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Flying Speed in Drosophila melanogaster Selected for Fast Flight

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Flying Speed in *Drosophila melanogaster* Selected for Fast Flight

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Flying Speed and Power in *Drosophila melanogaster* Selected for Wind Tunnel Flight

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Flying Speed in *Drosophila melanogaster* Selected for Fast Flight

ABSTRACT

The aim of this study was to quantify the increase in flying speed in two replicate lines of *Drosophila melanogaster* (AA1 and AA2), after approximately 520 generations of selection for fast flight in a wind tunnel. A previous study had been done with the same goal (Simons, Weber & Walker; unpublished). The present study revisited two critical aspects of the previous one: (1) the methodology of measurement, and (2) the question of appropriate controls to quantify the increase in flying speed. After considerable testing of alternative techniques and methods, we were able to make only a few minor improvements in methods, and our measurements of flying speed essentially confirm those of the previous study, for flies of the same lines. However, on the question of appropriate controls, the present study invalidates the previous one. The previous study used the unselected lines CN1 and CN2 as controls, to compare to the selected lines AA1 and AA2. This comparison was not unreasonable, because the selected lines AA1 and AA2 were derived from lines CN1 and CN2, respectively, and these in turn were replicate sublines, both derived from the same original large laboratory base population. In the present study we compared the performances of AA1, AA2, CN1, and CN2, as before, but we added two new, recently wild-caught lines of flies (K45 and N60) to the testing array. The recently wild-caught lines had been allowed to adapt for either one year (N60) or two years (K45) under the same laboratory culture conditions that have been used to maintain the CN lines for the last 20 years. These two new lines were expected to display flight characteristics that were still nearly optimal for survival in the wild, plus an enhanced ability to grow well under our standard lab culture conditions. Comparison to these new lines showed that the CN lines had declined significantly in flying speed since their establishment. Therefore, if they were used as controls, the gain in speed of the selected AA lines would be seriously overestimated. The AA lines and the CN lines must be regarded as two alternative treatments. The AA lines have been strongly selected for ~20 years for increased flying speed, while the CN lines have been cultured for the same length of time in a confined environment that probably has selected weakly *against* flying performance. The two treatments have in fact produced opposite effects on flying speed. Clearly, for both of these treatments, recently wild-caught lines are the only appropriate controls. This allows us to return to the original question of whether the long period of intense selection on the AA lines may have actually increased their flying speed above that of their own original wild ancestors. Using these new controls, we were able to demonstrate that the wind tunnel selected (AA) flies do in fact still show a highly significant superiority in flying speed over nearly-wild conspecific flies, in our testing system. This superiority can probably be accounted for by the increase in body weight of the selected lines.

INTRODUCTION

Insects exhibit flying abilities greater than would be predicted on the assumption of steady state dynamics, and employ alternative mechanisms to produce lift and thrust, such as clap and fling, and vortex shedding. They exhibit surprising abilities but those abilities seem to remain within constraints that scale closely with body size (Ellington, 1984). The fruit fly, *Drosophila melanogaster*, is a model for insect flight at small sizes, and has shown considerable response to selection for flight performance in a wind tunnel (Weber, 1996).

Weber (1996) demonstrated a rapid increase in mean apparent flying speed in selected lines, from an initial base population value of ~0.2 m/s, to ~1.7 m/s, after the first 100 generations of selection. Increased apparent flying speed was manifested by the distribution of flies in the wind tunnel as their average ending position moved incrementally further upwind, over multiple generations, under defined standard conditions. Working with these selected lines at about generation 160, in a laser-tracking apparatus during free flight in still air, Marden *et al.* (1997) showed that they typically flew at a significantly higher average velocity than controls, and also on a more level average flight trajectory. Both of these behavioral traits would be expected to improve their performance in the wind tunnel. However, the study by Marden *et al.* (1997) could not show any increase in the *maximum* demonstrated performance capability (top speed) in the selected lines. Instead, the selected (AA) lines and the control (CN) lines both exhibited approximately the same top speeds, when the upper performance limits in the fastest-flying individuals were compared. On the other hand, this study could not definitively reject the hypothesis of increased maximum flying speed in the selected lines, because the measurement environment lacked the same behavioral cues (primarily a bright light at the upwind end) that were always present in the wind tunnel selection environment.

Marden *et al.* (1997) concluded that the performance increase of the selected lines in the wind tunnel was most likely explained by behavioral changes in phototaxis, mean flying speed, and mean angle of flight, rather than by an increase in their top speed. No doubt the selection process did select for these behavioral differences. Increased attraction to light would have meant that selected lines were simply more motivated to fly up a selection wind tunnel. The mean angle of flight was close to horizontal in selected lines. If flies were selected to fly more directly into the direction of wind flow, i.e. horizontally, this could also have contributed to their improved success in navigating upwind in the Weber tunnel, again improving their performance by changing their behavior. The results of Marden *et al.* (1997) suggest that there may have been no increase in actual maximum flying speed.

The present study quantified the performance of Weber's selected lines, in a comparison using the same control lines (CN1 & CN2) but also several new control lines of recently wild-caught flies. Marden *et al.* (1997) measured flying speed in still air, at generation 160 of selection, on selected lines and original controls. Simons *et al.* (unpublished) measured flying speed in a laminar-flow wind tunnel at generation 480, on the same lines. We measured flying speed again, in the same laminar-flow wind tunnel at generation 550, on the same selected and control lines, along with the two new lines of wild-caught flies. We also attempted to refine the methods used by Simons *et al.* (unpublished). Our goal was to test both sets of previous results and their interpretations.

MATERIALS AND METHODS

Drosophila Stocks: The origins and selection treatments of lines CN1, CN2, AA1 and AA2 are given in Weber (1996). Briefly, the two wild-type lines (CN1 and CN2) were initiated from a base population founded in 1981from several collection sites in Lincoln, MA, and the two selected lines (AA1 and AA2) were derived from these lines. At the time the studies reported here were initiated, lines AA1 and AA2 had been subjected to ~520 generations of selection for fast flight in a wind tunnel. Line K45 was collected with traps on compost piles at Khadigar Organic Farm in Industry, ME, in July 2008. Line N60 line was collected with traps on compost piles at various sites in Natick, ME, in July 2009. Lines K45 and N60 had been mass-cultured in 30-40 vials on commercial potato flake medium (Carolina Biological) for less than 2 years and less than 1 year, respectively, at the time these tests were run, during the fall and winter of 2009/2010.

Flies for Testing: We used "counted-egg cultures" to produce flies for testing. This is a standard protocol in the Weber lab. The objective is to produce flies that have developed under identical and optimal conditions. All major environmental factors are standardized, including the density of larvae per vial. The process begins by setting up simultaneous cultures for all lines in 6 liter clear plastic pretzel jugs. The jugs have screw-on lids with plastic mesh windows for ventilation. A food mix of nutritional yeast and commercial potato-flake medium is used. Each jug is set up with 50 g of the dry mix plus 180 ml tap water, and about 500 flies. After 12 days at 26° C the progeny are harvested and placed on fresh medium of the same type. After two days on this medium the new young flies are in top condition and entering peak fecundity. At this point they are placed in new plastic bottles containing metal strips covered with colored agar plus yeast and cornmeal. After an hour or less these strips are covered with fresh eggs and can be removed and placed under a stereomicroscope. Sections of agar with 50 eggs apiece are cut out and placed in culture vials, on 4 g of commercial potato-flake medium mixed with 17 ml tap water. These vials are cultured at 26°C. This method provided flies grown under standardized conditions for testing. Eclosing males and females were collected and separated by sex. Flies were placed on media and aged for three to four days before testing. In pilot tests we found that average test performance plateaued between 4-8 days after eclosion.

The Wind Tunnel: We used a 6" low speed, open circuit wind tunnel, Model# 401(s) serial# 3850z. from Engineering Laboratory Design Inc. (P.O. Box 278 lake City, MN. 55041, <http://www.eldinc.com/cgi-bin/StandardWindTunnel.pl?id=4>). The tunnel consists of a clear Lexan center section with a hinged lid and an inside dimension of 15 x15cm. Attached to this is an incurrent siphon designed to produce laminar flow and an excurrent unit that contains the 20 amp motor and fan assembly. The fan motor is controlled by an S7 operator interface. Both ends of the clear center unit are covered by aluminum frames holding metal screen material.

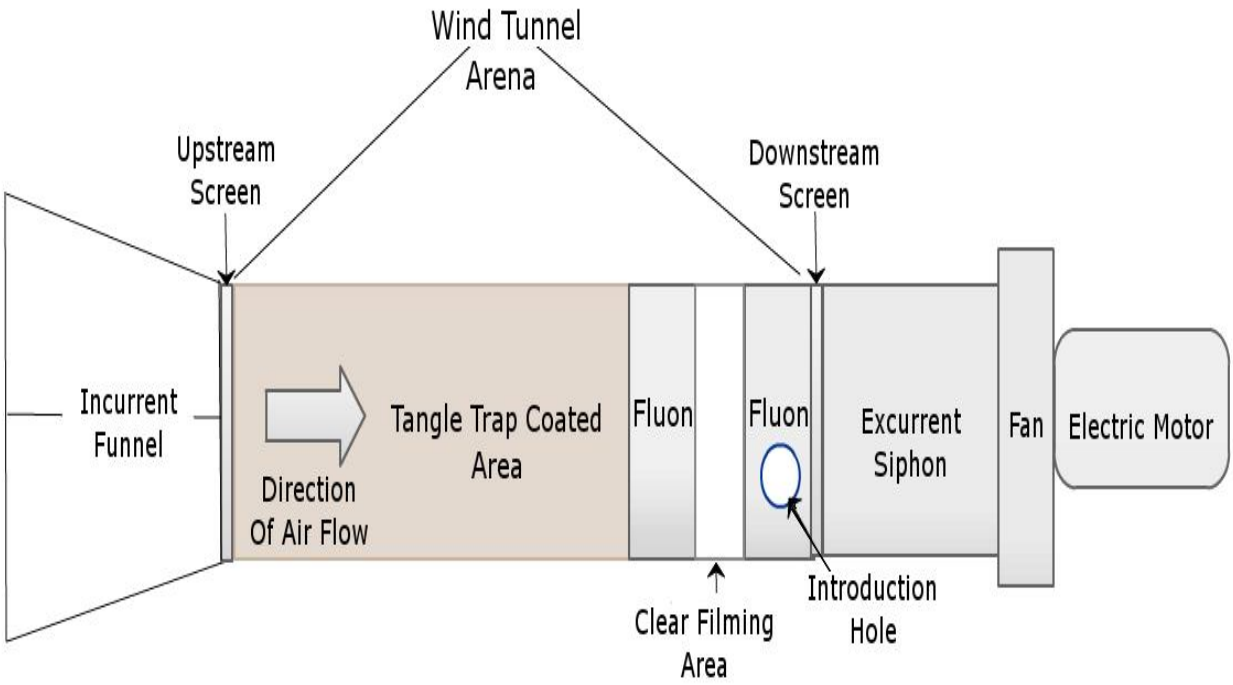


Figure 1. Diagram of the Wind Tunnel (Proportions not to Scale).

We observed flies preferentially crawling upstream in the tunnel against the airflow. In order to prevent this we coated the sides, top and bottom of the clear section with Fluon CD 090 (available from Bioquip). This non-stick surface prevents the insects from gaining purchase, so they can only move upwind by flying. The first 25cm of the tunnel was coated with the Fluon with the exception of a 4 cm filming window, 4 cm from the downwind end of the tunnel. Only flights that passed the entire window heading upstream were used for data.

To ensure that flies were only measured once, the walls of the tunnel farther upwind were covered with clear mylar membrane covered with a thin layer of Tangle-Trap insect coating manufactured by Contec Price. The Tangle Trap was spread using a six inch plastic putty knife. We cut the sheets to shape them, and then taped them down to a flat surface with masking tape along the two long edges. When the putty knife slid over the sheet it rode on the tape, laying down a layer of Tangle Trap that was the thickness of the tape. We attached the sheets to the top sides and bottom of the tunnel just upstream of the Fluon coated area using masking tape. Flies passing the Fluon coated area were trapped on contact with the Tangle Trap coating. After each individual sample run, flies were suctioned out of the tunnel to ensure that flies from one sample could not contaminate another. We used a Gist Manufacturing Corporation, model 022-v131-62772xsn:009, suction pump attached to 10mm OD plastic tubing. An empty plastic container acted as a sump for flies. There was a screen covered excurrent tube that connected to the suction pump. The incurrent tube emptied into the container and was used to suction up flies. Most flies dropped to the bottom of the container due to gravity. Both pieces of tubing entered through the cap by the use of plastic fittings. This allowed the container to be unscrewed from the cap and emptied.

It was desirable to introduce flies into the wind tunnel in a way that would minimize damage and escapes. The first thing tried was a 12mm x 70mm plastic test tube with one end removed. The inside of the tube was coated with Fluon with a screen placed over the removed end. We slipped 9mm tubing over the end with the screen. With this we could draw flies into the test tube by mouth suction and blow them into the tube. This set up allowed too many flies to escape and required a large puff of air that damaged flies.

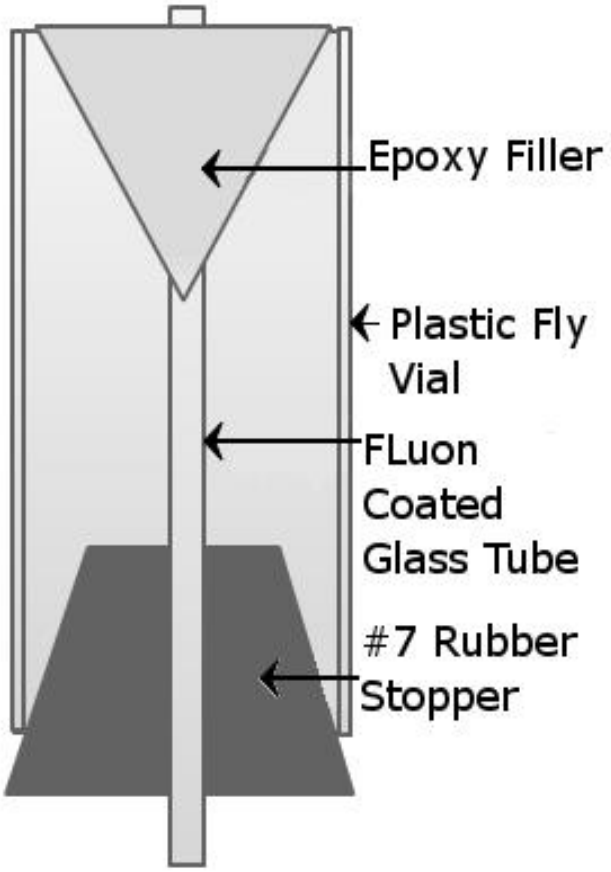


Figure 2. Diagram of the Fly Introduction Vial (Proportions not to Scale).

The measurement wind tunnel has a circular opening 34mm in diameter on the side, at the downwind end. We decided to use this to introduce the flies for each run. We constructed a device using a drosophila culture vial that had an outside diameter of 34mm (see figure #). With the lower end of the vial cut off it fit tightly into the tunnel opening. We cut a small polypropylene funnel to fit in the cut end of the vial with the narrow opening of the funnel centered in the opening of the vial. The space between the narrow opening of the funnel and the inside wall of the drosophila vial was filled in with 5 minute epoxy. We sanded the epoxy flush with the edge of both the vial and the cone. Except for the opening in the cone, the surface was flat. A piece of glass tubing with an outside diameter of 6mm passed through the cone and protruded 1 mm out the opening. The other end of the glass tube passed through a #7 rubber stopper and was covered with a piece of metal screen. The screen was held in place by 10mm plastic tubing that was slipped over it and secured with a stainless steel hose clamp. The inside of the glass tube and the surface of the epoxy were coated with Fluon. We introduced flies into the glass tube with the use of another polypropylene funnel. The funnel was cut to fit the 32mm

inside diameter of a culture vial and the narrow end inserted into a #7 rubber stopper. Once the inside surface of the cone was coated in Fluon the entire unit was used to cap a vial of live drosophila. The flies were then tapped gently down, through the funnel and into the glass tube of the first unit. The introductory unit was then slid into the opening of the wind tunnel until its forward face was flush with the tunnel wall. We then used a small puff of air to introduce the flies into the wind tunnel.

Measurement of Flying Speed: We used preliminary experiments to help determine the optimum length of test intervals. Flies were subjected to tunnel speeds of 31 Hz (134.43 cm/sec) and dialed down to 3 Hz (12.99 cm/sec, in 2 Hz steps. Each step lasted 3 minutes with the number of successful flights recorded every minute of the 3 minute step. We found that most flies flew in the first minute of each three-minute interval. Extending the test period beyond one minute would contribute little usable data and risk fatiguing the flies.

We also used preliminary experiments to determine appropriate initial wind speed settings for each line. The initial wind speeds we decided to use were 35 Hz (151.5 cm/sec) for the selected lines (AA1 and AA2); 30 Hz (129.9cm/sec) for the wild-type lines (K45 and N60); and 25 Hz (108.25 cm/sec) for the control lines (CN1 and CN2). These settings were 5 Hz higher than the maximum speed that flies of each line had been observed to fly against, insuring that the flies were initially pushed to the back screen of the tunnel, but by approximately the same margin above their highest speed, to avoid unduly tiring the flies.

In the actual measurement runs, wind speeds were decreased in regular steps of 2 Hz, at one-minute intervals, while applying regular physical stimulation (as described below) to encourage flies to take off. During each interval we recorded the images of flies passing upstream in the tunnel through a 5cm clear section that was 10cm upwind from the starting point.

Recording: All flights were recorded using a GFM electronics V502G-J66 digital camcorder with a 7.4mm F3.2 lens. We mounted the camera on a Diagnostic Instruments SM20 series adjustable boom stand. The boom stand was stabilized on a Kinetic Systems vibration damping table top. The camera was kept at a constant distance from the wind tunnel using a piece of aluminum angle attached to the camera mount. We would place the end of the angle up against the side of the tunnel positioning the lenses 40 cm from the outside wall of the wind tunnel. The angle also served to position the mount constantly. We marked the wind tunnel where the angle made contact allowing us to reposition the camera in the same place for every filming session.

All recordings were made using the VGA setting on the camera with a resolution of 640 x 480 pixels at 30 frames per second. Recordings were made on PNY Optima secure digital cards with a 2GB capacity. Each line was recorded on a different card each night. We transferred video clips to a windows PC using a Kingston Media external SD card reader. Videos were recorded in .avi format.

We used a flip card to record tunnel speed on the camera. A flip card set up was used with the tunnel setting printed on it. When a new tunnel speed was used the card was flipped to the appropriate speed visible in the video. Another card with the date line and sex of the flies being used was held up and recorded before each run. A Fischer scientific timer was also mounted to be visible in frame. The timer was reset and started at the beginning of every one-minute interval. The timer provided a visual indication on video of the time remaining in the test period.

We recorded all videos in .avi file format. Video recordings were then imported to a PC using a Kingston Media external SD card reader. We opened videos using ImageJ version 1.42 software available from National Institutes of Health (NIH) at <http://imagej.nih.gov/ij/>. Files were opened using virtual stack with compression and converted to gray scale.

Successful Flights: A successful flight was defined as a recorded flight that passed from right to left past the clear-walled recording section without touching a tunnel surface along the way. We were not able to obtain a successful flight recording for every individual that was introduced into the tunnel.

The camera field of view extended from the very top of the tunnel to the very bottom, so it was impossible for flies to fly above or below the field of view.

Missing Runs: The number of successful or useable flights was often much lower than the number of flies introduced. Flies could be lost in the transfer process from the vial to the wind tunnel (this was the main source of severe undercounts); also some individuals were able to evade the camera and the Fluon and still end up in the Tangle-Trap; and some individuals would simply refuse to fly at all (especially in the CN lines).

A second problem arose from occasional formatting problems with SD cards, causing runs that did not record properly. These runs did not yield any data at all and show up as missing samples here and there. Each run required about half an hour of continuous recording. Therefore each run was recorded on a separate SD card, so each missing run represents a problem with an individual card. Nights were only used when all the lines being compared were present in that night. This prevents the variation between nights from biasing for, or against, a line.

Determining Source Frames: The video frames used for measurements were chosen by reviewing the video of each successful flight. Our initial plan was to take an average measurement for each fly based on three consecutive intervals, between four consecutive frames. In the end we used only the single maximum speed for each fly, from one interval between two consecutive frames. Excel worksheets with the full data set were imported into Jmp8 statistical software. The summary function in Jmp was used to pull out the highest speed recorded for each fly. This data was then saved as an Excel sheet and reopened in Excel. We used the MID function to extract the wind tunnel speed, line, and date from the file name.

Measuring from Frames: We measured the distance traveled between frames by visually determining the centroid of the fly in the frame. Using the linear measurement tool, the centroid was clicked on and the frame then advanced. We moved the linear tool to the centroid in the second frame and ctrl + M was used to take and record the linear measurement. When each set of three measurements was taken they were saved using a unique file name. The naming convention was as follows; line, sex, tunnel setting in Hz, date, and order number of the fly at that speed. For example, AA1f.25.01.09.10-01 would be a female fly from the AA1 select line flying in the tunnel at the 25Hz setting on 1/9/2010 and the first to fly at that speed.

We pasted each file into Microsoft Excel using a program in visual basic written by Joseph Sungail (University of New England, Marine Science Masters graduate) that would tag each line of data with its file name, then copy it to a spreadsheet. Each line had a header with its file name, the angle of flight, and the distance traveled. The wind tunnel speed (Hz), date, line and sex were extracted from the file name and placed in separate columns using the MID command in Excel.

Calibration of the Wind Tunnel: It was necessary to correlate wind speeds with the Hz setting on the wind tunnel. We carried out calibration using a Phantom high speed digital camera with 50mm telocentric lenses. The camera was hooked up to a Dell laptop using a Fire Wire connection. Films were recorded using the Phantom proprietary software. The camera was mounted on a Diagnostic Instruments SM20 series adjustable boom stand. We lighted the tunnel using a high intensity halogen spotlight. The wind flow in the tunnel was visualized by burning a stick of incense in the air stream. We determined visually where the stream of smoke would pass in the wind tunnel. We focused the camera by moving it in and out on the boom stand until the smoke stream was in focus. Then we suspended graph paper in the tunnel at this point, parallel to the direction of wind flow. We moved the paper towards the camera until it was in focus so that it was on the same plane and distance from the camera as the smoke stream. We video recorded the paper at 1000 frames/sec with the same lighting as the smoke and saved the file.

The smoke was then recorded with the tunnel running at various Hz settings. Detectable landmarks were put into the smoke stream by banging on the wind tunnel. This caused the incense stick to bounce

slightly and a bump in the smoke stream moved down the tunnel at the same speed as wind flow. We made films at 5, 12, 15 and 20 Hz. The smoke was recorded and saved as cine files using the proprietary Phantom software. All cine files were converted into .avi format using the same software. The files were transferred onto a rewritable CD for transfer to another PC.

.Avi files were opened on ImageJ software available from NIH. The files were opened without converting to virtual stack and in gray scale. The .avi files with the graph paper were used to calibrate the software. We used the linear measuring tool to measure the pixels between the lines on the graph paper. In ImageJ the measurement of pixels can be set to represent a linear distance. The distance measured between lines was designated as 0.5cm and this calibrated the measuring tool to measure in centimeters from that point on. The films recording smoke movement were opened using the same settings. By advancing frame by frame we could track the "bump" in the smoke as it moved in the air stream. A starting point was designated on the apex of one of these bumps using the linear measurement tool in ImageJ. Then the video was advanced 10 frames and the measuring tool was used to determine the distance the bump traveled. This distance was divided by 10 to get an average distance traveled per frame in centimeters. Five measurements were taken per film and their measurements averaged. This average was then multiplied by 1000 frames per second to get the speed in centimeters per second. Centimeters per second were divided by the Hz setting the smoke was filmed at to derive centimeters per second per HZ. This was an average of 7.388 cm/sec/Hz across the different speeds.

Derivation of Velocity From Data: The distance traveled over the time elapsed provided a velocity vector of the flight. Along with the angle of flight and the velocity of wind in the tunnel we could derive the flies' actual velocity. Measurements made in ImageJ provided a speed in the distance traveled over time; distance traveled being the measurement from the video, and the time being the time elapsed per frame. Velocity combined with the angle from horizontal provides vector d. The actual flight speed T can be derived using the pythagorean theorum. The actual velocity of the flight T is equal to the square root of L^2+h^2, where $L = w + d'$ and $h = d \sin \theta$. θ is the flight angle and $d' = d \cos \theta$. T can be turned into a velocity by dividing it by the seconds per frame (0.066 seconds). W is the wind speed in cm/sec/0.066seconds; this is the distance traveled by the wind in time elapsed per frame. The actual velocity, T, is the speed the fly would have been traveling had it not been impeeded by the wind.

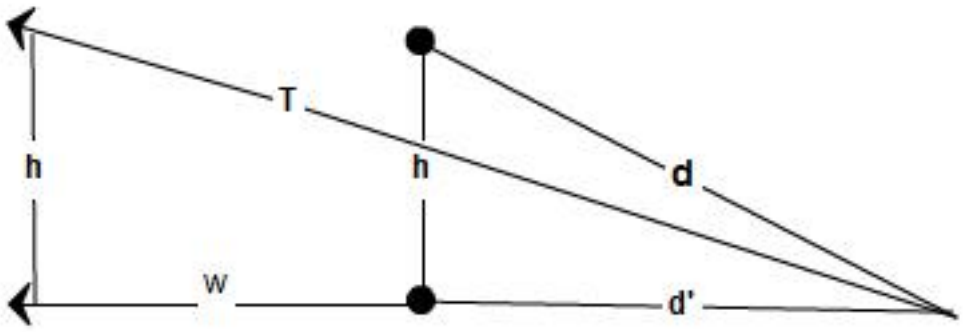


Figure 3. Diagram of Actual Velocity (T) versus measured Vector (d).

Data handling: Data was grouped according to the date and the line. Average speeds were computed by first computing the average for a line on a date. Overall averages for a line were then computed as an average of these averages.

Methods for Motivating Flight: Insects have a "hunker down" response to wind. They tend to crouch and wait out wind gusts. We considered and tested multiple ways to overcome this tendency and also to motivate the flies to fly rapidly once in flight.

1) Attraction to light. Light attracts flies. However, we decided to avoid that particular motivator. We already knew that the selected lines AA1 and AA2 had greatly elevated phototaxis. Wild Drosophila naturally exhibit positive phototaxis and can be selected to increase this trait (Hadler 1964). When selected for burst speed flight performance in Weber's (Weber 1997) wind tunnel, the flies were attracted to the terminal end of the tunnel using an incandescent light. This means that while being selected for increased flight performance the flies were also being selected for increased phototaxis. Using light to motivate flies could bias the performance toward the selected lines of flies. We wanted to measure any increase in performance in the selected lines that could not be due to light. In order to minimize the effect of phototaxis we covered the top panel of the tunnel with black construction paper and restricted the room lighting to overhead fluorescent light.

2) Attraction to Food. We tested a live culture of yeast as an attractant to both sexes of flies. Drosophila is attracted to the smell of yeast with the presence of food. The culture was started by dissolving 3, 1 oz. packets of Fleishman's dry active yeast and 600 milliliters dry volume of granulated cane sugar in 3 liters of warm tap water. We allowed the yeast to activate and grow for one half hour at room temperature before testing. The mixture was made in an open-top plastic container and placed in the intake of the wind tunnel. We mounted a small fan to blow on the yeast tub to disturb the boundary layer of air above the culture and ensure that scent was introduced into the tunnel. We tested this by placing AA1 select line flies in the wind tunnel with 3 oz. of yeast, warm water and 300 ml of sugar mixed as an attractant. Flies were tested using the procedure outlined in preliminary performance testing. Fresh mixture was made prior to each run. Preliminary testing showed increased performance when using actively growing yeast.

3) Attraction of Males to Virgin Females. We tested the scent of virgin females as an attractant for male flies. Male flies only were used in the flight portion of this trial. Virgin females were isolated and placed in a plastic box, with both ends removed, and the open ends covered with fine tent screen. This container was suspended in the intake of the wind tunnel where the wind flow would carry their scent through the tunnel. We tested the effect by placing AA1 select line flies in the wind tunnel with 3 oz. of yeast, warm water and 300 ml of sugar mixed as an attractant. Flies were tested using the technique outlined in preliminary performance testing. The first test with recently-eclosed females showed no significant increase in performance. A second test was also performed using "desperate" virgin females that had been held in culture without males for a week. The idea was that females nearing the end of their reproductive life might possibly produce more of the pheromones used to attract mates, since they are much more receptive to males by that point. Flies were again tested using the procedure outlined in preliminary performance testing. The males in this second trial of virgins again showed no significant increase in performance and this method was not used to attract flies in any other flight tests.

4) Startle Reaction—Banging. We built a device to deliver an impact of consistent magnitude to the rear screen of the wind tunnel. The device consisted of a piece of aluminum angle stock that attached to the wind tunnel and acted as a hinge. A piece of 1 inch mild steel square tubing acted as a travel arm. The arm rotated around a 1/4 inch fine thread bolt through both the tubing and the angle stock which was secured by a nut and washers. A threaded hole was cut in the opposite end of the tubing and a 3 inch piece of 1.4 inch threaded rod was threaded into it. Pieces of scrap steel pipe were put over the threaded rod to act as a weight. The striker was made from a brass machine screw attached

by its head to the steel tubing with JB weld epoxy. We positioned the striker so that it would strike the center of the frame of the screen that covered the exit of the tunnel. A strike transferred a shock downward through the screen that most flies would be standing on during the test. This technique was tested using the protocol outlined in preliminary flight testing. There was no significant increase in flight speeds as compared to flights without the banging. This device was not used in the final measurements.

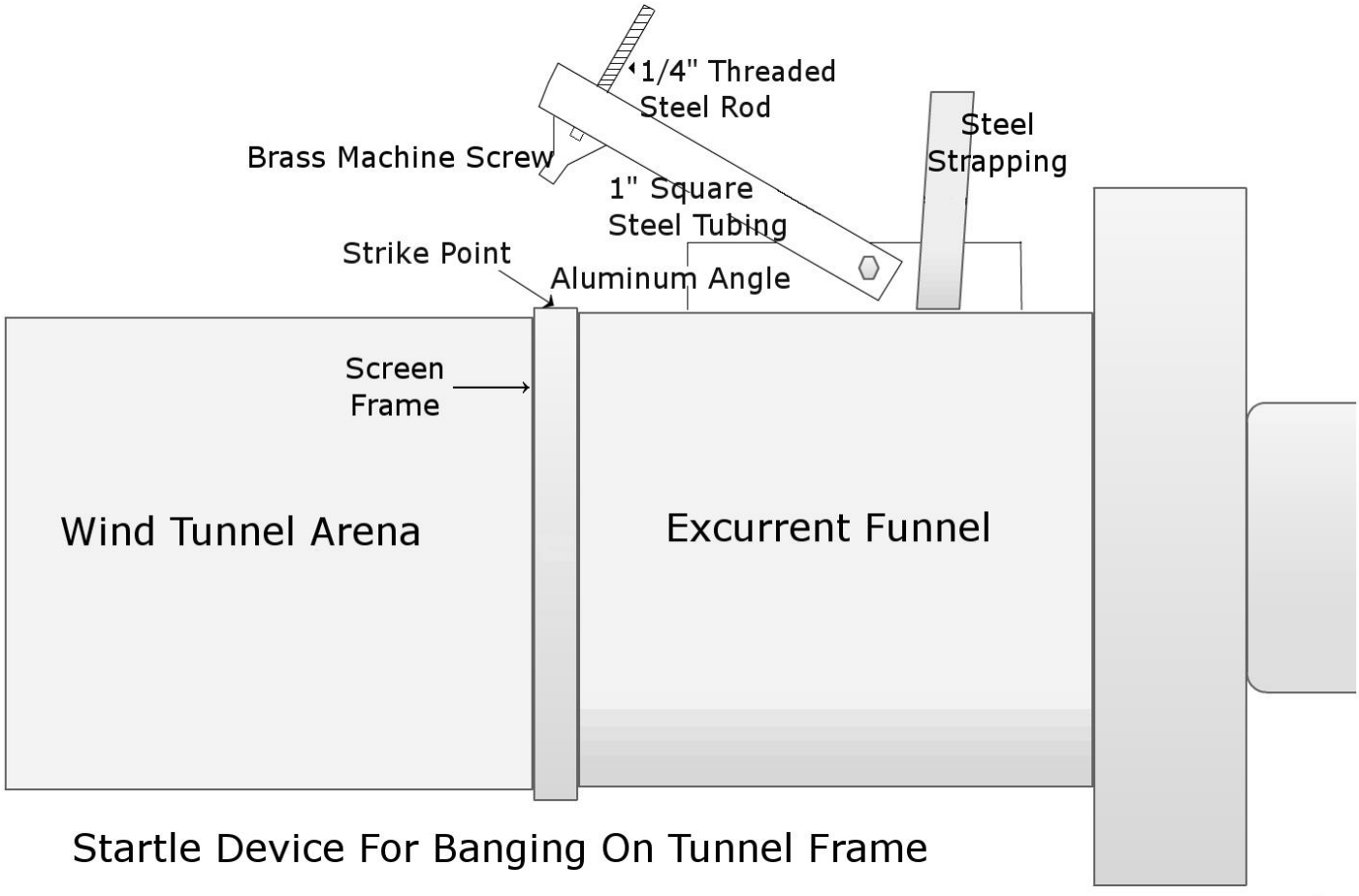


Figure 4. Startle Device for Banging on Tunnel Frame (Proportions not to Scale).

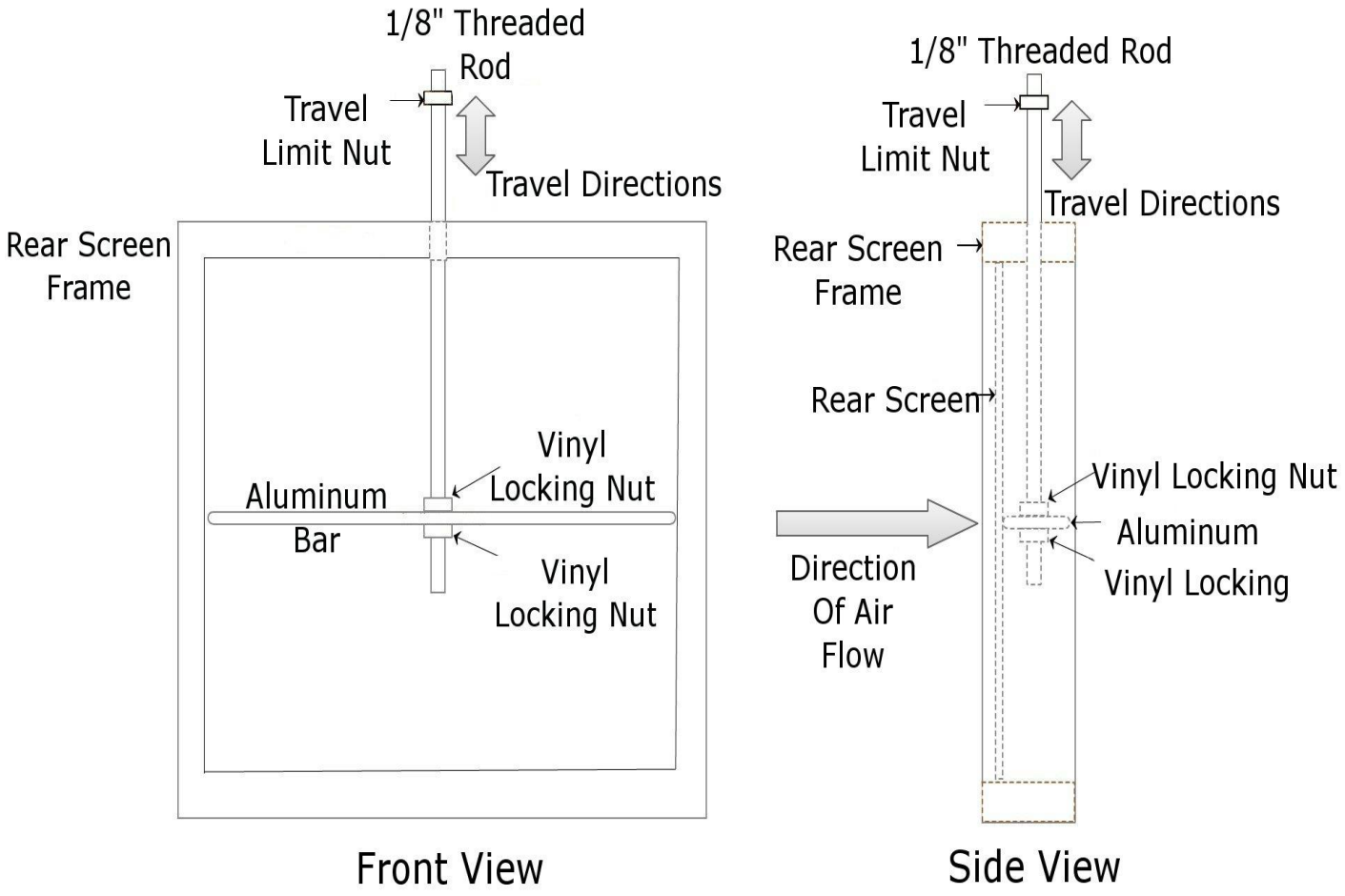


Figure 5. Front and Side Views of the Scraping Device. The rod was moved up and down, scraping the flat aluminum stock across the back of the screen material.

5) Startle Reaction—Scraping. We drilled the frame supporting the rear screen to accept a piece of 1/8 inch threaded rod. The threaded rod was passed through the hole and behind the screen. A 2mm x 20mm x 100mm strip of aluminum was drilled and attached to the rod with metal and vinyl locking nuts. We then replaced the screen frame in the wind tunnel. It was possible to move the threaded rod up and down, scraping the aluminum strip on the backside of the screen. The unit was moved up and down through its full range of motion, three times after every change in wind tunnel setting. We tested this technique by placing AA1 select line male and female flies in the wind tunnel with 3 oz. of yeast, warm water and 300 ml of sugar mixed as an attractant. Flies were tested using the procedure outlined in preliminary performance testing. Preliminary testing showed a significant increase in flight speeds when this approach to mechanical stimulation was used

RESULTS

There were 16 complete sets of measurement runs. A complete set of runs included 12 samples of flies—separate samples of males and females from each of the six lines: AA1, AA2, CN1, CN2, K45 and N60. Thus the 16 sets of runs included a grand total of 192 samples. All 12 samples in each complete set were cultured simultaneously under the same standardized conditions, and all were measured in a single evening.

The sex that was to be measured first was determined by a coin toss; then all the samples of that sex were completed first. Within sexes, the order that each line was run was determined using an online number generator.

Each sample contained about 30 flies. However, because of various difficulties the number of flies in each sample that yielded useable videos was rarely more than 25, and often much less (mean sample size and sd of measured sample = 15.89 ± 2.84 for females and 16.49 ± 0.96 for males). In a few samples, the number of measured flies was lower than three. Out of 192 samples, 29 or 15% had less than three useable videos. These samples were omitted from all calculations.

We also considered the effect of dropping all the data from a whole set, if it had one or more unusable or missing samples. To include any data from an incomplete set, where one or more lines were not represented, might have biased the comparisons. In Table 1 we compare the means of all lines and sexes with and without the data from incomplete sets. Table 1A shows the means where only the samples with less than three flies were omitted. Table 1B shows the means if whole sets with any unusable or missing samples excluded. The means in the two tables are not significantly different.

Table 1: Summary of Results

Table 1A: Grand means of all sample means, from all sets, including runs from sets with some missing runs. (Values extracted from Tables 2 & 3.)

	Males	(n)	(N)	SD	Females	(n)	(N)	SD
AA1	175.3	15	17	19.68	187.1	13	19	19.68
AA2	178.1	13	14	24.59	169.7	13	16	17.80
CN1	82.7	15	16	16.83	94.5	12	17	16.83
CN2	102.5	14	16	24.81	120.7	15	20	11.32
K45	120.4	12	15	21.54	124.5	15	12	17.59
N60	133.6	13	16	16.67	121.3	13	16	15.62

Table 1B: Grand means of sample means , including *only* runs from sets with no missing runs. (Values extracted from Tables 4 & 5.)

	Males	(n)	(N)	SD	Females	(n)	(N)	SD
AA1	171.6	8	18	19.33	177.2	7	22	11.19
AA2	169.8	8	16	17.37	169.9	7	15	12.63
CN1	80.9	8	17	15.12	90.7	7	16	18.63
CN2	107.2	8	16	26.47	119.3	7	17	14.96
K45	125.5	8	15	6.88	121.0	7	12	14.53
N60	136.0	8	16	14.09	119.5	7	15	12.47

n=Number of included sample means; N=Rounded mean number of flies/sample;
SD= Standard deviation of sample means.

The full data set is shown in Tables 2 and 3. These tables include all 16 sets, and all data from all samples, including the samples with less than three flies (these are not used in any calculations), for males (Table 2) and females (Table 3). These tables are presented graphically in Figures 6 and 7.

Table 2. Mean maximum speeds of male flies in centimeters/second.

Date	AA1m		AA2m		CN1m		CN2m		K45m		N60m	
	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N
8/6/2009	170.95	11	-	-	97.78	3	58.37	7	65.7	13	109.31	8
8/12/2009	165.2	24	137.69	14	73.62	18	73.48	22	133.52	11	130.39	11
11/21/2009	157.26	23	-	-	73.93	21	92.21	17	145.11	14	151.38	21
11/30/2009	158.38	12	167.52	25	74.74	25	108.5	19	133.71	11	139.62	19
12/5/2009	177.37	21	159.88	15	81.26	21	128.43	12	117.53	16	141.15	10
12/12/2009	170.73	25	186.26	13	96.47	13	117.77	12	127.32	14	145.62	27
12/19/2009	184.15	14	191.68	19	106.68	12	100.91	21	51.74	1	147.93	30
12/27/2009	155.64	16	200.14	19	75.72	18	104.39	16	-	-	130.52	16
1/3/2010	215.69	6	224.02	3	102.88	21	113.94	15	94.17	12	-	-
1/9/2010	180.84	20	182.08	17	56.28	9	127.06	17	129.8	21	133.96	15
1/16/2010	134.57	9	192.35	21	108.67	17	144.31	25	116.64	20	160.81	17
1/24/2010	195.09	14	153.69	12	71.01	18	95.69	16	116.92	11	109.02	9
1/31/2010	-	-	-	-	81.31	17	107.95	14	-	-	-	-
2/6/2010	184.11	17	143.83	13	54.86	5	-	-	135.43	16	109.54	14
2/14/2010	188.75	23	196.69	3	-	-	-	-	-	-	-	-
2/21/2010	190.49	19	179.19	14	85.49	17	62.5	6	128.65	17	127.18	17
Mean	175.28	17	178.08	14	82.71	16	102.54	16	120.38	15	133.57	16
St. Dev.	19.68	5.85	24.59	6.29	16.83	6.17	24.81	5.33	21.54	3.42	16.67	6.64

N is the number of measurable flights for each sample. The table shows all means for all lines from all 16 sets, including those samples with fewer than three measurable flights. However, the grand means and standard deviations do not include the data from the samples with fewer than three flights.

Table 3. Mean maximum speeds of female flies in centimeters/second.

Date	AA1f		AA2f		CN1f		CN2f		K45f		N60f	
	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N
8/6/2009	160.50	20	150.16	10	64.44	8	113.60	7	95.81	12	133.20	14
8/12/2009	169.82	20	165.05	13	67.40	10	101.11	16	117.13	12	108.12	12
11/21/2009	167.19	24	177.44	22	-	-	118.50	25	163.76	6	118.80	15
11/30/2009	177.7	13	159.71	23	87.93	12	-	-	124.54	18	121.59	25
12/5/2009	178.36	25	181.15	21	90.501	17	107.89	24	116.68	11	113.41	20
12/12/2009	180.72	26	179.83	23	116.04	20	109.75	20	124.22	15	130.20	15
12/19/2009	206.55	23	143.72	1	113.03	24	117.96	46	130.33	20	161.49	16
12/27/2009	219.7	4	220.89	1	115.28	23	134.65	20	-	-	-	-
1/3/2010	200.28	21	178.43	8	-		114.93	20	113.03	13	-	-
1/9/2010	170.22	22	159.43	10	97.79	16	126.71	30	144.38	3	100.39	16
1/16/2010	193.03	23	184.34	17	100.57	28	138.07	6	120.99	17	129.15	17
1/24/2010	224.19	2	125.71	8	84.29	7	120.55	17	98.86	6	121.01	19
1/31/2010	-	-	191.31	18	-	-	126.94	21	114.86	18	112.52	14
2/6/2010	-	-	184.14	18	98.23	25	129.77	24	129.21	12	104.80	14
2/14/2010	187.44	17	169.42	11	98.42	14	138.01	14	127.51	11	122.05	13
2/21/2010	220.34	3	218.05	2	-	-	111.98	11	146.75	13	-	-
Mean	187.07	19	169.70	16	94.49	17	120.69	20	124.54	12	121.29	16
St. Dev.	19.68	7.49	17.80	5.77	16.83	7.03	11.32	9.79	17.59	4.81	15.62	3.48

N is the number of measurable flights for each sample. The table shows all means for all lines from all 16 sets, including those samples with fewer than three measurable flights. However, the grand means and standard deviations do not include the data from the samples with fewer than three flights.

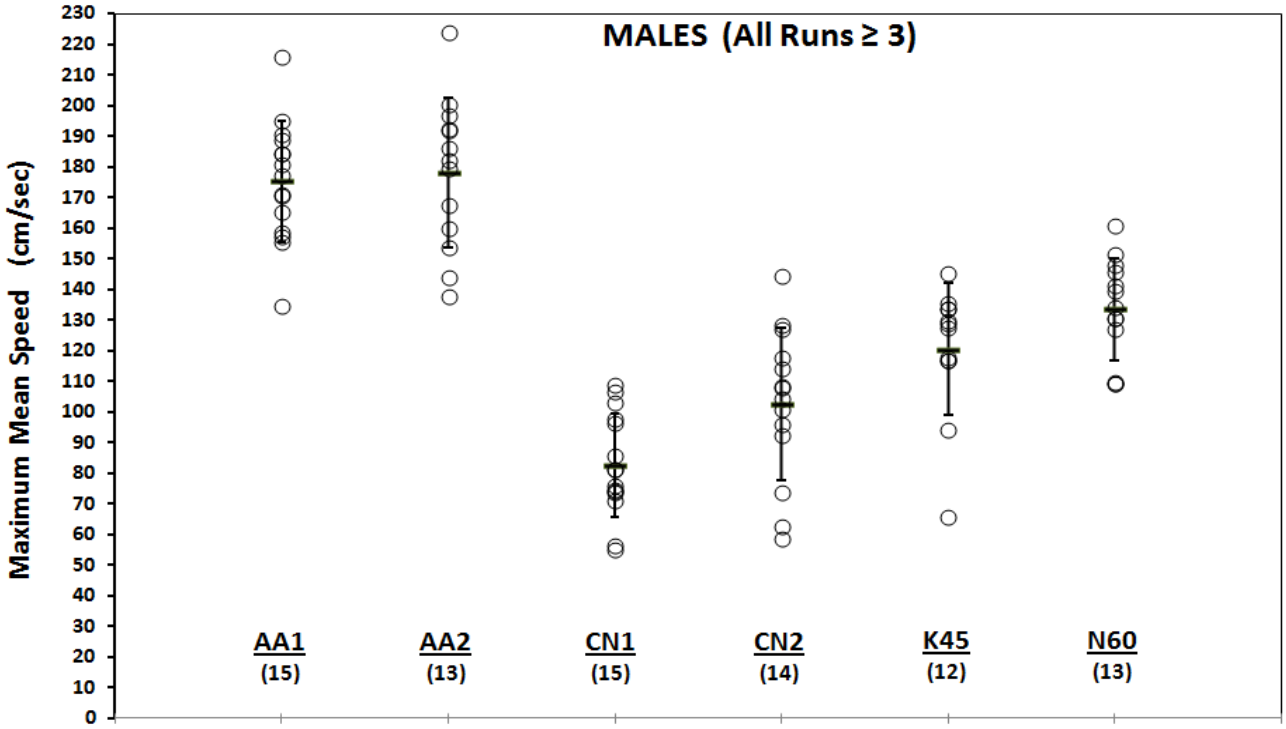


Figure 6. Sample means of the maximum speeds of male flies, in cm/sec, for all six lines. Each data point represents the mean fastest speed (*i.e.*, the mean of the single fastest speed recorded during the flight of each fly) in one sample, including all samples with measurable flights of 3-30 individuals. The total number of such samples for each line is shown in parentheses. The total number of measurable flights per sample is shown in Table 2. Vertical lines show ± one standard deviation.

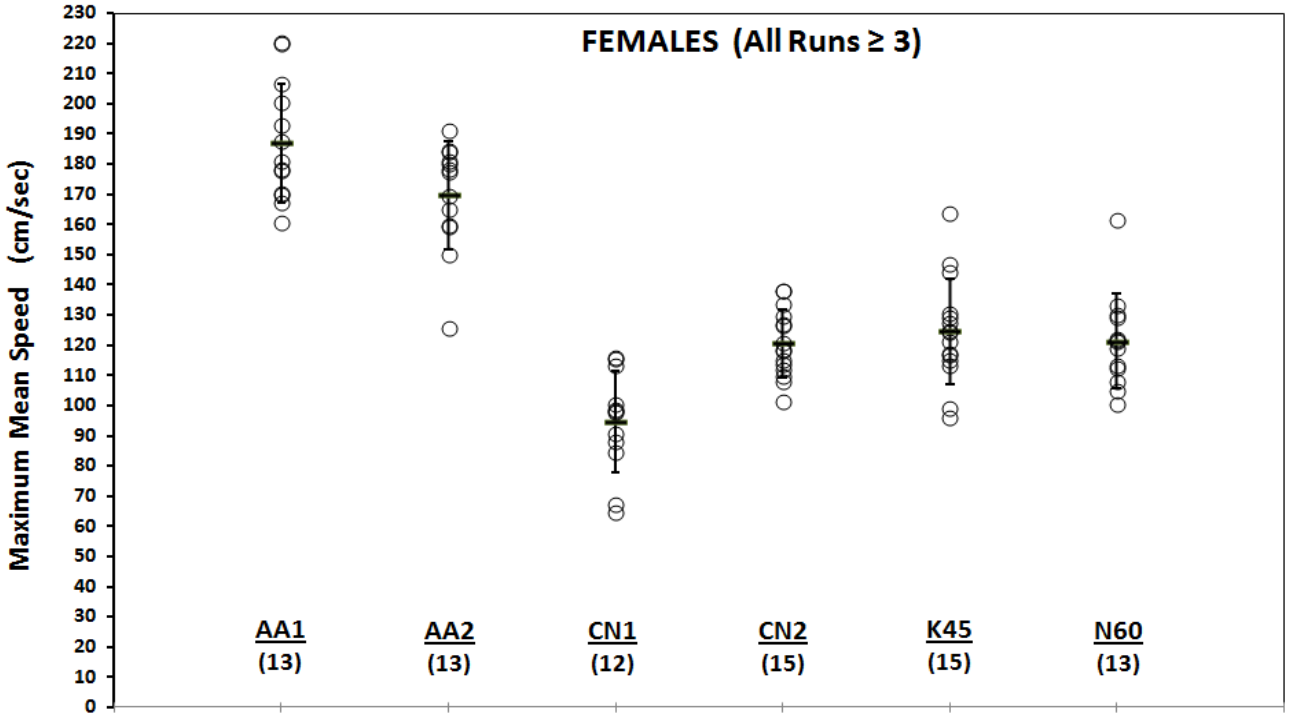


Figure 7. Sample means of the maximum speeds of female flies, in cm/sec, for all six lines. Each data point represents the mean fastest speed (i.e., the mean of the single fastest speed recorded during the flight of each fly) in one sample, including all samples with measurable flights of 3 to 30 individuals. The total number of such samples for each line is shown in parentheses. The total number of measurable flights per sample is shown in Table 3. Vertical lines show ± one standard deviation.

Tables 4 and 5 show only the data from evenings when all six runs of one sex were useable. These include only 8 sets for males, and only 7 sets for females. These tables also include only the samples with three or more flies, for males (Table 2) and females (Table 3). These means are not much different from the previous means but would have to be considered as a fairer comparison between lines. These tables are presented graphically in Figures 8 and 9.

Table 4. Means for nights when all six runs for males were useable.

Date	AA1m		AA2m		CN1m		CN2m		N60m		K45m	
	mean max. Speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N
8/12/2009	165.20	24	137.69	14	73.62	18	73.48	22	130.39	11	133.52	11
11/30/2009	158.38	12	167.52	25	74.74	25	108.50	19	139.62	19	133.71	11
12/5/2009	177.37	21	159.88	15	81.26	21	128.43	12	141.15	10	117.53	16
12/12/2009	170.73	25	186.26	13	96.47	13	117.77	12	145.62	27	127.32	14
1/9/2010	180.84	20	182.08	17	56.28	9	127.06	17	133.96	15	129.80	21
1/16/2010	134.57	9	192.35	21	108.67	17	144.31	25	160.81	17	116.64	20
1/24/2010	195.09	14	153.69	12	71.01	18	95.69	16	109.02	9	116.92	11
2/21/2010	190.49	19	179.19	14	85.49	17	62.50	6	127.18	17	128.65	17
Mean	171.58	18.00	169.83	16.38	80.94	17.25	107.22	16.13	135.97	15.63	125.51	15.13
St. Dev.	19.33	5.39	17.37	4.18	15.12	4.49	26.47	5.69	14.09	5.50	6.88	3.79

Mean maximum speeds in centimeters/second for all lines on each date with all six lines represented by samples with ≥ 3 measurable flights, for male flies. N is the number of measurable flights for each line on each night.

Table 5. Means for nights when all six runs for females were useable.

Date	AA1f		AA2f		CN1f		CN2f		N60f		K45f	
	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N	mean max. speed	N
8/6/2009	160.50	20	150.16	10	64.44	8	113.60	7	133.20	14	95.81	12
8/12/2009	169.82	20	165.05	13	67.40	10	101.11	16	108.12	12	117.13	12
12/5/2009	178.36	25	181.15	21	90.50	17	107.89	24	113.41	20	116.68	11
12/12/2009	180.72	26	179.84	23	116.04	20	109.75	20	130.20	15	124.22	15
1/9/2010	170.22	22	159.43	10	97.79	16	126.71	30	100.39	16	144.38	3
1/16/2010	193.03	23	184.34	17	100.57	28	138.07	6	129.15	17	120.99	17
2/14/2010	187.44	17	169.42	12	98.41	14	138.01	15	122.05	10	127.51	11
Mean	177.16	21.86	169.91	15.14	90.74	16.14	119.31	16.86	119.50	14.86	120.96	11.57
St. Dev.	11.19	3.13	12.63	5.27	18.63	6.64	14.96	8.69	12.47	3.29	14.53	4.39

Mean maximum speeds in centimeters/second for all lines on each date with all six lines represented by samples with ≥ 3 measurable flights for female flies. N is the number of measurable flights for each line on each night.

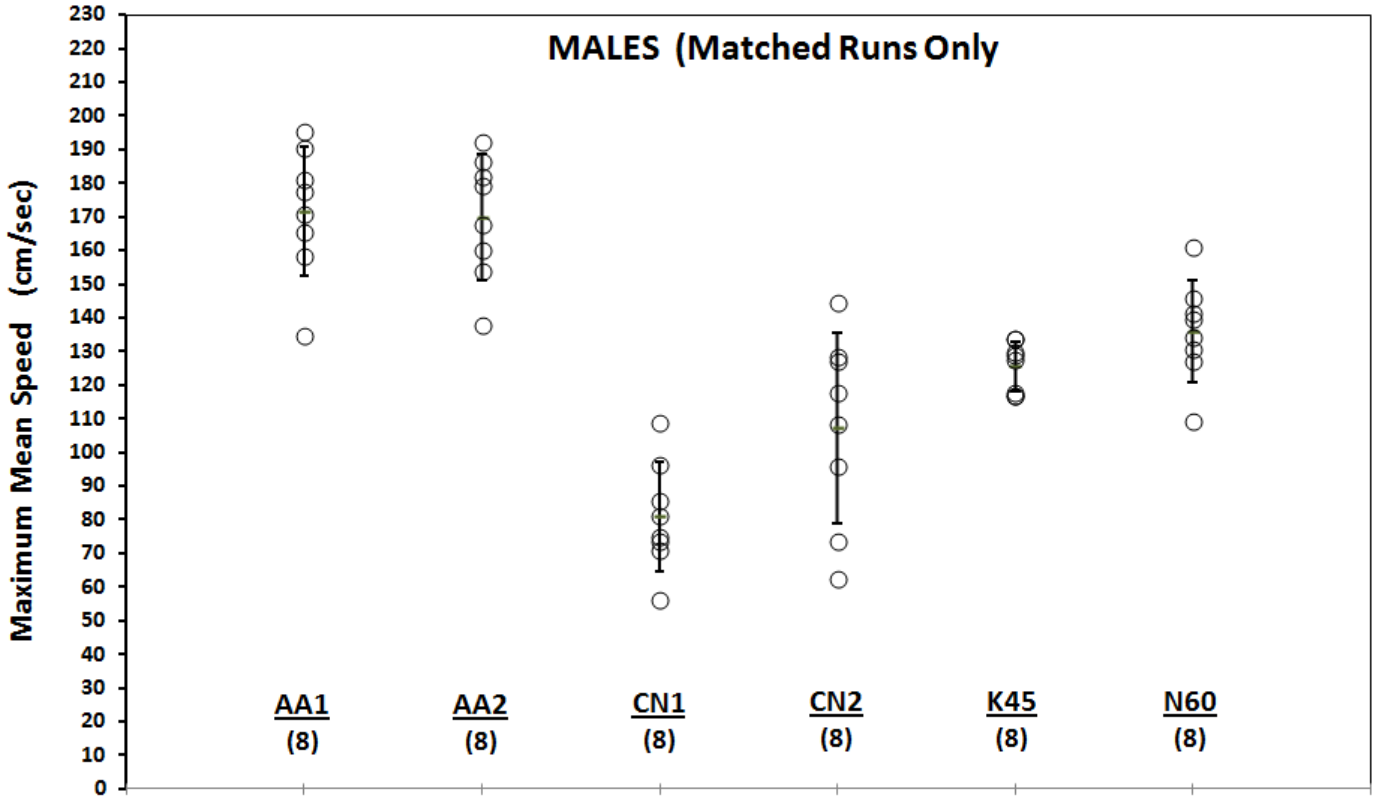


Figure 8. Comparison of the mean maximum speeds of male flies in each line, using only data from complete sets, i.e., sets with samples of ≥3 flies for all lines. Each data point represents the average maximum speed of individuals of a line for a specific set on a specific date. Data from Table 4. Vertical lines show ± one standard deviation.

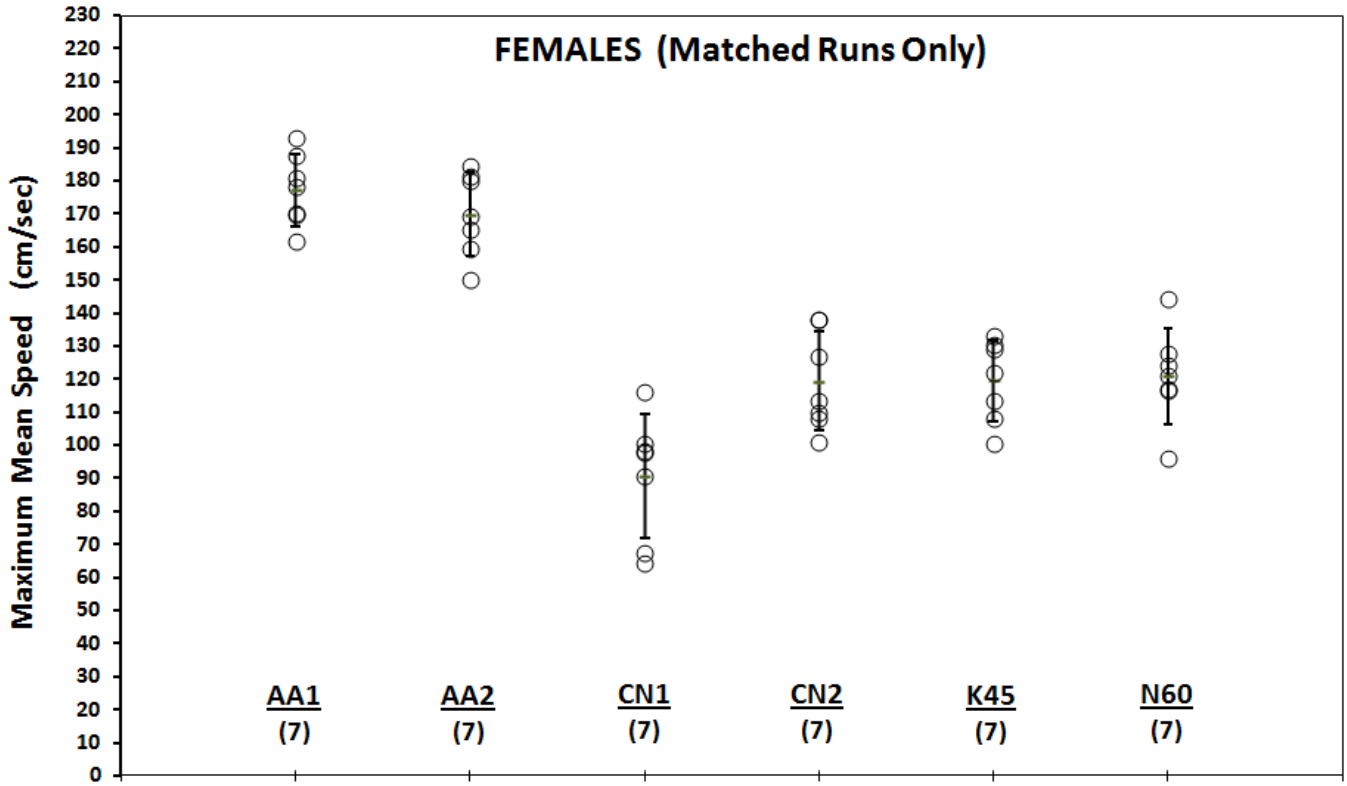


Figure 9. Comparison of the mean maximum speeds of female flies in each line, using only data from complete sets, i.e., sets with samples of ≥3 flies for all lines. Each data point represents the average maximum speed of individuals of a line for a specific set on a specific date. Data from Table 5. Vertical lines show ± one standard deviation.

Tables 6 and 7 show only the data from lines AA1, AA2, N60, and K45, from evenings when all four of these lines provided useable data in one sex. Lines CN1 and CN2 are omitted here, because our data show they are inappropriate as controls. By restricting the data to these four lines, we increase the number of useable sets to nine for males, and also nine for females, still including only samples with three or more flies. These means are the most accurate for comparing selected and wild-type lines, to estimate the true response to selection. These tables are presented graphically in Figures 10 and 11.

Table 6. Means for all nights with useable runs for males of both selected and both wild lines.

Date	AA1m		AA2m		N60m		K45m	
	mean max speed	N	mean max speed	N	mean max speed	N	mean max speed	N
8/12/2009	165.20	24	137.69	14	130.39	11	133.52	11
11/30/2009	158.38	12	167.52	25	139.62	19	133.71	11
12/5/2009	177.37	21	159.88	15	141.15	10	117.53	16
12/12/2009	170.73	25	186.26	13	145.62	27	127.32	14
1/9/2010	180.84	20	182.08	17	133.96	15	129.80	21
1/16/2010	134.57	9	192.35	21	160.81	17	116.64	20
1/24/2010	195.09	14	153.69	12	109.02	9	116.92	11
2/6/2010	184.11	17	143.83	13	109.54	14	135.43	16
2/21/2010	190.49	19	179.19	14	127.18	17	128.65	17
Mean	172.98	18	166.94	16	133.04	15	126.61	15
St. Dev.	18.56	5.4	19.41	4.3	16.62	5.5	7.64	3.8

Average maximum speeds of male flies for select and wild type lines on each night with all lines represented. N is the number of measurable flights for each line on each night.

Table 7. Means for all nights with useable runs for females of both selected and both wild lines.

Date	AA1f		AA2f		N60f		K45f	
	mean max speed	N	mean max speed	N	mean max speed	N	mean max speed	N
8/6/2009	160.50	20	150.16	10	133.20	14	95.80	12
8/12/2009	169.82	20	165.05	13	108.12	12	117.13	12
11/21/2009	167.19	24	177.44	22	118.80	15	163.76	6
11/30/2009	177.70	13	159.71	23	121.59	25	124.54	18
12/5/2009	178.36	25	181.15	21	113.41	20	116.68	11
12/12/2009	180.72	26	179.83	23	130.20	15	124.22	15
1/9/2010	170.22	22	159.43	10	100.39	16	144.38	3
1/16/2010	193.03	23	184.34	17	129.15	17	120.99	17
2/14/2010	187.44	17	169.42	11	122.05	13	127.51	11
Mean	176.11	21	169.61	17	119.66	16	126.11	12
St. Dev.	10.25	3.9	11.81	5.7	10.83	4.0	18.95	4.9

Average maximum speeds of female flies for select and wild type lines on each night with all lines represented. N is the number of measurable flights for each line on each night.

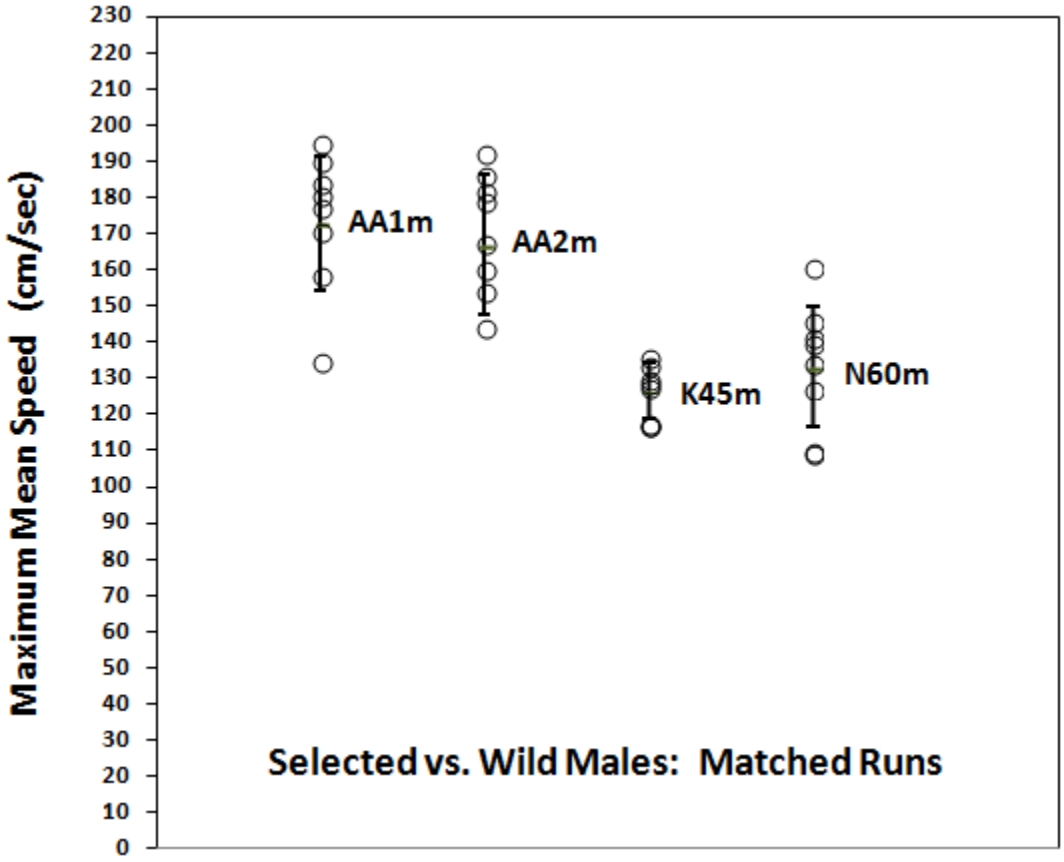


Figure 10. Comparison of the average maximum speed of selected line (AA1 & AA2) and wild type (K45 & N60) males flies in each line on nights with samples of ≥3 flies for all four of these lines. Each data point represents the average maximum of a line for a specific night. Vertical lines show ± one standard deviation

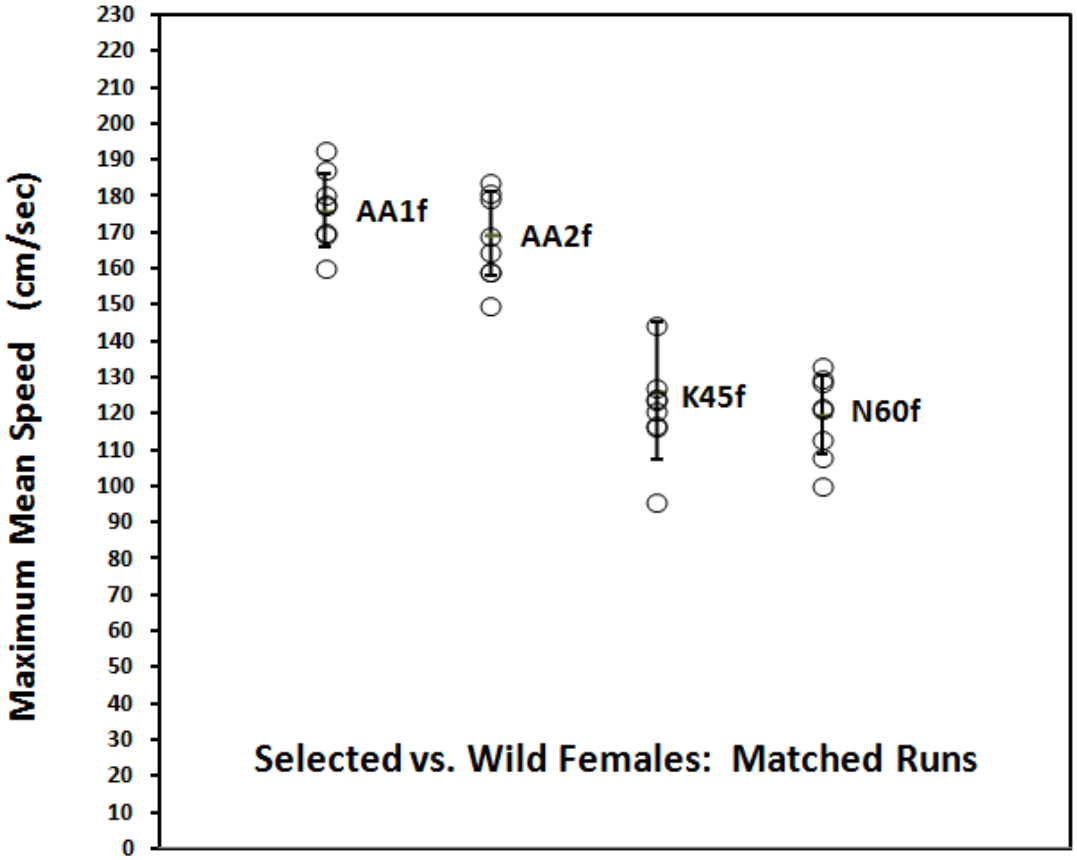


Figure 11. Comparison of the average maximum speed of selected line (AA1 & AA2) and wild type (K45 & N60) females flies in each line on nights with samples of ≥3 flies for all four of these lines. Each data point represents the average maximum of a line for a specific night. Vertical lines show ± one standard deviation.

The selected lines (AA1 and AA2) show much faster flying speeds than any of the unselected lines (CN1, CN2, K45 and N60), in both sexes (Figures 8 and 9; Tables 4 and 5). These speeds represent the male grand means of the sample means of maximum flight speed attained by individuals for each line: AA1 (171.58 ± 19.33) AA2 (169.83 ± 17.37), CN1 (80.94 ± 15.12), CN2 (107.22 ± 26.47), N60 (135.97 ± 14.09), K45 (125.51 ± 6.88). Comparisons of each pair of means using the Tukey-Kramer HSD test show significant differences at an alpha of 0.05. Comparisons of the AA and CN lines show P-values <0.0001. The N60 line flies differed significantly from the CN1 (P=<0.0001) and CN2 (P=0.0375) lines. The N60 line was significantly different from the AA1 (p=0.0050), AA2 (p=0.0086) but not the K45 (p=0.8667) line. The K45 line differs significantly from the AA1 line (p=<0.001), AA2 (p=<0.001) lines and the CN1 control line (p=<0.001). K45 did not differ from the CN2 line (p=0.3739). There is no significant difference between each of the two AA lines (p=0.9999) Control (CN) lines showed no statistical difference (p=0.0711).

Using only data from the nights where all lines of females are represented, the selected AA lines show improved performance over CN lines, CN, K45 wild type line and N60 wild type lines. The AA lines had the highest performance averaging 177.16 ± 11.19 for the AA1 line and 169.91 ± 12.63 for the AA2 line followed by the K45 line at 120.96 ± 14.53 and the N60 line at 119.50 ± 12.47. The control lines CN1 and CN2 had the lowest performance at 90.74 ± 18.63and 119.31 ± 14.96 (Table 5). These results represent the mean of the mean maximum flight speed for each line on each useable night. Comparisons using Tukey-Kramer HSD t-test of both AA lines and the CN1 lines show

significant differences at an alpha of 0.05 with P-values <0.0001. The CN2 line was statistically different from the AA1 line (p=<0.001) and the AA2 line (p=<0.001). The N60 line flies differed significantly from the AA1 (p=<0.001) and AA2 (p=<0.001) lines. The N60 line was significantly different from the CN1 line (p=0.0076) but not the CN2 line (p=1.000) and the K45 line (p=0.999). The K45 line differs significantly from the AA1 select line (p=<0.001) and the AA2 line (p=<0.001). K45 did differ from the CN1 line (p=0.0042). K45 did not differ from the CN2 control line (p=0.4041). There is no significant difference between each of the two AA lines (p=0.930) or between the control (CN) lines (p=0.0755).

The comparison among all lines makes it evident that control lines CN1 and CN2 had lost performance ability and could not be used as controls to estimate the performance gain in the selected lines AA1 and AA2. We conclude that the more legitimate comparison is between the selected lines (AA1 & AA2) and the new wild-type lines (K45 and N60) (Tables 6 and 7; Figures 10 and 11).

In a comparison of mean maximum speed in males for selected (AA) and wild type lines (K45 and N60) on nights with data for all four lines, the selected (AA) lines showed a mean performance of 172.98 ± 18.56 cm/sec for the AA1 line and 166.94 ± 19.41 cm/sec for the AA2 line. The wild-type lines showed a mean performance of 126.61 ± 7.64 cm/sec for line K45 and 133.04 ± 16.62 cm/sec for line N60. Using Tukey-Kramer HSD t-test at an alpha of 0.05 we found a significant difference between the selected AA1 line and the wild type K45 (p=<0.0001) and N60 (p=<0.0001) lines. The AA2 lines were also significantly different from the K45 (p=<0.0001) and N60 (p=0.0001) lines. There was no significant difference between the two AA lines (p=0.893) or the K45 and N60 lines (p=0.824).

In a comparison of mean maximum speed in females, selected (AA) lines and wild type lines (K45 and N60) on nights with data for all four lines, selected (AA) lines showed improved performance over K45 and N60 wild type lines. Selected (AA) lines had a mean performance of 176.11 ± 10.25 cm/sec for the AA1 line and 169.61 ± 11.81 cm/sec for the AA2 line. Line K45 mean performance was 126.11 ± 18.95 cm/sec and line N60 mean performance was 119.66 ± 10.83 cm/sec. Using Tukey-Kramer HSD test at an alpha of 0.05 we found a significant difference between the selected AA1 line and the wild type K45 (p=<0.0001) and N60 (p=<0.0001) lines. The AA2 lines were also significantly different from the K45 (p=<0.0001) and N60 (p=<0.0001) lines. There was no significant difference between the two AA lines (p=0.736) or the K45 and N60 lines (p=0.739).

Body weights were obtained for all lines and sexes on 15 of the 16 sets of evening runs. These body weights show a relation to flying speed. Figure 12 shows the full data set. The body weights of all the unselected lines (CN1, CN2, K45, and N60) are closely comparable to each other, but the flying speeds are not, because of the lower performance of the CN1 line. The CN lines do not show quite the same relation between body weight and flying speed, because they have lost flying speed (CN1 in particular) but not body weight. Figure 13 shows the same data as Figure 12, minus the data for lines CN1 and CN2. This compares only "normal" flyers (K45 and N60) to selected flyers (AA1 and AA2). A regression line is shown for each sex. Bigger flies fly faster, but the dependence of speed on body weight is different in males and females.

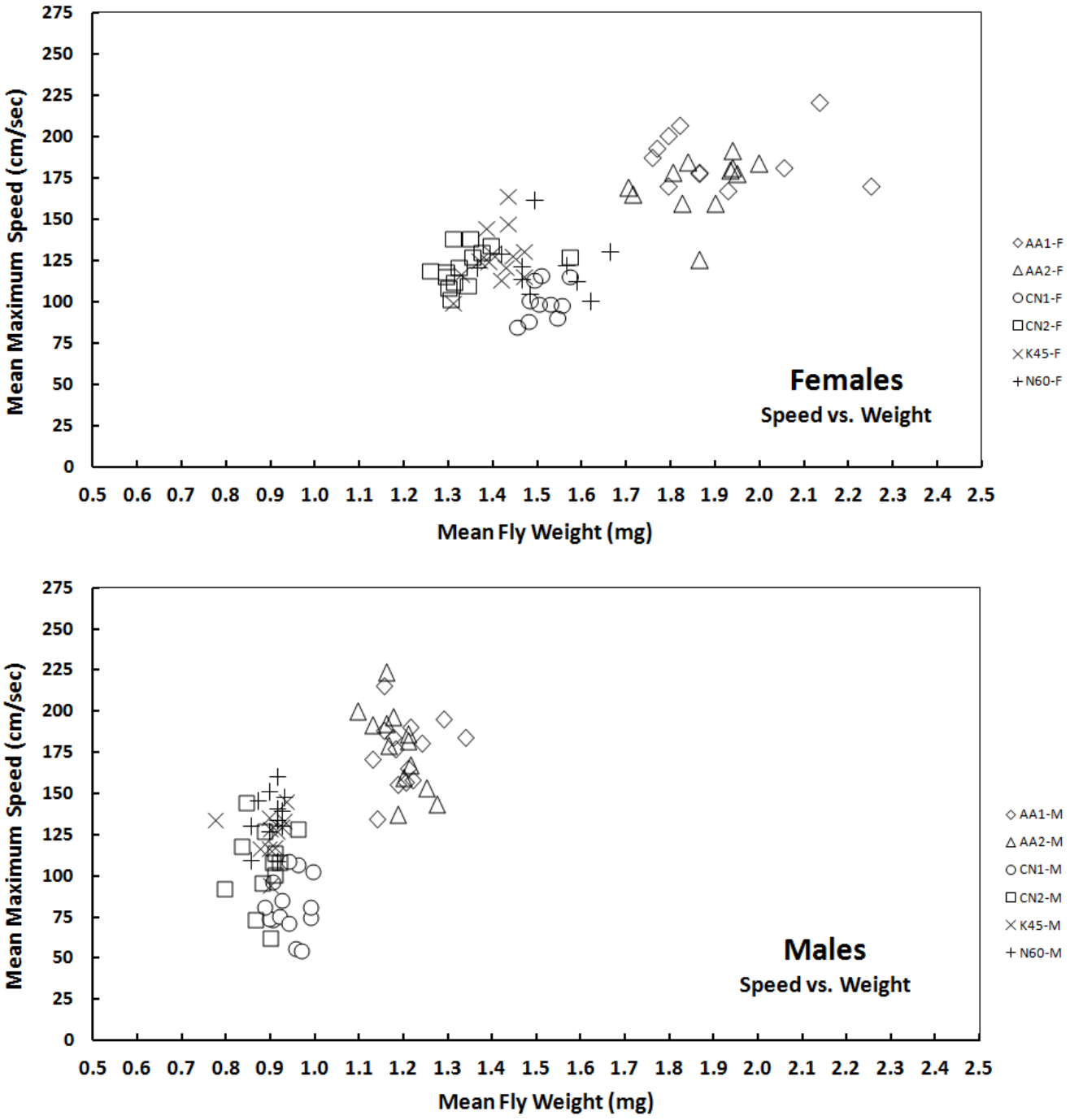
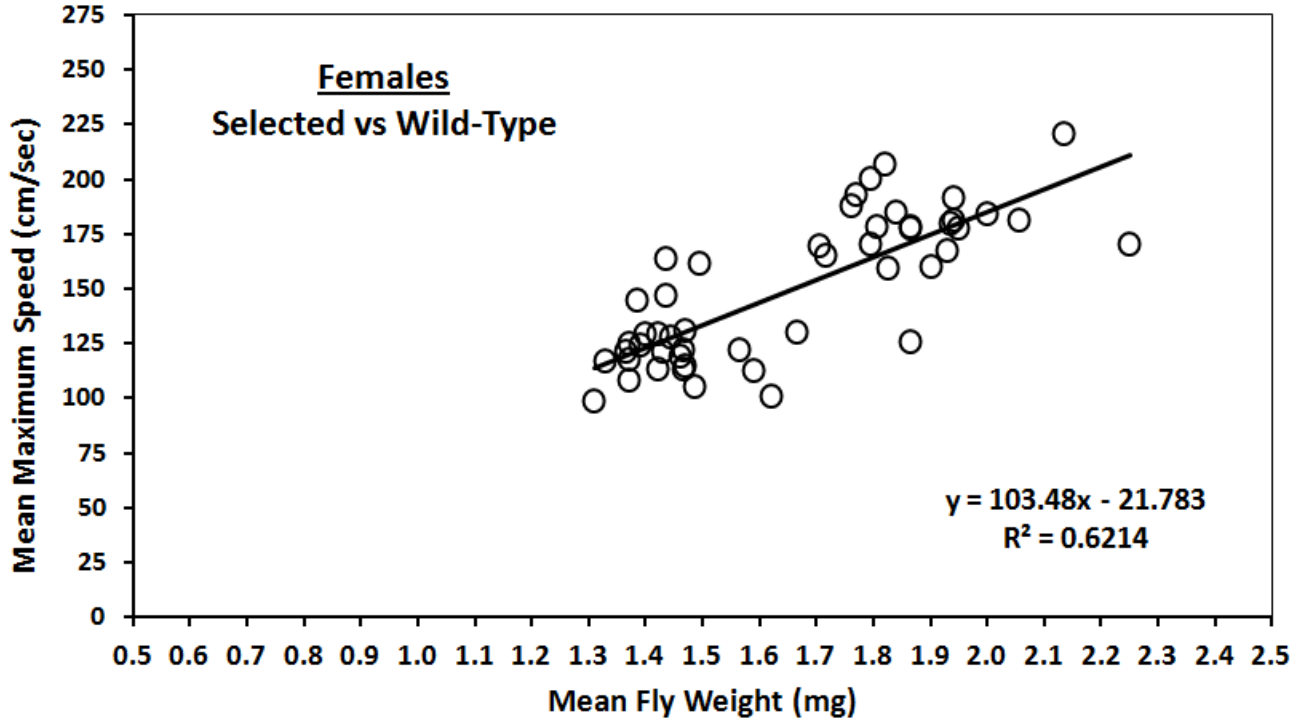


Figure 12. Mean sample flying speeds as a function of mean sample body weights for males and females of all six lines.



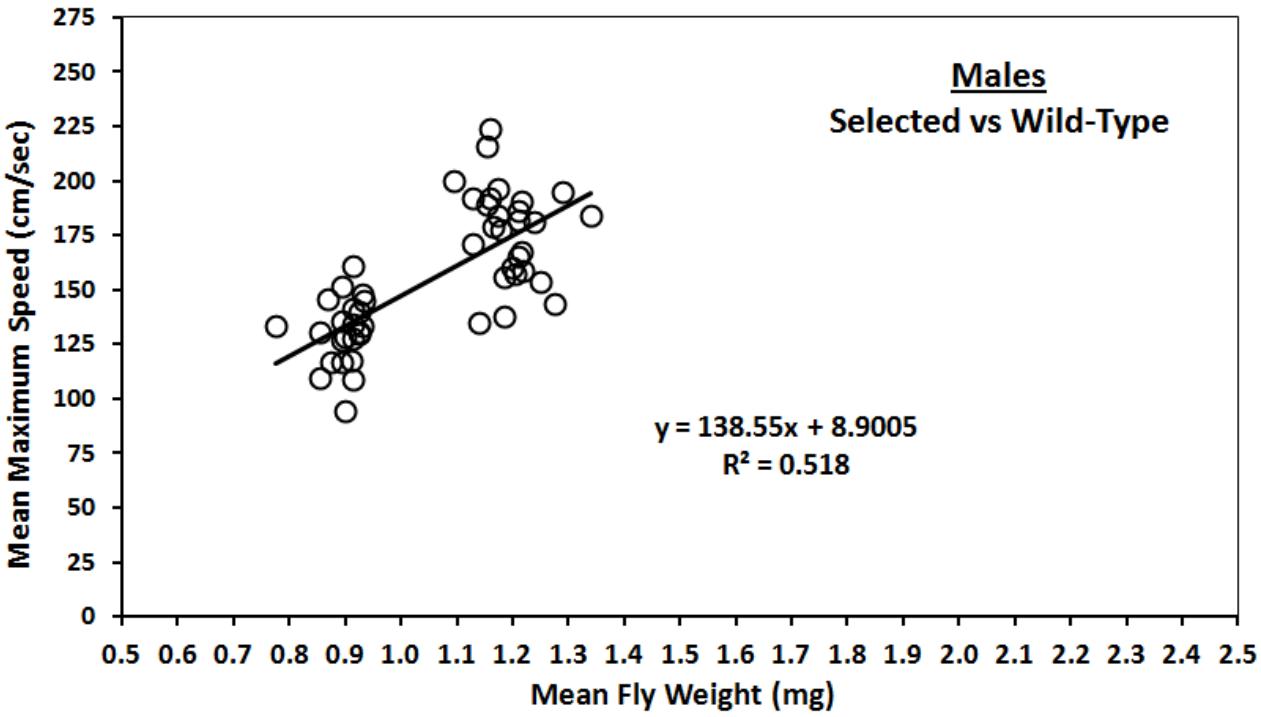


Figure 13. Mean sample flying speeds as a function of mean sample body weights, with regression lines, for males and females of lines AA1, AA2, N60 and K45, leaving out lines CN1 and CN2.

DISCUSSION

In this study it was necessary to separate the trait of flying speed *per se* as much as possible from other traits that may contribute specifically to the flying speed of selected flies, but not controls. We would want the selected flies to show their top speed under conditions where they have the same motivations to fly as the controls, in order to avoid exaggerating the increase in flying speed.

The primary example of such a confounding trait would be phototaxis. The wind tunnel uses light as an attractant in selecting for wind tunnel performance, so that positive phototaxis has been a selected trait. Hadler (1964) demonstrated that phototaxis in flies is a selectable trait. The studies of Marden *et al.* (1997) and Weber *et al.* (2002) showed that in fact, the wind tunnel flies respond to light more than control flies. To address this issue, we minimized lighting in our testing wind tunnel, limiting it to overhead fluorescent lighting, and also covering the top of the tunnel with opaque black paper. Light entered the testing wind tunnel only indirectly through the sides, and this would not preferentially encourage upwind flight in the selected lines. Also the runs were all done at night so directional natural light was not a factor.

Drosophila also exhibit positive anemotaxis and will generally trend towards upwind movement (Johnston 1982; Budick & Dickinson, 2006). Johnston (1982) demonstrated that this upwind tendency is selectable and inheritable. It is likely that positive anemotaxis was selected for in the wind tunnel. Weber (1997) tested this effect in the tunnel by exposing the flies to light without wind, finding that selected (AA) lines demonstrated increased anemotaxis compared to the unselected ancestral (CN) lines. However, the levels of increased performance in the *Drosophila* lines created in this study were greater than the levels of anemotaxis found by Weber (1997).

Line	This Study	SD	N_a	Simons *et al*	SD	N_b
AA1m	175.59	19.65	15	181.7	12.84	15
AA1f	177.16	10.36	14	190.8	17.47	14
AA2m	189.72	20.58	13	170.5	11.68	13
AA2f	174.3	23.85	16	172.4	16.61	14
CN1m	76.19	25.75	15	114.9	17.34	11
CN1f	94.49	16.12	12	120.8	20.01	10
CN2m	105.93	21.3	14	119.3	22.04	16
CN2f	120.62	10.84	15	140.6	17.67	12
N60m	125.16	38.9	13			
N60f	121.286	36.79	12			
K45m	114.9	36.79	13			
K45f	124.54	16.99	15			
	Total N_a		167	Total N_b		105.00

Table 8. Summary of results from this study compared with results from Simons et al. (unpublished) N_a=the number of sample means contributing to the line mean; where each sample mean is the mean of individual top speeds. N_b= the number of individual fly top speeds contributing to the mean. Speeds are based on data from tables 2 and 3 above, showing data from all nights.

	This Study	Simons *et al*
Number of Flights/Fly	One Flight/Fly	Eight flight attempts or 20 min./fly
Direction of Wind Speed Steps	Downward From Maximum	Upward From Minimum
Size of Wind speed Step Changes	Decrease by 8.66 cm/sec (2hz) after 1 min. time increments	Increased by 14.25 cm/sec after each successful flight
Derivation of Top Speed	Digitally measured between frames of video	Measured as top speed setting on wind tunnel that individual will still fly successfully
Startle Mechanism	Bar scraped across back of downwind screen	Insect pins poked through the downwind screen
Odor of Active Yeast Culture	Active yeast culture placed at intake of wind tunnel. A small fan introduced the odor of yeast growing on glucose into the tunnel mouth	Active yeast culture placed at the intake of the wind tunnel
Basic Speed Data	Sample mean of top speeds of each individual	Individual top speed of each individual
Measurements Per Line	16 Samples	10-16 individuals/line
Flies Per Measured Run	Up to 30	1
Total Flies Measured	1465	105
Basic Weight Data	Sample mean weights of 30 flies	Individual weights
Generation of Selection	560	460
Wind Speed Calibration	Puff of smoke. Speed measured with high speed video	Puff of smoke. Speed measured with high speed video
Culturing of Tested Flies	Standard counted-egg culture	Standard counted-egg culture

Table 9. Summary of methods comparing this study with Simons et al. (unpublished)

Another inadvertently selected behavior that may be inherited when selecting increased flight performance in *Drosophila* is the startle response. The propensity to initiate flight when startled may be selectable. Therefore, flies with a lower threshold of initiating flight may be more likely to successfully travel upwind in a selection wind tunnel. This trait is affected by the presence of P(GT1) transposons, displaying a genetic component that can be selected (Yamamoto et al. 2011). However, inadvertent selection of the startle response could not influence the results of this experiment, because the filming area was upwind of the area in which flies would initiate flight. Even if flies were more likely to initiate flight, due to a selected startle response, they had to have the increased performance in flight ability to successfully travel upstream in the wind tunnel before they could be recorded and measured. It was not evident in the sample sizes of the different lines that any one line was more likely to attempt flight. All lines had similar numbers of successful flights.

Our study was comparable to the Simons et al. (unpublished) study , but differed from it in important ways. Both studies used the same wind tunnel which was calibrated in the same way. Both used the same counted egg techniques of fly rearing. Both used the same yeast smell to attract flies. However, the Simons et al. (unpublished) study was designed to measure individual flies, one at a time. In their study the fly was first anesthetized with CO_2 and then introduced into the tunnel. The wind settings of the tunnel were then increased until the fly preferred to settle on the downwind screen. The researchers then induced the fly by poking at it from behind with a set of blunt insect pins. If the fly was able to fly up-wind this setting was recorded as a success. The wind tunnel speed setting was then increased by 14.25 cm/sec and the process was repeated until either the fly was no longer able to fly or 20 minutes had passed. This technique had the potential to fatigue and dehydrate the fly during the time it was in the tunnel. The anesthetization was also a potential variable; there was no easy way to know if the fly had fully recovered from anesthetization. The whole process could have been stressful to the insect. The design of our experiment was meant to minimize sources of fatigue or impairment. In the Simons et al. (unpublished) study the flies were also treated individually when being weighed. Each fly was weighed after being anesthetized, before being placed in the wind tunnel. This had the potential to introduce error since the weight of the fly aproached the minimum weight that the balance could resolve. The selected flies used in both studies were separated by about 100 generations of selection. The data were treated differently as well. The Simons et al. (year) study generated individual top speeds, while our study was based on the average best speed per individual of a sample from each line. This means that the previous study drew from a much smaller data set and measured a total of 105 individual flies while our study measured 1465 in 192 samples (Table 8).

In spite of these differences in methods, both studies produced remarkably similar results for the performance of the selected (AA) lines and the control (CN) lines. The major difference between the studies was that we also measured K45 and N60 wild type lines. These lines had not been living under captive conditions for as long as the control lines. This means that they more accurately reflect the condition of true wild type flies. Because of the decreased performance of the CN lines it is evident that there has been negative pressure on them and that these lines represent a treatment themselves. The two control lines show divergent performance implying that their flight ability is affected only by drift or very weak negative selection. The control flies have been raised in culture vials where flight was not a factor in reproductive success. Flight was not needed to reach either food or mates. Within the culture vial, there would likely have been weak selection against flight performance. Maintaining flight muscle would be costly for an organism that does not need it to be successful. In any case, the CN lines are not appropriate controls because their performance has degraded almost as much as the selected lines have improved.

Marden et al. (1997) determined a maximal flying speed of 85 cm/sec in the selected lines of flies. This is much less than either the Simons et al. (unpublished) study or our own. There are differences in the methodology of the three studies. The Marden et al. (1997) study measured flight

speeds in a still air situation without any motivational stimulus at all. It is unclear from the Marden et al. (1997) paper how the flies were cultured before being used. We cultured our flies in a very uniform way using counted-egg cultures. This technique guarantees that flies are reared at populations that do not overuse their media ensuring that the flies are near their optimum condition and body weight. *Drosophila* do not moderate their egg laying according to the density of adults and will lay more eggs than the amount of food can support (Gilpin, 1974). Our selected lines of flies, which tend to have the highest body weights, were larger in our study than in the Marden et al. (1997) study (Marden & Wolf, & Weber, 1997), thus implying that ours were in better condition. The flies used in the Simons et al. (unpublished) study and in our study had also been under selection for, respectively 260 and 360 more generations than the ones used in the Marden et al. (1997) study.

Marden et al. (1997) pointed out that flight performance, as measured by power output, scales with body size and mass of the flight muscle (Chai, Chen, & Dudley, 1997; Dudley, 1995; Marden, Wolf, & Weber, 1997; Pennycuick, Fuller, & Mcallister, 1989). The flies that were used in the Marden et al. (1997) study are stated to have had an average mass of 1 mg. It is not mentioned if this was for males or females, selected or control lines, or an average of all, or just in fact a round number representing a rough estimate. They mention that in preliminary tests they found that males and females flew at the same speeds, so that in their actual published measurements they did not distinguish the sexes, only the lines. In their study, selected and control flies shared an apparent maximum velocity of 85cm/sec. Our female flies had an average mass of ~1.4 mg for the CN lines and ~1.9 mg in the selected lines. The body mass of our selected lines can be compared to the mass of *Drosophila virilis*, a larger species with a published body weight of 2 mg (Vogel, 1966). The flying speed of *D. virilis* is given as 225 cm/sec (Vogel, 1966). The average speed for the selected (AA) line flies was 172 cm/sec. The increase in performance that we have measured falls within the range of performance that would be predicted by the increase in weight.

We did not determine whether the increase in body weight was only due to an overall increase in body size, or whether there was in fact an increase in the relative mass of muscle produced. It would also be quite interesting to repeat these measurements in a system that uses light as an attractant in order to elicit the highest flying speed from the AA flies.

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