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Persistent one-way walking in a circular arena in *Drosophila melanogaster* Canton-S strain

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Abstract

We describe persistent one-way walking of *Drosophila melanogaster* in a circular arena. Wild-type Canton-S adult flies walked in one direction, counter-clockwise or clockwise, for minutes, whereas white-eyed mutant w^{1118} changed directions frequently. Computational analysis of locomotor behavior showed that counter-clockwise walking and clockwise walking were the two main components of locomotion, and that pausing was more frequently associated with directional persistence but not directional change. Genetic analysis revealed that while wild-type genetic background primarily controlled the number of counter-clockwise walking and clockwise walking during five minutes of locomotion, the *white* (w^+) gene promoted persistent one-way walking by increasing the number of pauses, increasing the maximal duration of one-way walking, and reducing the path length in five minutes. These results support a pleiotropic function of w^+ in promoting persistent one-way walking in addition to eye pigmentation.

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Introduction

Walking locomotion in *Drosophila melanogaster* contains many distinctive features. Negative geotaxis and positive phototaxis are the classical stereotypes of locomotion in adult flies [1, 2, 3]. Directional persistence and local wall attraction are the two main features of walking in open field arenas [4]. Wing-clipped flies walk back and forth towards inaccessible visual targets in Buridan's paradigm [5, 6, 7]. In addition, adult flies are unwilling to walk through confined tunnels, a phenomenon termed claustrophobia [8]. While restrained in small arenas, flies perform relentless walking for hours [9, 10].

A common interest is to understand the genetic basis for walking behavior in *Drosophila*. Two widely used strains, wild-type Canton-S and white-eyed mutant w^{1118}, have different walking performance observed in several experimental settings. Canton-S flies walk towards light more often than w^{1118} flies [11]. In circular arenas, Canton-S flies have higher boundary preference than w^{1118} flies [12, 10, 13]. Canton-S recover walking after anoxia faster and more consistently than w^{1118} flies [14]. When rigidly restrained, Canton-S flies show spontaneous and rhythmic motor activities that can be recorded extracellularly from the brain, whereas w^{1118} flies have greatly reduced rhythmic motor activities [15]. These findings raise a concern: either the *white* (w^+) gene, which is null-mutated in w^{1118} flies, or its genetic background, is primarily responsible for the different walking performance of Canton-S compared with w^{1118} flies.

w^+ is a classic eye-color gene discovered by Thomas Hunt Morgan in 1910 [16]. The product of w^+ is a subunit of transmembrane ATP-binding cassette (ABC) transporter, which loads vesicles/granules with biogenic amines [17], second messenger [18], metabolic intermediates [19, 20] and pigment precursors [19, 21, 22, 23]. Increasing evidence has supported the proposal that w^+ has pleiotropic housekeeping functions in addition to eye pigmentation [24, 25, 26, 18, 17, 20, 14, 27, 28, 29]. We hypothesized that w^+ modulates locomotor behavior and promotes persistent walking performance.

In this study, we describe persistent one-way walking of Canton-S flies in circular arenas. Pre-

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liminary observations show that Canton-S flies walk in one direction, counter-clockwise or clockwise, for minutes in the arenas, whereas w^{1118} flies change directions frequently. We extracted the behavioral features of walking in the arena, and show that counter-clockwise walking and clockwise walking are the two main locomotor components, and that pausing is mainly associated with directional persistence but not directional change. We further show that while wild-type genetic background primarily suppresses the number of counter-clockwise and clockwise walks, w^{+} promotes persistent one-way walking by increasing the number of pauses, increasing the maximal duration of counter-clockwise or clockwise walking, and reducing path length in five minutes.

Results

Persistent one-way walking of wild-type flies in circular arenas

A male, wild-type Canton-S fly walked in one direction, counter-clockwise or clockwise, for minutes in a circular arena (1.27 cm diameter 0.3 cm depth). This persistent one-way walking was consistent between individuals (Video S1). Male flies of a white-eyed mutant w^{1118}, however, changed direction frequently and failed to maintain the walking direction for at least a minute (Video S2). 3D walking trajectories of Canton-S flies, represented as a time series of connected X-Y positions per 0.2 s, showed a regular, coil-like shape during 60 s locomotion (Fig. 1a). The trajectories of w^{1118} flies displayed a shape of collapsed coil with visually increased irregularity (Fig. 1b). The 2D path of Canton-S showed a strong preference for the perimeter of arena, whereas the 2D path of w^{1118} flies displayed reduced preference for perimeter and frequent crossing of the central arena for the 60 s period. The persistent one-way walking was also observed in Canton-S females compared with w^{1118} females (Fig. S1). Several different wild-types, including Oregon-R, Hikone-AS and Florida-9, showed a similar performance of persistent one-way walking in circular arenas. Several white-eyed mutants (w^{1}, w^{a} and w^{cf}) displayed irregular trajectories similar to w^{1118} flies (Fig. S2). Therefore, wild-type flies showed persistent one-way walking in

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the circular arenas.

Counter-clockwise walking and clockwise walking were the main components of locomotion in a circular arena

Using a fly-tracking protocol [10] and software R [30], we computed the walking directions of flies and re-constructed the components of locomotor behavior.

Four walking structures could be identified in a fly. They were counter-clockwise walking (CCW), clockwise walking (CW), pausing and unclassified activities. Canton-S showed a walking pattern that was clearly recognizable. Several flies walked in one direction, inter-spaced with a few pauses, throughout 300 s. In contrast, w^{1118} flies displayed a complicated pattern with frequent transit between walking structures (Fig. 2a).

CCW and CW were the two main locomotor structures, comprising the most proportion of time in Canton-S (median 0.92, interquartile range (IQR) 0.87 - 0.96, n = 31) (Friedman test with Dunn's multiple comparison) as well as w^{1118} flies (median 0.82, IQR 0.78 - 0.86, n = 48) (Friedman test with Dunn's multiple comparison). Pausing comprised a relative small proportion of time in Canton-S (median 0.06, IQR 0.03 - 0.12, n = 31) and w^{1118} flies (median 0.07, IQR 0.5 - 0.09, n = 48). Times spent on unclassified activities in Canton-S (median 0.009, IQR 0.005 - 0.013, n = 31) and w^{1118} flies (median 0.10, IQR 0.08 - 0.11, n = 48) were also small (Fig. 2b).

During 300 s locomotion, the time for CCW (median 187.6 s, IQR 65.4 - 244.8 s, n = 31) and the time for CW (median 60.6 s, IQR 9.0 - 207.4 s, n = 31) were statistically the same in Canton-S flies (P = 0.1470, Wilcoxon matched pairs test). The time for CCW (median 123.1 s, IQR 105.4 - 134.2 s, n = 48) and the time for CW (median 129.2 s, IQR 111.7 - 138.9 s, n = 48) were also the same in w^{1118} flies (P = 0.4327, Wilcoxon matched pairs test) (Fig. 2c). There was no preference for CCW or CW in the circular arenas in either strain.

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Pausing was associated with directional persistence

Flies paused often in the arenas (see Fig. 2a). It is possible that a fly pauses and changes walking direction. We examined whether the pausing was associated with directional change or directional persistence of flies in the arenas.

Canton-S flies paused several times within a period of 100 s. There was no substantial difference of walking direction before and after a pause. Similarly, w^{1118} flies showed no apparent change of walking direction before and after a pause (Fig. 3a). Within 5 min, the number of pauses with directional persistence (median 4, IQR 3 - 6, n = 31) was higher than those with directional change (median 0, IQR 0 - 1, n = 31) in Canton-S ($P < 0.0001$, Wilcoxon matched pairs test) (Fig. 3b). The number of pauses with directional persistence (median 8, IQR 5 - 10, n = 48) was also higher than those with directional change (median 1, IQR 0 - 1, n = 48) in w^{1118} flies ($P < 0.0001$, Wilcoxon matched pairs test) (Fig. 3c). Thus, pausing was associated with directional persistence of walking in the arena.

Wild-type genetic background suppressed the number of counter-clockwise and clockwise walks

Canton-S and w^{1118} flies have different alleles of *w* and genetic background. We examined the contributions of w^{+} and its genetic background to persistent one-way walking.

We first compared the walking activities of w^{+} F1 and w^{1118} F1 male flies. w^{+} F1 was the progeny of w^{1118} males and Canton-S females, and reciprocally, w^{1118} F1 the progeny of Canton-S males and w^{1118} females. These two types of males had different *w* alleles and roughly the same genetic background. Both w^{+} F1 and w^{1118} F1 flies changed walking directions frequently in the circular arenas during 300 s locomotion (Fig. 4a). The number of CCW and CW episodes in w^{+} F1 (median 34.5, IQR 29.3 - 46.0, n = 24) was statistically the same as that in w^{1118} F1 (median 43.0, IQR 31.5 - 47.5, n = 32) ($P = 0.1234$, Mann-Whitney test) (Fig. 4b). Likely, wild-type

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genetic background but not w^+ was responsible for the number of CCW and CW episodes in the arena.

We further examined the walking activities in w^+ F10 and w^{1118} F10 male flies. These two types of males were generated by serial backcrossing between Canton-S and w^{1118} for ten generations [14]. w^+ F10 flies, which carried w^+ in isogenic background, had the number of CCW and CW episodes (median 44.0, IQR 34.0 - 53.0, n = 47) markedly greater than Canton-S (median 5.0, IQR 2.0 - 8.0, n = 31) ($P < 0.0001$, Mann-Whitney test) (Fig. 4c). Additionally, w^{1118} F10 flies, which carried w^{1118} allele in wild-type genetic background, had the number of CCW and CW episodes (median 8.0, IQR 4.0 - 10.0, n = 43) remarkably lower than w^{1118} flies (median 61.0, IQR 50.0 - 70.8, n = 48) ($P < 0.0001$, Mann-Whitney test) (Fig. 4d). These data confirmed that the wild-type genetic background suppressed the number of CCW and CW episodes in the circular arena.

w^+ promoted pausing during locomotion

Preliminary observations indicated that w^+-carrying flies (including Canton-S) paused more often than w^{1118}-carrying flies (including w^{1118} strain) (see Fig. 2 and Fig. 4), suggesting that w^+ promoted pausing. We examined the effect of w^+ on the number of pauses during 300 s locomotion in the arena.

The number of pauses in w^+ F1 (median 10.5, IQR 5.0 - 13.0, n = 24) was greater than that in w^{1118} F1 (median 4.5, IQR 3.0 - 6.0 , n = 32) ($P = 0.0002$, Mann-Whitney test) (Fig. 5a). These data supported the suggestion that w^+ increased the number of pauses during locomotion.

The number of pauses in w^+ F10 (median 13.0, IQR 10.0 - 26.0, n = 47) was greater than that in w^{1118} (median 9.0, IQR 6.0 - 11.0, n = 48) ($P < 0.0001$, Mann-Whitney test). The number of pauses in Canton-S (median 7.0, IQR 6.0 - 8.0, n = 31) was also greater than that in w^{1118} F10 (median 3.0, IQR 2.0 - 4.0, n = 43) ($P < 0.0001$, Mann-Whitney test) (Fig. 5a). The genetic background was virtually the same between w^+ F10 and w^{1118}, as well as between Canton-S and

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w^{1118} F10 flies. Therefore, w^+ promoted pausing during locomotion in the arena.

w^+ increased maximal duration of counter-clockwise or clockwise walking

The maximal duration of CCW or CW could reflect the persistence of one-way walking in the arena. We explored the effect of w^+ on the maximal duration of CCW or CW within 300 s locomotion. Flies carrying different *w* alleles but nearly identical genetic background were compared.

The maximal duration of CCW or CW in w^+ F1 flies (median 32.0 s, IQR 24.0 - 49.0 s, n = 24) was longer than that in w^{1118} F1 flies (median 27.2 s, IQR 21.3 - 35.1 s, n = 32) (P = 0.0434, Mann-Whitney test). The maximal duration of CCW or CW in w^+ F10 flies (median 21.4 s, IQR 17.6 - 32.8 s, n = 47) was greater than that in w^{1118} flies (median 18.6 s, IQR 14.9 - 22.3 s, n = 48) (P = 0.0030, Mann-Whitney test). The maximal duration of CCW or CW in Canton-S (median 184.6 s, IQR 129.2 - 235.4 s, n = 31) was markedly longer than that in w^{1118} F10 flies (median 97.8 s, IQR 76.4 - 156.2 s, n = 43) ($P < 0.0001$, Mann-Whitney test) (Fig. 5b).

Clearly, Canton-S had the ability to walk in one direction for around 185 s in a circular arena.

These data indicated that w^+ increased the maximal duration of counter-clockwise or clockwise walking in the arena.

w^+ reduced 5-minute path length

Because of persistent turning, the speed of one-way walking along the curved perimeter would be slower than the speed of crossing of central region. A consequence of increased persistence of one-way walking and increased pausing could be a reduction of path length over a time. The 3D trajectories were displayed, and 5-min path length was compared between flies carrying different *w* alleles with nearly identical genetic background.

A w^+ F1 fly had a much more regular coil-shape of 3D trajectory compared with a w^{1118} F1 fly during 60 s locomotion (Fig. 6a). 5-min path length in w^+ F1 flies (median 173.4 cm, IQR 164.9 - 205.2 cm, n = 24) was significantly shorter than that in w^{1118} F1 flies (median 265.3 cm, IQR 250.4

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- 286.5 cm, n = 32) ($P < 0.0001$, Mann-Whitney test). 3D trajectory of a w^+ F10 fly had likely improved regularity compared with a w^{1118} fly. 5-min path length in w^+ F10 (median 164.4 cm, IQR 145.7 - 177.7 cm, n = 47) was shorter than that in w^{1118} flies (median 225.9 cm, IQR 210.1 - 240.4 cm, n = 48) ($P < 0.0001$, Mann-Whitney test) (Fig. 6b). 3D trajectory of a Canton-S fly was much more regular in the coil-shape relative to a w^{1118} F10 fly. 5-min path length in Canton-S (median 199.4 cm, IQR 177.0 - 228.3 cm, n = 31) was clearly shorter than that in w^{1118} F10 flies (median 262.0 cm, IQR 226.1 - 279.0 cm, n = 43) ($P < 0.0001$, Mann-Whitney test) (Fig. 6c). Therefore, w^+ reduced path length during 5-min locomotion in the arena.

Discussion

We report a phenomenon of persistent one-way walking in a circular arena in adult *Drosophila melanogaster*. It is striking that a Canton-S fly is able to walk in one direction, counter-clockwise or clockwise, for around 185 seconds without a change of direction. Few articles have described this phenomenon in flies. Using the techniques of behavioral computation and genetic manipulation, we show that both w^+ and its genetic background of Canton-S flies contribute to persistent one-way walking in a circular arena.

The extraction of locomotor components of walking behavior provides rich information on the behavioral elements, the frequency of each element, and how they transit from one to another over time. It has been shown that Canton-S and w^{1118} males increase locomotion in circular arenas as a response to spatial restriction, and maintain active walking for at least an hour [10]. Here we examine the details of locomotion and find that, common to fly strains, CCW (counter-clockwise walking) and CW (clockwise walking) are the two main locomotor components, and there is no preference for CCW or CW in the arena. Furthermore, the wild-type genetic background suppresses the number of CCW and CW episodes. The feature of reduced CCW and CW episodes within 5-min is transferable, from Canton-S to w^{1118}-carrying flies with wild-type genetic background, indicating a strong association between genetic background and the number

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of CCW and CW episodes.

A fly pauses during locomotion in the arena. Behavioral computation reveals that pausing is associated with directional persistence but not directional change. Thus, pausing indicates a state that flies rest and retain a memory of walking direction, rather than a state in which flies are unsure of their direction. Cocaine-treated Canton-S flies show a general tendency to rotate in one direction both before and after an immobility [31], a finding similar to our observation of pausing associated with directional persistence in circular arena. There are at least two consequences of pausing: reduction of transit between CCW and CW episodes, and increase of persistence in CCW or CW direction. With nearly the same genetic background, w^+-carrying flies pause more often than w^{1118}-carrying flies in the arenas. These observations suggest that w^+ promotes persistent one-way walking. Further examination of behavioral structures of walking reveals that, w^+-carrying flies have increased maximal duration of a single episode of CCW or CW, much more regular walking trajectories, and reduced 5-min path length compared with w^{1118}-carrying flies. These data firmly support the suggestion that w^+ modulates walking performance and promotes persistent one-way walking.

It is intriguing that in general, a Canton-S fly is able to walk in one direction in a circular arena for around 185 seconds, while a w^{1118} fly only 19 s. We have previously shown that Canton-S flies have spontaneous and rhythmic motor activities with a periodicity of around 19 s, but this periodicity is lost or greatly reduced in w^{1118} flies [15]. Whether rhythmic motor activities promote persistent one-way walking in Canton-S flies is currently unclear.

For over a hundred years, it has been believed that w^+ controls eye color in *Drosophila*. This has led to an application of mini-*white*, a short form of w^+, as a marker gene indicating successful transformation of a transgene. This application heavily relies on the function of w^+ in eye pigmentation. However, mini-*white* causes abnormal courtship in male flies [24, 25], and confers male-female copulation success in a manner that is mini-*white* copy number dependent [28]. Additionally, w^+ promotes fast and consistent locomotor recovery from anoxia [14, 29]. Wild-type

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flies have enhanced memory of thermal stimulus [32], and increased vesicular content of biogenic amines [17] compared with *w* mutant flies. The extra-retinal function that the White protein transports cyclic guanosine monophosphate (cGMP) has been reported [18, 27]. Together, these studies suggest that w^+ has pleiotropic functions in housekeeping rather than a function responsible for eye color. In the current study, we provide further evidence to support this suggestion. That w^+ increases the maximal duration of one-way walking and promotes pausing, which is primarily associated with directional persistence, and that Canton-S flies are able to walk in one direction in circular arenas for around 185 seconds, firmly support a pleiotropic effect of w^+ in promoting persistent one-way walking.

During persistent one-way walking flies move forward. We did not observe flies walking backward persistently in the arenas. The phenotype of walking backwards has been attributed to the moonwalker descending neuron (MDN) [33]. Thus, the neural or molecular mechanisms for persistent one-way walking might not involve such a neuron. In *Drosophila* larvae, persistent and directional locomotion is observed in the mutants of *Hyperkinetic*, *Shaker* and *quiver* [34, 35]. These genes are potential candidates that might mediate persistent one-way walking of adult *Drosophila* in small circular arenas.

Methods

Flies

Fly strains and their sources were: Canton-S (Bloomington Stock Center (BSC) # 1); w^{1118} (L. Seroude laboratory); Oregon-R (BSC # 2376); Hikone-AS (BSC # 3); Florida-9 (BSC # 2374); w^1 (BSC # 145); w^a (BSC # 148) and w^{cf} (BSC # 4450). Flies were maintained with standard medium (cornmeal, agar, molasses and yeast) at 21-23 °C with 60-70 % relative humidity. An illumination of light/dark (12/12 h) cycle was provided with three light bulbs (Philips 13 W compact fluorescent energy saver) in a room around 133 square feet. Flies were collected within 0 - 2 days

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after emergence. We used pure nitrogen gas to anesthetize flies during collection. Collected flies were raised in food vials at a density of 20 - 25 flies per vial for at least three additional days. A minimum of three days free of nitrogen exposure was guaranteed before testing. The ages of tested flies were 4 - 9 days old. Unless otherwise indicated, male flies were used for experiments. To avoid natural peak activities in the mornings and evenings [36]), experiments were performed during the light time with three hours away from light on/off switch.

Locomotor assay

Locomotor assay was performed by following a reported protocol [10]. In general, flies were loaded into circular arenas (1.27 cm diameter and 0.3 cm depth) with one fly per arena. The depth of 0.3 cm was considered to allow flies to turn around but suppress vertical movement. We machined 128 arenas (8 $\times$ 16) in an area of 31 $\times$ 16 cm^2 Plexiglas. The bottom side of arena was covered with thick filter paper allowing air circulation. The top was covered by a slidable Plexiglas with holes (0.3 cm diameter) at one end for fly loading. The Plexiglas with arenas was secured in a large chamber (48.0 $\times$ 41.5 $\times$ 0.6 cm^3). A flow of room air (2 L/min) was provided to remove the effect of dead space [37]. A time of 5-min was allowed for flies to adapt to the experimental settings. Locomotor activities were video-captured at 15 frames per second, and stored for post analysis. Fly positions (the locations of center of mass) with 0.2 s interval were computed by custom-written fly tracking software [10]. The positions were used for subsequent movement analysis, including the construction of time-series of 3D trajectory and computation of counter-clockwise and clockwise walking directions.

Construction of time-series of 3D trajectory

Time-series of 3D trajectories were constructed by plotting the sequential X-Y positions against time. Briefly, for each fly, a dataset containing information of 1500 positions, corresponding to 300 s locomotion, was used for trajectory construction. The Cloud function from an R pack-

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age "Lattice" [38]) was applied for 3D data visualization. The arena size remained unchanged throughout this study. Therefore, for simplification, we omitted the *x*, *y* axes (representing position coordinates) and *z* axis (representing time), and provided a color key as an indicator of time. 3D trajectory and walking directions were measured from the camera view.

Computation of counter-clockwise and clockwise walking directions

Computation of walking direction (counter-clockwise or clockwise) was performed as previously described [39]. To improve the estimation, we separated pausing from counter-clockwise or clockwise walking. A "pausing" was defined as at least five consecutive relocations with step size $<$ 0.28 mm [39].

There were three main procedures for computing walking directions: (1) Compute angular coordinates of fly positions by a trigonometric function atan2(y, x). (2) Calculate parameter ω - the angular displacement per 0.2 s. To avoid the big jump of ω value due to radian rotation, we calculated ω twice using radian interval (0, 2π] and (-π, π], and chose the one with smaller absolute value. (3) Determine the walking direction as counter-clockwise walking ($\omega > 0$) or clockwise walking ($\omega < 0$).

We defined a "counter-clockwise walking" as at least five consecutive displacements with $\omega >$ 0, and a "clockwise walking" at least five consecutive displacements with $\omega < 0$. Classified data were further treated to allow 1-2 steps of pausing or backward walking (with $|\omega| < 0.21$) without an apparent change of direction. The activities that were not categorized as "counter-clockwise walking", "clockwise walking" or "pausing" were assigned as "unclassified".

Statistics

Data processing and visualization were conducted by using software R [30] and these supplementary packages: gdata, lattice [38] and adehabitatLT [40]. Data normality was examined by D'Agostino & Pearson omnibus normality test. Nonparametric tests (Mann-Whitney test, Wilcoxon

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matched pairs test and Friedman test with Dunn's multiple comparison) were performed for the comparison of medians between groups. Data were presented as scattered dots and median. A $P < 0.05$ was considered significant.

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Acknowledgments

This work was funded by Natural Sciences and Engineering Research Council of Canada (NSERC) grant (RGPIN 40930-09) to R.M.R. The authors declare no conflict of interest.

Supporting Information

Video S1: Walking activities of Canton-S flies in the circular arenas

Video S2: Walking activities of w^{1118} flies in the circular arenas

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Figure legends

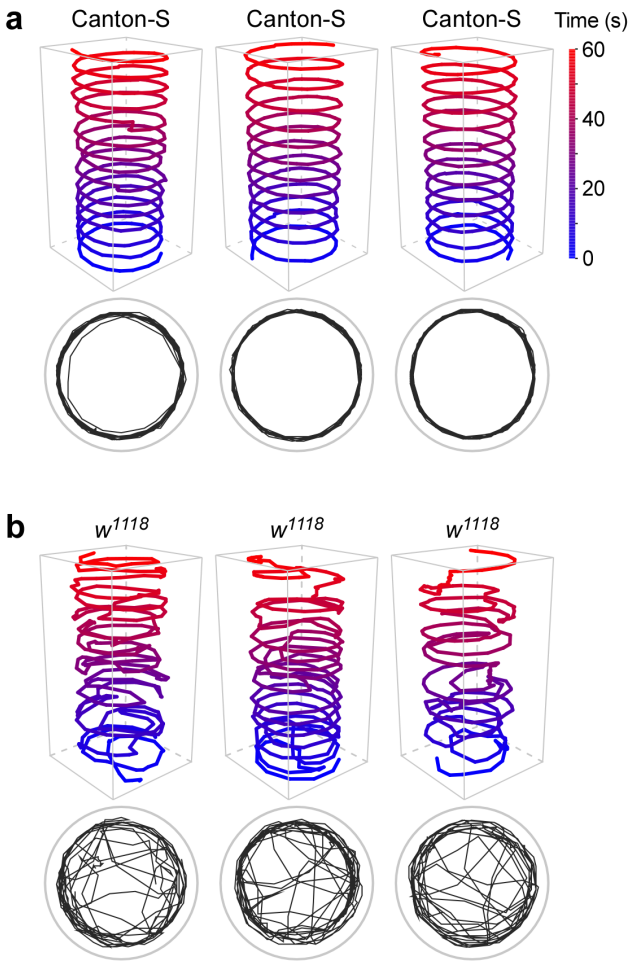

Figure 1: **Persistent one-way walking of Canton-S flies in circular arenas.** (**a**) 3D walking trajectories and 2D path of Canton-S flies in the circular arenas (1.27 cm diameter 0.3 cm depth) during 60 s locomotion. There is only one fly in each arena. Color key indicates the time, and gray circle the arena edge. (**b**) 3D walking trajectories and 2D path of w^{1118} flies during 60 s locomotion.

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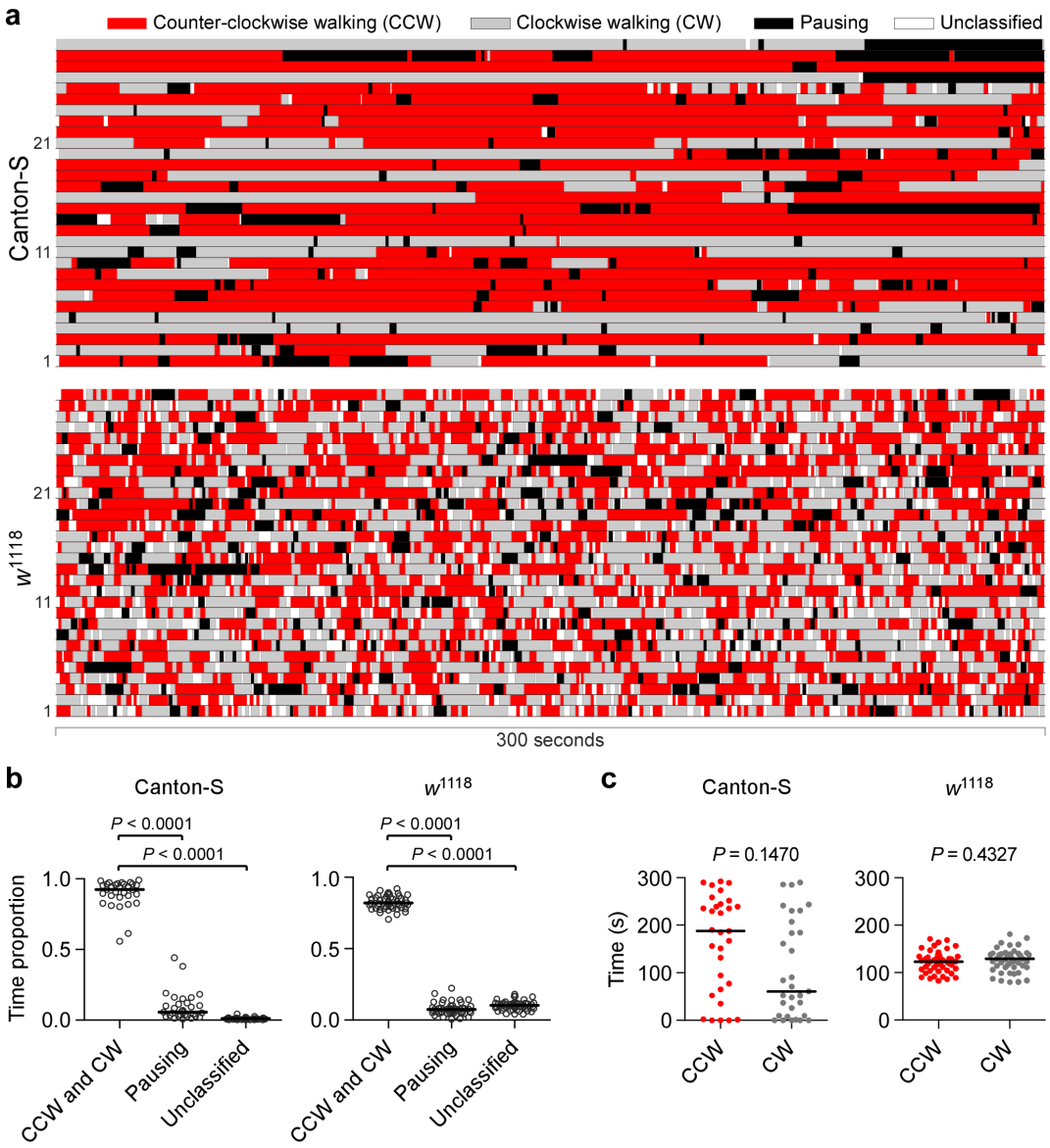

Figure 2: **Walking structures of flies in the circular arenas.** (**a**) Schematic structures of walk during 300 s locomotion. Shown are the counter-clockwise walking (CCW, in red), clockwise walking (CW, grey), pausing (black) and unclassified (white) activities from 30 Canton-S and 30 w^{1118} flies. (**b**) Time proportion for CCW and CW in Canton-S (left) and w^{1118} flies (right). Data are presented as scattered dots and median (black line). *P* values are from Friedman test with Dunn's multiple comparison. (**c**) Time for CCW (red) and CW (grey) during 300 s locomotion in Canton-S (left) and w^{1118} flies (right). *P* values are from Wilcoxon matched pairs test.

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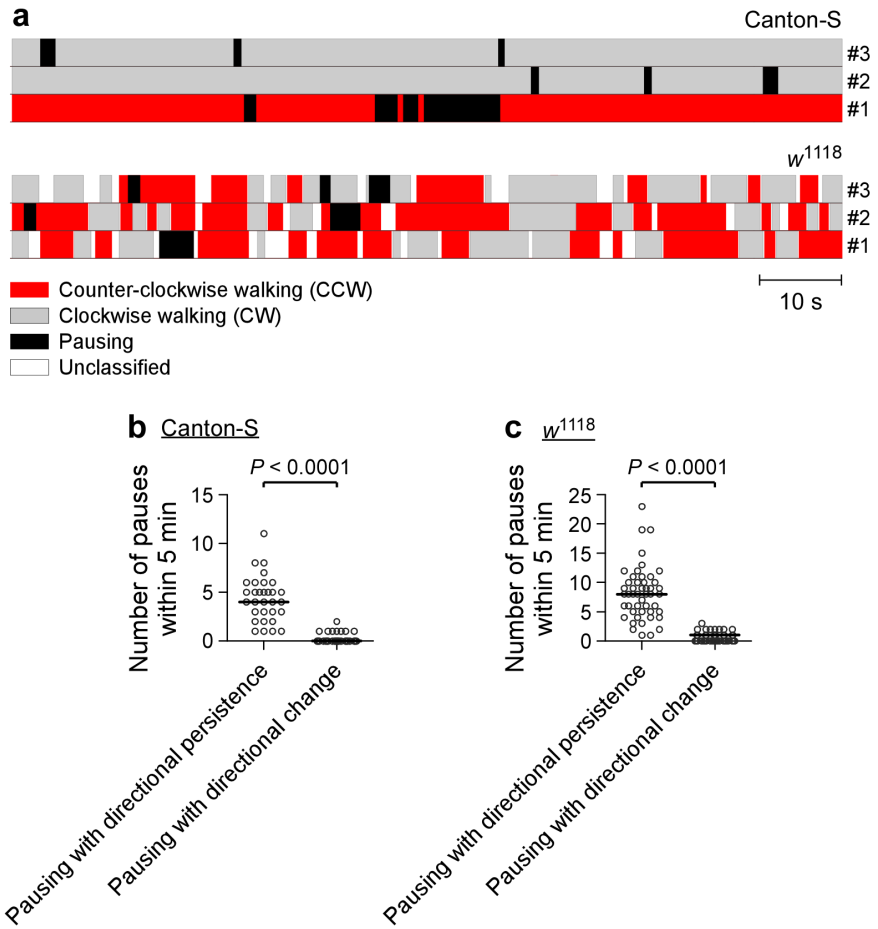

Figure 3: **Pausing associated with directional persistence.** (**a**) Representations of pausing and several walking structures in Canton-S and w^{1118} flies. Shown are the 100 s activities of flies in the circular arenas. Walking structures are counter-clockwise walking (CCW, red), clockwise walking (CW, grey), pausing (black) and unclassified (white) activities. (**b**) Association between pausing and directional persistence in Canton-S flies. *P* value from Wilcoxon matched pairs test. (**c**) Association between pausing and directional persistence in w^{1118} flies. *P* value from Wilcoxon matched pairs test.

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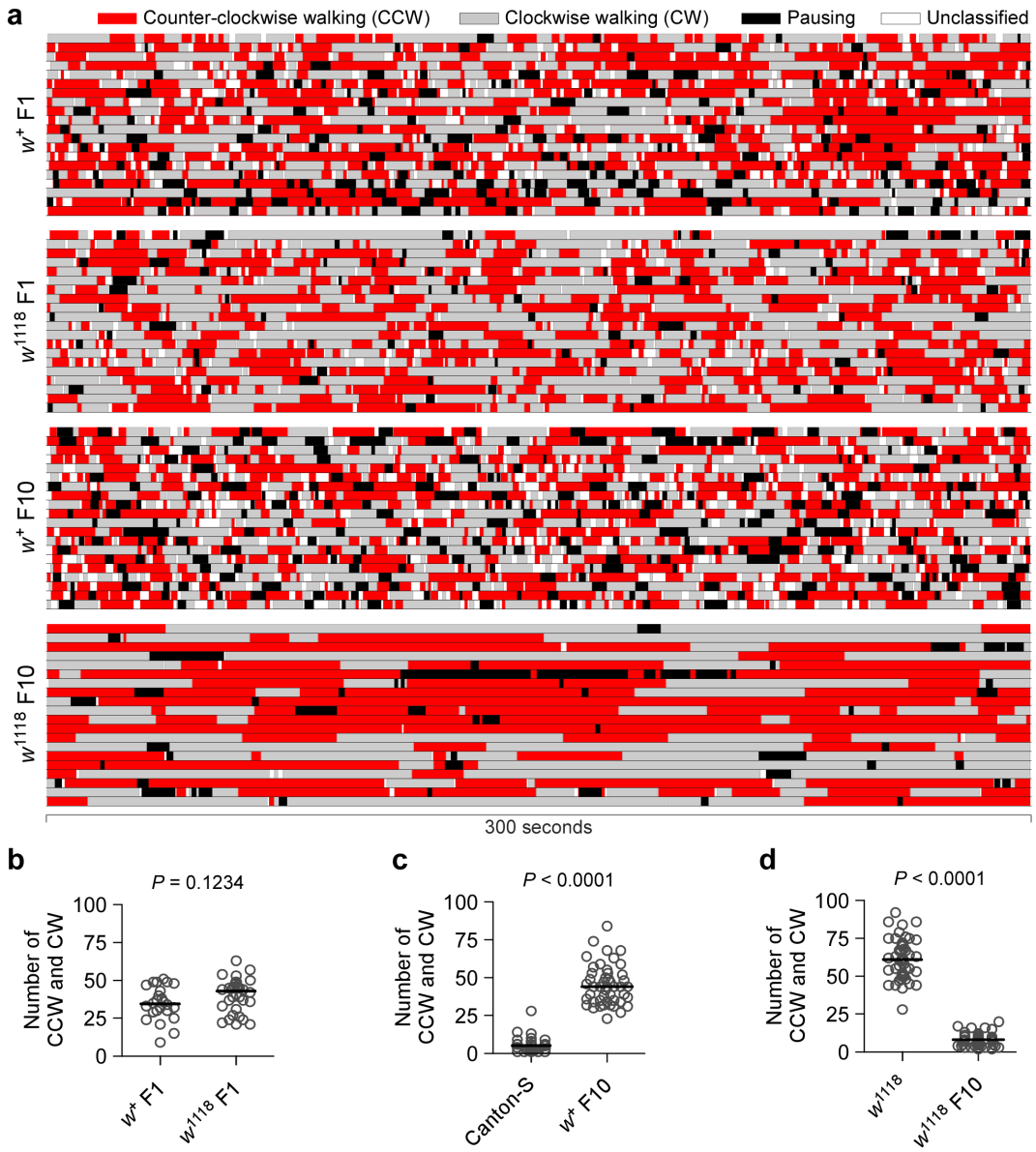

Figure 4: **Wild-type genetic background suppressed the number of counter-clockwise and clockwise walking.** (**a**) Schematic structures of walking during 300 s locomotion in w^{+}F1, w^{1118}F1, w^{+}F10 and w^{1118}F10 flies. Shown are counter-clockwise walking (CCW, red), clockwise walking (CW, grey), pausing (black) and unclassified (white) activities from 20 flies of each genotype. (**b**) Number of CCW and CW during 300 s locomotion in w^{+} F1 and w^{1118} F1 flies. (**c**) Number of CCW and CW within 300 s locomotion in Canton-S and w^{+} F10 flies. (**d**) Number of CCW and CW within 300 s locomotion in w^{1118} and w^{1118} F10 flies. Data are presented as scattered dots and median (black line). *P* values are from Mann-Whitney test.

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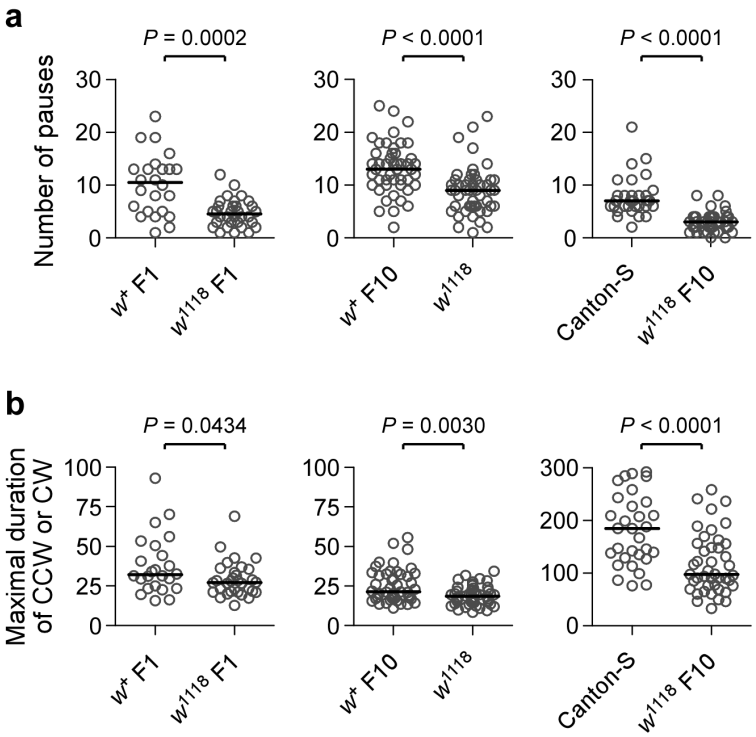

Figure 5: ***w*$^{+}$ promoted pausing and persistent one-way walking.** (**a**) Number of pauses during 300 s locomotion in different flies. (**b**) Maximal duration of CCW or CW within 300 s locomotion in different flies. The genotypes are indicated. Data are presented as scattered dots and median (black line). *P* values from Mann-Whitney test.

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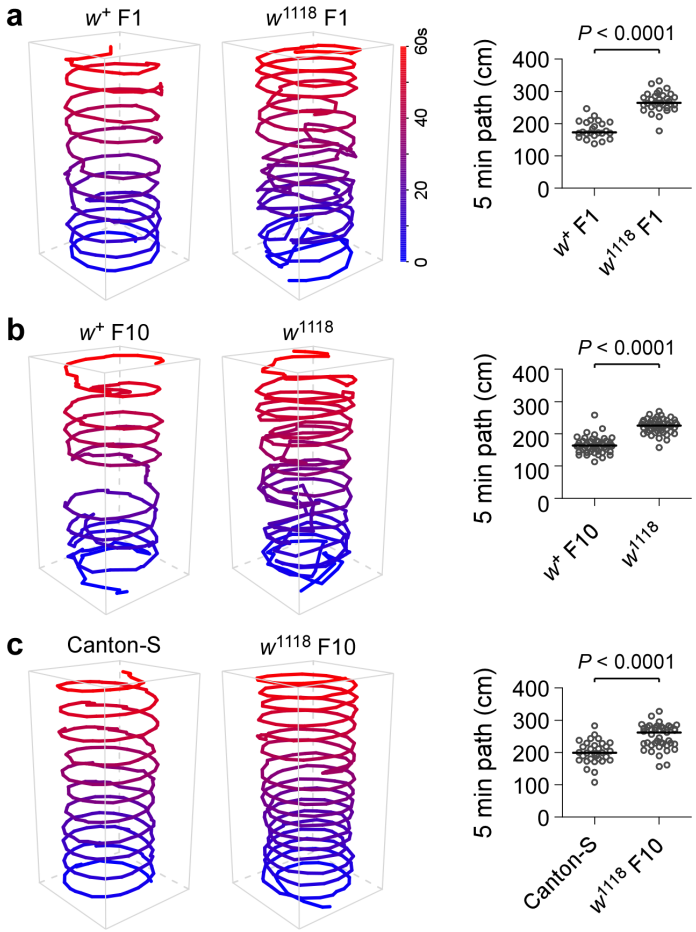

Figure 6: ***w*$^{+}$ reduces path length in five minutes.** (**a**) Representations of 3D trajectories in w^{+} F1 and w^{1118} F1 flies. Analysis of 5-min path length is provided. Color key indicates the time. (**b**) 3D trajectories and analysis of 5-min path in w^{+} F10 and w^{1118} flies. (**c**) 3D trajectories and analysis of 5-min path in Canton-S and w^{1118} F10 flies. *P* values are from Mann-Whitney test.

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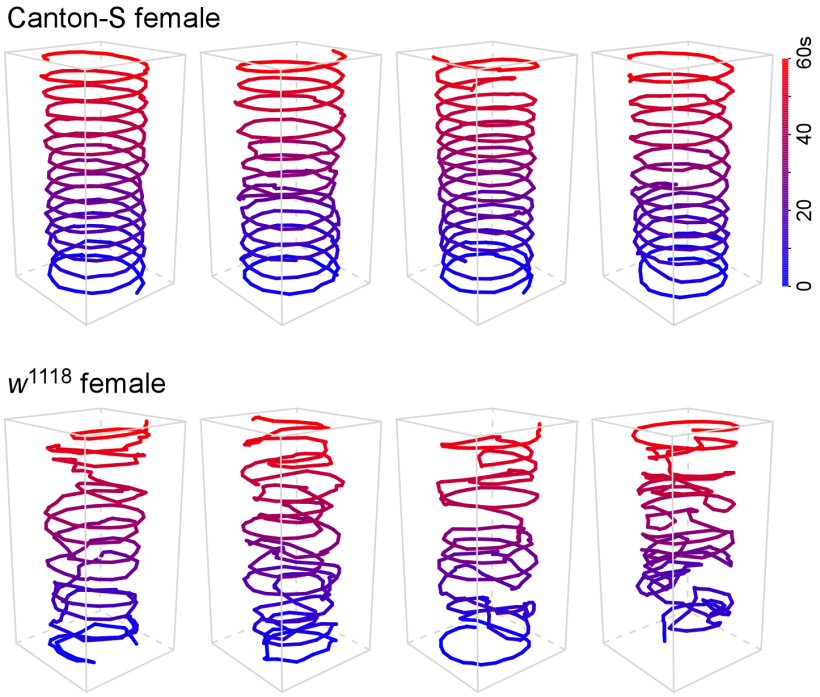

Figure S1: **Walking trajectories in Canton-S and w^{1118} female flies.** Persistent one-way walking was observed in Canton-S (upper panel), and it was likely lost in w^{1118} (lower panel) flies.

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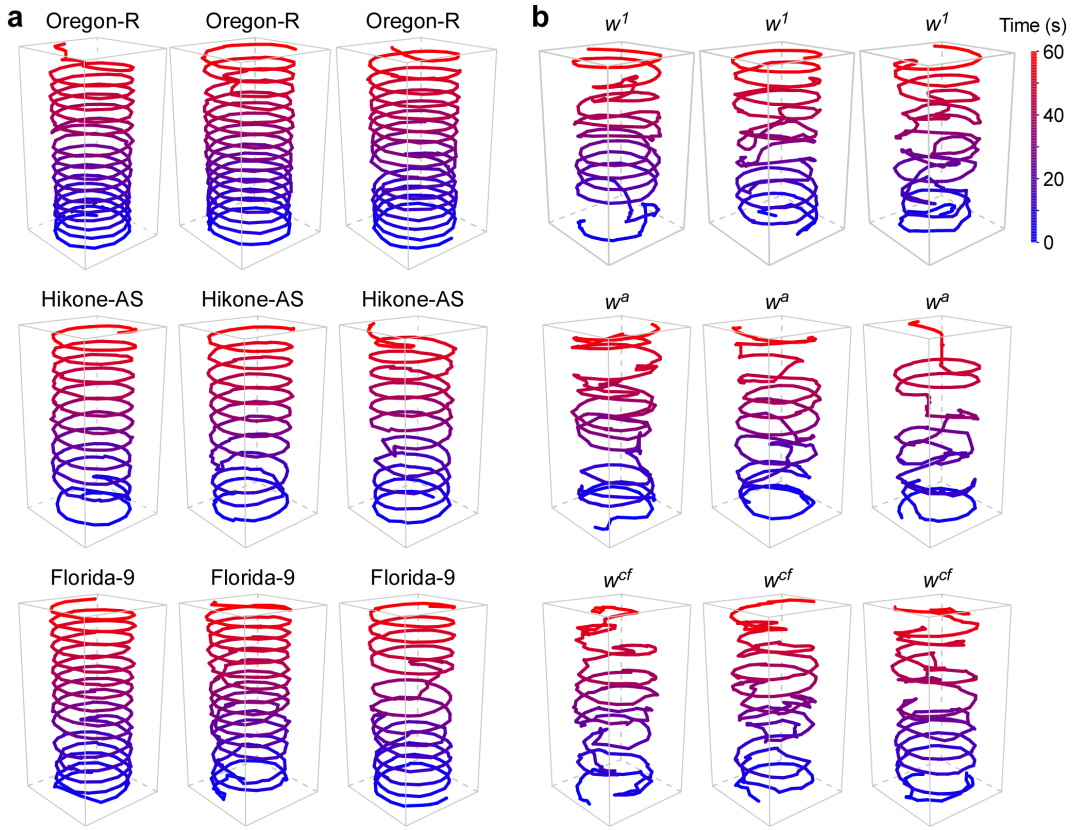

Figure S2: **Walking trajectories of several wild-types and *w* mutants.** (**a**) Walking trajectories of Oregon-R, Hikone-AS and Florida-9 in the circular arenas during 60 s locomotion. (**b**) Walking trajectories of w^1, w^a and w^{cf} in the circular arenas during 60 s locomotion. Color key indicates the time.