1 **Title: Laterally biased diffusion of males of the water flea *Daphnia magna***

2 **Running title: Swimming behaviour of daphnids**

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4 **Kenji Toyota^{1,2,3*}, Masaki Yasugi⁴, Norihisa Tatarazako⁵, Taisen Iguchi⁶, Eiji Watanabe^{7,8*}**

5 ¹Marine Biological Station, Sado Center for Ecological Sustainability, Niigata University, Sado,

6 Niigata 952-2135, Japan.

7 ²Department of Biological Sciences, Faculty of Science, Kanagawa University, Hiratsuka,

8 Kanagawa, Japan.

9 ³Department of Biological Science and Technology, Faculty of Industrial Science and Technology,

10 Tokyo University of Science, Katsushika, Tokyo, Japan.

11 ⁴Faculty of Engineering, Utsunomiya University, 7-1-2 Yoto, Utsunomiya, Tochigi 321-8585, Japan.

12 ⁵Department of Science and Technology for Biological Resources and Environment, Graduate

13 School of Agriculture, Ehime University, Matsuyama, Ehime 790-8577, Japan.

14 ⁶Graduate School of Nanobioscience, Yokohama City University, Yokohama, Kanagawa 236-0027,

15 Japan.

16 ⁷Laboratory of Neurophysiology, National Institute for Basic Biology, Higashiyama 5-1,

17 Myodaiji-cho, Okazaki, Aichi 444-8787, Japan.

18 ⁸Department of Basic Biology, Faculty of Life Science, SOKENDAI (Graduate University for

19 Advanced Studies), 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan.

20

21 *Correspondences and requests for materials should be addressed to K.T.

22 (toyotak@cc.niigata-u.ac.jp) and E.W. (eiji@nibb.ac.jp).

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24 **Key words (3-6 words): *Daphnia magna*, behaviour, sex difference.**

1 **Summary statements (15-30 words)**

2 We analysed the swimming behaviours of adult water flea *Daphnia magna*, and found apparent
3 sexual differences: laterally biased diffusion of males in contrast to the nondirectional diffusion of
4 females.

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7

1 **Abstract**

2 The water flea *Daphnia magna* is a representative example of zooplankton living in freshwater
3 environments. They primarily propagate via asexual reproduction under normal and healthy
4 environmental conditions. Environmental stimuli that signal a shift to disadvantageous conditions
5 induce *D. magna* to change their mode of reproduction from asexual to sexual reproduction. During
6 the sexual reproduction phase, they produce special tough eggs (resting eggs), which can survive
7 severe environmental conditions. Despite our increased understanding of their mating behaviours,
8 the sex-specific characteristics of swimming behaviours among daphnid species are poorly
9 understood. In this study, we analysed the swimming patterns and dynamics of female and male
10 adult *D. magna*. First, we found laterally biased diffusion of males in contrast to the homogeneous,
11 nondirectional diffusion of females. Second, computer modelling analysis using a discrete-time
12 Markov chain simulation, in which the frequencies of turning behaviour were evaluated as
13 probability distributions, explained the greater diffusion of males in the horizontal direction. Under
14 the presumption that high diffusion in the horizontal direction increases the probability of
15 encountering a distant mate, these findings led us to hypothesise that male *D. magna* increase
16 genotype heterogeneity by effectively selecting the probability distributions of certain motion
17 parameters.

1 **Introduction**

2 For animals, survival depends on the ability to engage in appropriate behaviours in
3 response to current circumstances. In various situations, animals must perform a range of behaviours,
4 such as feeding, escape, aggression and sexual behaviours, by appropriately regulating their motor
5 systems. Daphnids (referred to as water fleas), which are cladoceran crustaceans living in freshwater
6 ecosystems, are no exception. Although they cannot perform smooth motion in the same manner as
7 higher-order animals, such as humans, they can engage in appropriate behaviours to search for food,
8 escape from predators and find mates using primitive sensors and motors (Ebert, 2005). In this study,
9 we studied the swimming behaviours of *Daphnia magna* as a representative example of daphnid
10 species (Figure 1). It is one of the most readily available and large species of daphnids, and it
11 occupies a key position in food webs of shallow ponds.

12 The jumping-like motion of daphnids stems from the beating of their second antennae,
13 which do not act as sensory organs but rather function in a manner similar to the oars of a boat (refer
14 to supplemental movies 1 and 2). Their rapid downbeat produces a swift upward movement, whereas
15 motionless organisms rapidly sink due to gravity (Haury and Weihs, 1976; Matsunaga and Watanabe,
16 2012). This rhythmic swimming pattern results in directed movement. In addition, these organisms
17 occasionally drastically change their direction via irregular and disordered motion of their second
18 antennae (Garcia et al., 2007). By simply repeating downbeats, succumbing to the pull of gravity and
19 occasionally performing turning behaviour, they perform vertical and horizontal migration towards
20 or away from a specific location in a pond or a lake. Such swimming patterns in a random fashion
21 are widely observed in zooplankton species (Seuront and Strutton, 2003). Although these organisms
22 freely move in water under constant conditions in the absence of specific physical stimuli, such as
23 water flow or directional light, the motion of zooplankton is fundamentally explained by random
24 walks or Levy flights (Bartumeus et al., 2003; Garcia et al., 2007; Kjørboe and Bagøien, 2005;

1 Seuront et al., 2004a; 2004b; Visser and Thygesen, 2003), which are representative types of Markov
2 chain (MC) processes. The movement vector (motion direction and movement length) at each step is
3 stochastically determined. In addition to the random walk theory, mathematical models based on
4 continuous stochastic differential equations closely approximate zooplankton behaviours (Grünbaum,
5 2000; Øien, 2004; Schimansky-Geier et al., 2005). Biologists might expect that these internal
6 stochastic processes found in zooplankton play an important role in their biological behaviours, such
7 as foraging (Bartumeus et al., 2003; Garcia et al., 2007).

8 Among the variety of characterised behaviours of cladocera species, including daphnids,
9 mating behaviours, such as encounters, grasping and copulation, have received growing attention
10 (Brewer, 1998; Kerfoot, 1980; Winsor and Innes, 2002), given that daphnids have evolved a unique
11 reproductive system for increasing the encounter probability between female and male individuals in
12 a population. Their method of reproduction is termed cyclical parthenogenesis, in which
13 parthenogenetic (asexual) and sexual phases are interchanged in response to external environmental
14 stimuli, such as day length, temperature, nutrient availability and crowding (Banta and Brawn, 1929;
15 Hobæk and Larsson, 1990; Kleiven et al., 1992; Smith, 1915). Under favourable growth conditions,
16 they parthenogenetically produce diploid eggs, giving rise to genetically identical female offspring.
17 When environmental conditions deteriorate, they produce males and sexual females bearing haploid
18 diapause eggs, permitting sexual reproduction and increasing genetic diversity. Resting eggs are
19 resistant to harsh conditions, such as drying, freezing and exposure to the digestive enzymes of fish
20 (Jarnagin et al., 2000). Despite this accumulated knowledge on daphnid mating behaviours, little
21 information concerning sexual differences in swimming behaviours among daphnid species is
22 available (Brewer, 1998). The goal of the present study was to elucidate the sex-specific swimming
23 patterns of adult daphnids and to reproduce these observed behavioural patterns via computer
24 modelling. We found laterally biased diffusion of males in contrast to the nondirectional diffusion of

females. Computer simulation showed that the frequencies of turning behaviour are involved in male-specific behaviour.

Results

Overview of swimming patterns

Figures 2A and 2B show the swimming trajectories of 28 female and 28 male *D. magna* individuals. They travelled on average 111.52 ± 2.42 mm (females) and 96.17 ± 2.68 mm (males) per individual from their starting position to their ending position in 8.53 s (256 frames), with mean speeds of 13.07 ± 0.028 mm/s and 11.27 ± 0.31 mm/s, respectively. In Figures 2A and 2B, consecutive coordinates were normalised to the given halfway point (the 129th coordinate). To reflect all data of the coordinates of the trajectory in a single graph, the trajectory graph was transformed into a probability density plot (Figures 2C and 2D). Next, we performed kernel density estimation. Whereas females exhibited a relatively symmetric distribution of movement in all directions, males exhibited a biased movement distribution in the horizontal direction. The horizontal-to-vertical ratio of high-density areas (density of 0.6 and greater; greenish yellow to white in Figures 2C and 2D) was 1.35 in females and 2.10 in males. The highest density for females was 1.16-fold greater than that for males. Furthermore, a comparison of the area with a density of at least the highest density for males (density of 1.1 and greater) revealed that the area for females was 4.29-fold larger than that for males.

Mean squared displacement and autocorrelation function

Next, we analysed the MSD of the motion of *D. magna*. Figure 3 shows the MSD derived from the trajectories of *D. magna* females and males. The MSD was calculated independently in

1 vertical and horizontal directions, and best-fit lines were calculated using a power-law
2 approximation. The power-law exponent in the vertical direction was 1.58 for females (least mean
3 square = 0.97) and 1.39 for males (0.97). The power-law exponent in the horizontal direction was
4 1.29 for females (0.93) and 1.82 for males (0.99). The male-to-female ratio of the power-law
5 exponent was 0.88 in the vertical direction and 1.41 in the horizontal direction. The MSD plot of
6 females moving in horizontal directions appears to consist of two time scales divided at
7 approximately 5,000 ms (Figure 3, bottom, red). However, to compare diffusion rates of *D. magna*
8 females and males, each of them was simply represented by a single power-law exponent. All
9 power-law exponents larger than 1.0, indicating diffusion of both *D. magna* females and males, were
10 labelled as ‘superdiffusion’ in both directions. The motion analysis data also indicated that
11 horizontal diffusion rates of males were higher than those of females irrespective of the lower mean
12 speed of males.

13 The estimate for the correlation timescale obtained from the velocity autocorrelation
14 function is shown in Figure 4. In both cases (for females and males as well as for vertical and
15 horizontal diffusions), distinct periodicity and autocorrelation characteristics were not observed. In
16 many cases, a persistent random walk (or correlated random walk) was used for modelling the
17 trajectories of the moving animals, given that the velocities of moving animals are persistent, in
18 general (Codling et al., 2008). However, the present models include a fundamentally uncorrelated
19 random walk because of the low autocorrelation characteristic. Using statistical MC models based on
20 the discrete probability distributions of the velocities, move-step lengths, and SCISs (turns), we
21 attempted to identify the parameter element contributing to the higher diffusion rate of *D. magna*
22 males in the horizontal direction. The MC3 model referencing the discrete probability distribution of
23 SCIS is a type of persistent random walk, given that the size values of SCIS are coupled with the
24 persistence of the movement direction.

1

2 ***MCI***

3 Assuming the MC in reference to frequency distributions based on velocity, computer
4 simulation was performed using the MC method. Computational results are shown in Figure 5. The
5 power-law exponent in the vertical direction was 1.43 for females (least mean square = 0.99) and
6 1.17 for males (0.99) and that in the horizontal direction was 1.02 for females (0.98) and 1.89 for
7 males (0.99). The male-to-female ratio of the power-law exponent was 0.82 in the vertical direction
8 and 1.85 in the horizontal direction.

9

10 ***Frequency of move-step length and MC2***

11 The frequency distribution of the swimming speed (move-step length, mm/video frame; n
12 = 7,168 for each sex) is shown in Figure 6. The results clearly illustrate a high frequency in the
13 lower move-step length, which gradually decreased as the move-step length increased. In both
14 female and male individuals, the frequency distributions were well fitted by exponential functions
15 (Figure 6, right). The slope of the regression lines for the vertical direction was -5.03 for females
16 (least mean square = 0.97) and -6.08 for males (0.99). The slope of the regression lines for the
17 horizontal direction was -4.55 for females (0.98) and -5.59 for males (0.96). For both directions, the
18 slope of the regression line was greater for females than for males. Significant differences in the
19 mean move-step lengths in each direction were observed between females and males (females and
20 males in the vertical direction: 0.26 ± 0.0027 mm/video frame and 0.21 ± 0.0022 mm/video frame,
21 respectively; and females and males in the horizontal direction: 0.28 ± 0.0030 mm/video frame and
22 0.25 ± 0.0027 mm/video frame, respectively; $p < 0.05$, unpaired *t*-test). In both directions, the mean
23 move-step lengths of females were longer than those of males.

MC simulations using model 2 (MC2), which was derived from swimming speed, were conducted. Plus or minus signs were randomly assigned to randomly selected move-step lengths, the re-signed values were used as a data set for swimming velocity and MSDs were calculated. The power-law exponent in the vertical direction was 0.92 for females (least mean square = 0.98) and 0.92 for males (0.90). The power-law exponent in the horizontal direction was 1.07 for females (0.97) and 1.13 for males (0.97). The male-to-female ratio of the power-law exponent was 1.00 in the vertical direction and 1.06 in the horizontal direction.

Size of a cluster with an identical sign and MC3

In addition to regular jumping motions, *D. magna* occasionally makes a large change in the direction of movement (left/right or up/down) via turning behaviour. These turning phenomena can be represented by the direction of movement (left/right or up/down). We accordingly calculated the sign of the direction of movement. Dominant and recessive directions were defined for each data set, with the dominant direction represented by a plus sign (Figure 7A, see Methods for details). Although the turning behaviours have a large effect on the global trajectory of *D. magna*, capturing the turning motion from local trajectories based on the time unit of the video frame is difficult. For this reason, to visualise the turning motion, we used a parameter termed SCIS, (Figure 7A, see Methods for details). The frequency distribution of the parameter SCIS is shown in Figure 7B. A long-tailed distribution (arrow) was found for positive-sign movements of males. In Figure 7C, the dominant area of the frequency distribution is shown on logarithmic scales. The power-law exponent of the frequency distribution in the vertical direction was -3.15 for females (least mean square = 0.98) and -3.12 for males (0.98). The power-law exponent in the horizontal direction was -3.22 for females (0.96) and -1.87 for males (0.99). The mean distance in the horizontal direction was 118.84 ± 18.32 mm for males, significantly greater than that for females (25.63 ± 4.54 mm). In contrast, the

1 mean length of the movements of males in the vertical direction was 29.84 ± 5.41 mm, not
2 significantly different from that of females (22.92 ± 2.63 mm).

3 Assuming an MC in reference to the SCIS frequency distributions, MC simulations using
4 model 3 (MC3), which was derived from SCIS, were performed to calculate MSDs (Figure 8). The
5 power-law exponent of the MSD in the vertical direction was 1.79 for females (least mean square =
6 0.98) and 1.82 for males (0.99). The power-law exponent in the horizontal direction was 1.38 for
7 females (0.98) and 1.96 for males (0.99). The male-to-female ratio of the power-law exponent was
8 1.02 and 1.42 in the vertical and horizontal directions, respectively. Characteristics of the two time
9 scales observed in the motions of females in the horizontal direction (Figure 3, bottom, red) appear
10 to be reproduced by the MC3 model. The power-law exponents of the MSDs of *D. magna* and three
11 MC models are summarised in Table 1.

12

13 **Table 1. Summary of three MC models.**

		<i>D. magna</i>	MC1	MC2	MC3
Female	V	1.58	1.43	0.92	1.79
	H	1.29	1.02	1.07	1.38
Male	V	1.39	1.17	0.92	1.82
	H	1.82	1.89	1.13	1.96
M/F	V	0.88	0.82	1.00	1.02
	H	1.41	1.85	1.06	1.42

14 The power-law exponents of MSDs of *D. magna* and three MC models are summarised. V: vertical
15 direction, H: horizontal direction, M/F: ratio of male divided by female.

16

17 ***Net-to-gross displacement ratios***

1 NGDRs (refer to Figure 9A) were calculated from three data sets: (1) the raw data derived
2 from *D. magna*, (2) MC1 and (3) MC3 (Figure 9B). NGDRs provide a measure of the relative
3 linearity of the swimming path of the plankton; lower NGDRs imply more curved trajectories.

4 The NGDRs of the raw data from females were 0.32 ± 0.027 in the vertical and $0.32 \pm$
5 0.050 in the horizontal direction. The NGDRs of the raw data from males were 0.33 ± 0.041 and
6 0.70 ± 0.053 in the vertical and horizontal directions, respectively. The male-to-female ratios of the
7 NGDRs were 1.03 in the vertical and 2.19 in the horizontal direction. These results indicate that
8 NGDR in the horizontal direction for males was significantly higher than the other NGDRs ($p <$
9 0.01 , unpaired t -test). Thus, the linearity of the swimming path of males in the horizontal direction
10 was significantly greater than that of the other direction and sex.

11 This specificity was reproduced in the two MC models. The NGDRs of MC1 for females
12 were 0.13 ± 0.015 and 0.078 ± 0.0087 in the vertical and horizontal directions, respectively, and
13 those for males were 0.11 ± 0.011 and 0.54 ± 0.013 , respectively. The male-to-female ratio of the
14 NGDR was 0.85 in the vertical and 6.92 in the horizontal direction. The NGDRs of MC3 for females
15 were 0.31 ± 0.029 and 0.28 ± 0.030 in the vertical and horizontal directions, respectively, and those
16 of males were 0.36 ± 0.025 and 0.77 ± 0.025 , respectively. The male-to-female ratios of the NGDR
17 were 1.16 in the vertical and 2.75 in the horizontal direction. These results indicate that based on the
18 MC models, the NGDR in the horizontal direction for males was distinctly higher than the other
19 NGDRs ($p < 0.01$, unpaired t -test).

20

21

22 Discussion

23 The motions of female and male *D. magna* individuals were tracked in two-dimensional
24 coordinates; then, three diffusion parameters (kernel density estimates, MSDs and NGDRs) were

1 calculated. All of these parameters showed that male adult *D. magna* individuals exhibit greater
2 diffusiveness in the horizontal direction than females. This male-dominant diffusion pattern was
3 computationally reproduced by the MC method in which the frequency map of the velocity was
4 considered as a probability distribution. Then, the factors that might have contributed to the
5 particularly high diffusiveness of males in random motions were analysed using the MC method.
6 The frequency maps of the migration length per unit time (absolute value of the moving velocity)
7 were nearly equal between females and males. The frequency distribution of the swimming speed in
8 females and males did not explain the higher diffusion of males in the horizontal direction. Because
9 these organisms often make drastic changes in the direction of movement, the frequency of turning
10 behaviour (SCIS) was calculated. When the frequency maps of SCIS were considered as a
11 probability distribution, the MC computational simulations reproduced the observed sexual
12 difference in diffusiveness, showing that male *D. magna* likely predominantly diffuse in the
13 horizontal direction by changing the frequency of turning behaviour. Regardless of the low
14 autocorrelation found for velocity, the sexual difference was reproduced in model MC3, which is a
15 type of persistent random walk. This result appears to show inconsistency. However, variation of
16 move-step length was not included in MC3 (move-step length was constant in MC3). Considerable
17 randomness and variation of move-step length may have contributed to the low autocorrelation.
18 Furthermore, two-valued directions (plus or minus) in SCIS possibly enhance the persistency. In
19 either case, it should be noted that SCIS analysis extracts the persistency from low-autocorrelation
20 data. For detailed evaluation of this question, comparison with other analyses using data with higher
21 spatial and temporal resolution or data to which a smoothing method applies will be helpful (Hen et
22 al., 2004; Postlethwaite and Dennis, 2013).

23 The three abovementioned indices of the autonomous free diffusion of *D. magna* describe
24 only a portion of their broad spectrum of ecological dynamics and were calculated using distinct

1 processes. Nonetheless, all of these indices commonly captured the greater diffusiveness of males in
2 the horizontal direction. Such preferred movement by males in the horizontal direction has been
3 observed in another daphnid species (*D. pulicaria*) (Brewer, 1998). Females exhibited a strong
4 vertical swimming component, ranging from -60 to $+75$ from the horizontal, whereas male
5 swimming behaviour was primarily linear and horizontally oriented, with swimming angles
6 deviating no more than ± 15 from the horizontal. Furthermore, very different motility was observed
7 between female and male copepod crustaceans (*Centropages typicus* and *Pseudocalanus elongates*)
8 (Kiørboe and Bagøien, 2005). Males were more directionally persistent than females based on
9 root-mean-square net distance. Thus, the comparatively high diffusion of males in the horizontal
10 direction may be a general phenomenon in zooplankton. Over 200 species of daphnids (Kotov et al.,
11 2012) and over 13,000 species of copepods (Boxshall and Defaye, 2008) have been discovered to
12 date; however, there are few zooplankton in which motion patterns have been investigated. Further
13 verification of their behaviour is required.

14

15 ***Random motion of D. magna***

16 In this study, we investigated the autonomous movement pattern of *D. magna* individuals
17 under uniform conditions with no water stream, a constant light cycle and temperature. To avoid
18 chemical and physical influences from animals of the opposite sex, the individual motion of
19 daphnids was measured while they were swimming together with those of the same sex. The
20 autonomous movement pattern of each zooplankton in such a uniform environment in the laboratory
21 was measured by various researchers. This characteristic was detected and expressed using various
22 mathematical models (Okubo and Levin, 2001; Seuront and Strutton, 2003), which are useful for the
23 analysis and comparison of the patterns of animal movement in any species.

1 In this study, MC simulation analysis was performed using speed distribution data from
2 raw tracking data considered as a probability distribution. As a result, the differences between males
3 and females mentioned above were reproduced. Thus, it can be inferred that it is appropriate to
4 analyse as a Markov process the difference in diffusion between males and females. Reproducibility
5 using the MC model indicates that only the present position is used as past information determining
6 the future motion of plankton and that the future motion follows specific probability distributions. In
7 our recent study, when visual stimulation was generated via a MC method in which speed
8 distribution data from raw tracking data of female *D. magna* were considered as a probability
9 distribution, visual stimuli promoted the feeding behaviour of medaka fish (*Oryzias latipes*)
10 (Matsunaga and Watanabe, 2012). This finding suggests that the Markov process observed in the
11 movement of *D. magna* is an important biological phenomenon. Furthermore, in this study,
12 movement of *D. magna* was separated into migration distance per unit time and frequency of turning
13 behaviour. The frequency of turning qualitatively explained the behaviour of males.

14 Turning behaviour has been observed in small organisms such as *Caenorhabditis elegans*
15 (Ohkubo et al., 2010; Srivastava et al., 2009), *Paramecium* (Nakaoka et al., 2009) and *Drosophila*
16 (Censi et al., 2013). These turning behaviours occur even under spatially and temporally uniform
17 conditions, indicating that these are self-motivated and voluntary behaviours. Theoretical studies
18 suggest that stochastic behaviours are important for efficiently searching for prey and mates
19 (Bartumeus et al., 2008). A similar turning phenomenon was observed in feeding female *D. magna*,
20 phytoplankton distributed throughout the aquatic systems of lakes and ponds. The male *D. magna*
21 could have adapted to the rapidly changing habitat environment by slightly reducing their probability
22 of turning. To clarify the biological meaning of this stochastic process, the presence of some internal
23 mechanism regulating the probability distribution of turning behaviour is suggested.

Sex-specific morphological characteristics might be associated with the generation of sex-specific behavioural differences. Indeed, mature male daphnids are distinguishable from females not only by their testes and sperm but also by several apparently distinctive morphological characteristics, such as a smaller body size, elongated first antennae and a copulatory hook at the first thoracic leg (Brewer, 1998; Mitchell, 2001). However, the causal relationships of the sexual differences between behavioural and morphological characteristics in daphnid species remain largely unknown.

Acquisition of genetic heterogeneity

As mentioned above, the higher diffusiveness of male zooplankton has been observed in multiple species, and the underlying dynamic regulation of turning has been reported in various animal species (Censi et al., 2013; Nakaoka et al., 2009; Srivastava et al., 2009). Thus, discussing the biological significance of this difference in diffusiveness would be useful, even though a conclusion on this issue is beyond the scope of the present study. Here we propose simply that the high diffusiveness of male *D. magna* is closely related to ensuring genotype heterogeneity.

As shown in Figure 2C, female *D. magna* exhibit heavier recursiveness to the point of origin, indicating that the motion of females exhibits less diffusion than that of males. Under normal conditions, female *D. magna* generate many offspring that are genetic clones of the mother. Because *D. magna* have no sex chromosome, both female and male offspring are genetically identical to the mother. Given that male *D. magna* individuals exhibit diffusion similar to that of females, males are more likely to encounter clones of their own family. We accordingly hypothesise that the comparatively high diffusive motion of male *D. magna* leads to displacement far from their generation, thus impeding contact with its family members. As a result, they can produce resting

1 eggs that exhibit high genetic variation. It appears reasonable that water fleas living in shallow ponds
2 concentrate their energy in horizontal directions.

3 Traditionally, behavioural patterns of daphnids have been demonstrated by population
4 (mass culture) levels under laboratory and/or natural environments (Beaver et al., 2018; Dodson,
5 1988). Recent technologies have enabled the individual-level behavioural analysis by tracking video
6 system, and it can be applied for the toxicological and risk assessment of chemicals as a novel
7 endpoint (e.g., swimming velocity and trajectory) in addition to, for examples, survival rate and
8 fecundity of *D. magna* (Bownik et al., 2019; 2020; Felice et al., 2019; Noss e al., 2013). Although *D.*
9 *magna* has been used in ecotoxicological studies as a representative surveillance organism to
10 monitor changes in water quality (Martins et al., 2007), little is known about the basic mechanisms
11 involved in its behaviour. In this study, we found that male individuals travelled in a diffuse
12 horizontal direction relative to females. Furthermore, this male-specific diffusion pattern was
13 reproduced by mathematical modelling using MC simulation, in which a frequency map of their
14 turning behaviour was used as a probability distribution. Although it is necessary to clarify strain
15 differences of behavioural patterns (Oda et al., 2007), current data provides an insight of new
16 endpoints to OECD Test Guidelines no. 211 using daphnids (OECD, 2012), enabling the validation
17 of chemical toxicity based on behaviour. Likewise, we recently found useful *Daphnia* strains that
18 can produce female or male in response to day-length differences in the *D. pulex* (Toyota et al.,
19 2015) and *D. magna* (Toyota et al., 2019; 2021). These strains will be used for comparative
20 behavioural analysis of inter- and intra-species between sexes, and moreover 3D tracking approaches
21 (Bianco et al., 2013) will shed light on the well-conserved innate behavioural patterns of daphnids.
22 Our findings provide the first insight into the behavioural characteristics of female and male adult *D.*
23 *magna* individuals and lay the foundation for advancing our understanding of the dynamics of
24 daphnid populations and the toxic effect of chemicals released into the aquatic environment.

1

2

3 **Materials and Methods**

4 ***Daphnia magna* strain and rearing conditions**

5 A *D. magna* strain (Belgium clone) was obtained from the National Institute for
6 Environmental Studies (NIES; Tsukuba, Japan) (Oda et al., 2006). Stock populations were
7 maintained as reported previously (Tatarazako et al., 2003). Briefly, they were housed in a 30 L
8 aquarium (containing 20 L of housing water) and maintained in an incubator at 24 ± 1 °C on a 14/10
9 h light/dark cycle (with lights on from 08:00 to 22:00). The housing water was prepared by mixing
10 artificial sea salt into deionised H₂O (18 g/60 L; Tetra Marine Salt Pro, Tetra Japan, Tokyo, Japan)
11 (Matsunaga and Watanabe, 2012). A chlorella (*Chlorella vulgaris*) suspension (0.3 mL/10 L;
12 Chlorella Industry, Tokyo, Japan) was added to the housing tank as a food source once a day at
13 08:30. This strain occasionally produces male offspring in addition to female offspring under
14 overpopulation conditions. Male juveniles were easily distinguished based on their elongated first
15 antennae (Olmstead and LeBlanc, 2000). Two- to three-week-old male and female animals were
16 used in subsequent experiments.

17

18 ***Motion recording***

19 Cuboid clear plastic aquaria [interior dimensions: 9.5 cm (width) × 1.0 cm (length) × 9.5
20 cm (vertical depth)] were used as test tanks for the motion analysis of *D. magna*. The aquaria were
21 filled with housing water to a vertical depth of 8.0 cm. The temperature of the tank water was
22 maintained at 25 ± 2 °C by air conditioning in the experiment room. The bottom of each tank was
23 covered with a black plastic mat to prevent light reflection. The sides of the tanks, excluding the side
24 on which the digital video camera (Himawari GE60; Library, Tokyo, Japan) was positioned, were

1 covered with black rubber to prevent excessive illumination and light reflection. The test tank was
 2 placed in a dark room. Illumination at the surface of the water was adjusted to 3,000 lx using three
 3 white fluorescent lamps placed outside the tank above the surface, excluding the side on which the
 4 video camera was positioned. Male or female ($n = 10\text{--}20$ each) *D. magna* individuals were
 5 transferred to the tank, and motion was recorded from the side of the tank using the digital video
 6 camera. The experiments on each female and male were repeated nine times (18 trials in total).
 7 Video images (640×480 pixels) were recorded at 30 frames per second (fps) and were quantified
 8 with DIPP-Motion 2D motion analysis software (DITECT, Tokyo, Japan). The coordinates of the
 9 centre of mass of the individuals were automatically tracked in each video frame. The coordinates
 10 were obtained as integer numbers (horizontal coordinates from 0 to 639; vertical coordinates from 0
 11 to 479). Three or four *D. magna* individuals were tracked in each experiment. Data points in which
 12 individuals collided against each other or the tank walls were excluded. Thus, sexual differences in
 13 swimming behaviours were studied in reference to the autonomous movement pattern of each
 14 individual in a uniform environment. Consecutive coordinates (257) for each daphnid were treated as
 15 a data set, and 28 data sets were collected for each sex. The frequency distribution of the velocities
 16 showed a single peak with wide tails located at either side (Supplemental Figure 1). The maximum
 17 values were 13 pixels (horizontal) and 13 pixels (vertical) for females and 9 pixels (horizontal) and
 18 10 pixels (vertical) for males. The minimal values were -14 pixels (horizontal) and -14 pixels
 19 (vertical) for females and -12 pixels (horizontal) and -9 pixels (vertical) for males. In the following
 20 analysis, these integral data were used and then finally converted to millimetre scale.

21

22 ***Kernel density estimation***

23 In total, 7,196 coordinates (240 sets, 257 consecutive coordinates per individual *D. magna*,
 24 $n = 28$ for each sex) of the calculated centre of mass of *D. magna* were obtained. The consecutive

1 coordinates were normalised to the given halfway point (the 129th coordinate), which was set as
 2 vertical coordinate 50 and horizontal coordinate 50 (Figure 1, top). Nonparametric kernel density
 3 estimation (Silverman, 1986; Fortmann-Roe et al., 2012; Worton, 2002) of the normalised trajectory
 4 data was performed using a quartic kernel function with a bandwidth of 12 mm. The kernel density
 5 estimator at coordinate $\mathbf{x}$ is given as follows:

$$6 \quad \hat{f}_h(\mathbf{x}) = \sum_{d_i \leq h} \frac{3}{\pi h^2} \left(1 - \frac{d_i^2}{h^2}\right)^2,$$

7 where h is a smoothing parameter termed bandwidth and d_i is the distance from coordinate $\mathbf{x}$ to
 8 coordinate $\mathbf{x}_i$. The kernel density estimator was applied to 12,100 (110 vertical $\times$ 110 horizontal)
 9 coordinates and the two-dimensional density map was visualised with terra colours using R software
 10 (<http://www.r-project.org/>).
 11

12 ***Motion analysis***

13 The movement distance per frame was calculated from a series of coordinates and
 14 converted to swimming velocity (mm/s), which was calculated separately for the horizontal axis
 15 (with increasing values to the right) and the vertical axis (with increasing values upward). The
 16 swimming speed was calculated as the absolute value of the swimming velocity. Mean squared
 17 displacement (MSD) was calculated from a series of coordinates as follows:

$$\text{MSD} \equiv \langle (\mathbf{x}(t) - \mathbf{x}_0)^2 \rangle,$$

18 where $\mathbf{x}_0$ is the initial position of a plankter and $\mathbf{x}(t)$ is the position of the individual at a specific time
 19 t .

20 The discrete autocorrelation Rs at lag n were computed as follows:

$$21 \quad R(n) = \frac{1}{N} \sum_{t=0}^{N-1} v(t)v(t+n),$$

22 where $v(t)$ is the velocity of an individual plankter at a specific time t . The normalised
 23 autocorrelation function (ACF) was calculated as follows:

$$ACF(n) = \frac{R(n)}{R(0)}.$$

1 Net-to-gross displacement ratios (NGDRs) (Figure 9) were computed as follows (Busley,
2 1984):

$$NGDR \equiv \frac{ND}{GD},$$

3 where ND (mm) and GD (mm) are the net and gross displacements, respectively, of an individual;
4 these measures correspond to the shortest distance between the starting and ending points of the
5 trajectory and the actual distance travelled by an individual, respectively. NGDRs were computed at
6 the finest available resolution (1/30 s) for each track. The statistical significance of the results of
7 NGDR was determined using unpaired (two-tailed) *t*-tests. The threshold for statistical significance
8 was $p < 0.05$. Values are reported as means $\pm$ SEM.

9 One-dimensional velocities in each set of 255 data have either plus or minus signs. The
10 most frequent sign was defined as the dominant direction. Dominant and recessive directions were
11 determined for each set of data, and the dominant direction was represented anew by a plus sign. The
12 movement distance per frame was converted to values accompanied by signs referring to the
13 dominant and recessive directions. The size of a cluster with an identical sign (SCIS) was calculated
14 as the duration for which identical signs were continuous (Figure 7A). Periods of zero speed were
15 assigned the sign of the preceding period.

16

17 ***Markov chain simulation***

18 Computer simulations using the MC method were performed. The virtual zooplankton
19 models were assumed to be uncorrelated random walks referencing the motion parameters of *D.*
20 *magna*. First, independent random variables, $v_1, v_2, v_3, \dots, v_{255}$, were taken with each variable

1 generated by a randomiser referencing the discrete probability distributions of velocities (model
2 MC1), move-step lengths (model MC2) or SCISs (model MC3). Then, we defined
3
$$x_0 = 0, x_n = \sum_j^n x_j,$$

4 where x_0 is the initial position of a model plankter and x_n is the position of the individual at a
5 discrete specific time j . Finally, the MSDs and NGDRs of each set of progressions $\{x_n\}$ were
6 calculated. Progressions $\{v_1, v_2, v_3, \dots, v_{255}\}$ for each MC model were generated as follows.
7 *MC1.* 1) The raw velocity data from 28 *D. magna* individuals were collected into a data set
8 (255 velocities per individual; $n = 7,140$ in total for each direction). 2) In total, 255 values were
9 randomly selected from each data set. The values were selected with repetitions. 3) A random
10 selection of values was repeated 28 times for each data set. The reselection of an identical data point
11 was not excluded.
12 *MC2.* 1) The raw move-step length data from 28 *D. magna* individuals were collected into
13 a data set. 2) In total, 255 values were randomly selected from each data set. 3) A random selection
14 of values was repeated 28 times for each data set. Values were selected with repetitions. 4) A plus or
15 minus sign was randomly assigned to the selected move-step length.
16 *MC3.* 1) The raw SCIS cluster data from 28 *D. magna* individuals were collected into a
17 data set. Each cluster included a sign (plus or minus) and a discrete size. 2) A cluster was randomly
18 selected from each data set. 3) The selected cluster was converted to a progression where 1 (if the
19 cluster sign was plus) or -1 (if the cluster sign was minus) was sequenced by the length of the cluster
20 size. 4) Steps 2 and 3 were performed again, and the first and second progressions were combined.
21 Clusters were selected with repetitions. 5) Steps 2–4 were repeated until the length of the
22 progression reached 255. 6) Members of the progression were multiplied by an arbitrary constant
23 value (2 pixels/video frame = 7.2 mm/s). 7) The generation of the progressions was repeated 28
24 times for each data set.

1

2

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8

9 **Competing interests**

10 The authors declare no conflicts of interest.

11

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14

15

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10

11

1 **Figure legends**

2 **Figure 1. *D. magna*.**

3 Sexually mature female (left) and male (right) *D. magna* (lateral view). Scale bar, 1 mm.

4

5 **Figure 2. Overview of swimming trajectories.**

6 Time series plot of female (A) and male (B) *D. magna*. In total, 7,196 coordinates (240 sets, 257
7 consecutive coordinates per individual, $n = 28$) of the calculated centre of mass of *D. magna* were
8 plotted along the vertical and horizontal axes. Consecutive coordinates were normalised to the given
9 halfway point (the 129th coordinate), which was set as vertical coordinate 50 and horizontal
10 coordinate 50. Each plot was annotated by arbitrary symbols with different colours (blue, red, green,
11 orange, violet or yellow). Trajectory graphs are represented as probability density plots for females
12 (C) and males (D). Kernel density estimation was performed using a quartic kernel function with a
13 bandwidth of 12 mm. The two-dimensional density map was visualised using terra colours. Scale bar,
14 10 mm.

15

16 **Figure 3. Mean squared displacement of *D. magna*.**

17 The mean squared displacements (MSDs) of female (red) and male (blue) *D. magna* are plotted
18 along the vertical (top) and horizontal (bottom) axes (logarithmic scales, $n = 28$). Best-fit lines using
19 a power-law approximation are shown in black. The power-law exponent (α) and least mean square
20 (R^2) are shown in the plots.

21

22 **Figure 4. Autocorrelation function of *D. magna*.**

23 Average normalised autocorrelation functions of female (top) and male (bottom) *D. magna* are
24 plotted along the vertical (left) and horizontal (right) axes ($n = 28$).

1

2 **Figure 5. Mean squared displacement of model MC1.**

3 The mean squared displacements (MSDs) of females (left) and males (right) of model MC1 are
4 plotted along the vertical (top) and horizontal (bottom) axes (logarithmic scales, $n = 28$). Best-fit
5 lines using a power-law approximation are shown in black. The power-law exponent (α) and least
6 mean square (R^2) are shown in the graphs. The data shown represent the means $\pm$ SEM.

7

8 **Figure 6. Frequency distribution of move-step length.**

9 The move-step lengths derived from 7,168 velocities are plotted in linear (left) and single
10 logarithmic (right, log-linear model) scale plots. The data for females (red) are superimposed on the
11 data for males (blue). Bin width, 0.12 mm. Best-fit lines using an exponent function are shown in
12 black. The exponent function and least mean square are shown in the plots.

13

14 **Figure 7. SCIS analysis.**

15 (A) Brief overview of the definition of the dominant and recessive directions and durations with an
16 identical sign. The major direction is defined as dominant and is indicated by the plus sign (refer to
17 Methods for details). (B) The frequency of the duration with an identical sign derived from 7,168
18 velocities is plotted at the bottom of the graphs. Frequencies greater than 1,500 ms and less than
19 -1,500 ms were added to the respective end of the bin. Bin width, 166.5 ms. The dominant area is
20 shown in dark grey, and the recessive area is shown in pale grey. In the insets, the tail regions of
21 graphs are enlarged. (C) The dominant area of B is shown on logarithmic scale. Female *D. magna* is
22 shown in red and male *D. magna* in blue. Best-fit lines using a power-law approximation are shown
23 in black. The power-law exponent (α) and least mean square (R^2) are shown in the plots. .

24

Figure 8. Mean squared displacement of model MC3.

The mean squared displacements for females (red) and males (blue) based on model MC3, which was derived from the duration with an identical sign, are plotted along the vertical (top) and horizontal (bottom) axes (logarithmic scales, $n = 28$). A constant arbitrary speed (7.2 mm/s) was used for this simulation. Best-fit lines using a power-law approximation are shown in black. The power-law exponent (α) and least mean square (R^2) are shown in the plots.

Figure 9. Net-to-gross displacement ratios of *D. magna* and two MC models.

(A) Brief overview of the definition of the net-to-gross displacement ratios (NGDRs). Net displacement (ND) is the straight line distance between the initial and final locations, and gross displacement (GD) is the sum of the distance D_i . (B) NGDRs observed in the raw data derived from *D. magna* are shown relative to those from MC1 and MC3. The term ‘fv’ indicates the vertical direction of females, ‘fh’ indicates the horizontal direction of females, ‘mv’ indicates the vertical direction of males and ‘mh’ indicates the horizontal direction of males.

Supplemental Figure 1. Frequency distribution of velocity.

A frequency histogram of the velocities derived from 7,168 vectors derived from female or male zooplankton. The velocity was calculated separately in the horizontal and vertical axes. Bin width = 1 pixel/video frame (0.12 mm/33.3 ms).

Supplemental Movie 1. Female *D. magna*.

An example of a female *D. magna*. Width of the movie is 640 pixels (76.8 mm), and height is 480 pixels (57.6 mm). Frame rate is 30 fps.

1 **Supplemental Movie 2. Male *D. magna*.**

2 An example of a male *D. magna*. Width of the movie is 640 pixels (76.8 mm), and height is 480

3 pixels (57.6 mm). Frame rate is 30 fps.

4