statistical and KL Divergence Analyses**

739

740 Chi-squared test and t-tests were used to test for differences in step kinematics between the different experimental setups
741 (**Figure 2**). Statistics were conducted on kinematic distributions containing data from all leg pairs and that were associated
742 with a walking speed between 6.5-10.1 BL/s. This walking speed range was chosen because it contained 50% of the data
743 across setups given their overlapping speed ranges. The chi-squared test determined whether the proportion of values of
744 a given discretely measured step metric (i.e. step frequency, stance duration, and swing duration) was the same between
745 freely walking flies and those in the other two setups. A Bonferroni correction of 18 was added to account for multiple
746 comparisons (6 legs and 3 setups). Therefore, a significant difference was determined to be $p < 0.0028$. Note that to make
747 the statistical comparisons of step frequency, stance duration, and swing duration between all three setups, we had to
748 interpolate the underlying signal for treadmill and freely walking flies from 180 fps and 150 fps, respectively, to 300 fps
749 (i.e. the tethered setup sampling rate). Finally, a t-test was used to determine significant differences between the mean
750 step lengths of freely, treadmill, and tethered walking flies. A Bonferroni correction of 18 was also applied.

751

752 To determine whether step kinematics of freely walking flies were more similar to those of treadmill or tethered walking
753 flies, we computed the relative KL divergence (**Figure S1**). KL divergence computes an unbounded similarity, in the form
754 of entropy, between two distributions, where a value closer to zero indicates greater similarity. Thus, we computed the
755 KL divergence between freely and treadmill step kinematic distributions, and freely and tethered distributions. Then, we
756 calculated a relative similarity score between the two sets of distributions by using the following equation:

757

758

$$\text{Similarity} = 1 - \frac{KL_{\text{freely-treadmill}}}{(KL_{\text{freely-treadmill}} + KL_{\text{freely-tethered}})}$$

759

760 A value of 1 indicated that freely and treadmill walking step kinematics were more similar than freely and tethered ones,
761 and vice versa.

762

763 Finally, a bootstrap statistical analysis was used to test if there were significant shifts in the mean anterior and posterior
764 extreme positions across the different belt conditions (i.e. tied, slow, and fast belt) of the split-belt task. A Bonferroni
765 correction of 36 was applied (6 legs, 3 belt conditions, and 2 axes of comparison), requiring a $p < 0.0014$ for a significant
766 difference.

767

768 **Kinematic Classification and Parameters**

769

770 *Swing and stance classification*

771

772 Leg tip velocity was used to classify leg swing and stance phases in all walking setups. We first computed the
773 instantaneous speed of each leg tip from their allocentric positions along the longitudinal and lateral body axes. For the
774 treadmill and tethered walking setups, a rotation matrix had to be applied to the position data to align all flies to a common
775 reference frame and to ensure symmetric contralateral leg movement with respect to the defined axes. The instantaneous
776 speed was then transformed into velocity by applying a negative sign to instances where the leg moved backward along
777 the longitudinal body axis in an egocentric reference frame. This forced the stance to be negative. To achieve this sign
778 application for freely and treadmill walking setups, where the heading of the fly is constantly changing, we rotated the fly
779 in each frame to a common heading. This made it easier to distinguish the period when the leg moved along the body.
780 The instantaneous velocities of the leg tips were then smoothed with a Gaussian kernel. The width of the kernel was
781 chosen such that the signal wasn't oversmoothed, but instantaneous tracking errors were mitigated. Swing was classified
782 as periods where the smoothed leg tip velocities were above and below manually chosen upper (treadmill: 5 mm/s, freely:
783 15 mm/s, tethered: 0 mm/s) and lower thresholds (all setups: -25 mm/s). Stance was classified as the period where the leg
784 tip velocities were between these thresholds. Finally, we corrected blips in the classification (e.g. converting a swing
785 period consisting of 1 frame into stance) and matched the stance and swing onsets of a given step.

786

787 We checked the accuracy of the swing and stance classifications by manually inspecting the raw high-speed videos. Note
788 that we also tried to perform swing and stance classification by thresholding the Hilbert transformed longitudinal body
789 axis position signal of each leg tip, and doing peak detection on that signal, but both methods performed more poorly than
790 the method described above. The Hilbert transform assumes that a signal is non-stationary, which is invalid when using a
791 leg position signal that dynamically moves in 3D. Peak detection also fails to compensate for the richness of leg
792 movement. Overall, our accurate classifications of swing and stance enabled precise quantifications of step kinematics
793 and inter-leg coordination.

794

795 *Forward walking bout classification*

796

797 Different forward walking bout classifiers were used for each walking setup. In the linear and split-belt setups, forward
798 walking bouts were periods lasting 200 ms where the fly walked in the middle of the chamber, had a heading angle within
799 -15 to 15 degrees with respect to the front of the chamber, and had a forward walking velocity (aligned to the driving axis
800 of the treadmill) greater than 5 mm/s. Flies were classified as walking in the middle of the chamber if their abdomen was
801 1.85 mm in front of the back of the chamber and the tarsi of their front legs were 1.08 mm behind the front of the chamber.
802 For freely walking flies, the thorax position, specifically the angle between sets of 3 position sample points, was used to

803 first isolate straight body trajectories in allocentric coordinates. A straight trajectory was defined as one where the angles
804 between thorax position sample points were less than 4.5 degrees. Once a straight trajectory was isolated, we classified it
805 as a forward walking bout if the corresponding fly's average body velocity was greater than 5 mm/s, the inter-quartile
806 range of the heading angles was less than 20 degrees, and the duration of the trajectory was greater than 200 ms. Lastly,
807 forward walking bouts of tethered flies were first identified by using a previously described behavioral classifier
808 (Karashchuk et al., 2021), but later refined to those greater than 200 ms in duration, having an average forward velocity
809 greater than 5 mm/s, a minimum instantaneous forward velocity of 0.5 mm/s, an average absolute rotational velocity less
810 than 25 degrees/s, and an absolute instantaneous rotational velocity less than 100 degrees/s. Across all setups, the first
811 and last steps of all legs were trimmed within identified forward walking bouts to compensate for the transitions into and
812 out of them.

813

814 *Forward walking step filtering*

815

816 Forward walking steps were filtered based on step frequency, stance duration, and swing duration in all walking setups.
817 Forward walking steps were considered to be those that had a step frequency between 5 and 20 steps/s, a swing duration
818 between 15 and 75 ms, and a stance duration less than 200 ms. These filtering thresholds were empirically determined
819 and based on previously published results of forward walking step kinematics in fruit flies (DeAngelis et al., 2019; Mendes
820 et al., 2014; Szczecinski et al., 2018; Wosnitza et al., 2013).

821

822 *Glossary of kinematic parameters*

823

824 **Body length:** the distance between the head and distal part of the abdomen.

825

826 **Body height:** the vertical distance between the ground and thorax.

827

828 **Step frequency:** the number of steps completed within a second.

829

830 **Stance duration:** the duration that a leg contacts the ground while walking.

831

832 **Swing duration:** the duration of the aerial phase of leg movement during walking.

833

834 **Step length:** the total distance a leg travels within a step (i.e. stance onset to the subsequent stance onset) in allocentric
835 coordinates.

836

837 **Step distance:** the total distance a leg travels within a step (i.e. stance onset to the subsequent stance onset) in egocentric
838 coordinates.

839

840 **Step speed:** the total distance a leg travels within a step in egocentric coordinates divided by the duration of the step.

841

842 **Anterior extreme position:** the position where a leg first contacts the ground (i.e. stance onset) in egocentric coordinates.

843

844 **Posterior extreme position:** the position where a leg first takes off from the ground (i.e. swing onset) in egocentric
845 coordinates.

846

847 **Number of legs in stance:** the number of legs contacting the ground at a given moment in time.

848

849 **L1 relative phase:** the relative offset in the stance onsets between the left front leg and the leg of interest with respect to
850 the left front leg's step cycle.

851

852 **Tripod step order:** the order in which the legs within a tripod group (i.e. ipsilateral front and hind legs and the
853 contralateral middle leg) first enter stance with respect to the left front leg's step cycle.