

## RESEARCH ARTICLE

# Rapid swimming and escape movements in the aquatic larvae and pupae of the phantom midge *Chaoborus crystallinus*

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**ABSTRACT**

Rapid locomotion in the aquatic larvae and pupae of the phantom midge *Chaoborus crystallinus* was analysed. A 10-mm long larva moved sporadically by rapidly curling into a tight circle and then unfurling. The most common movement (70% of all movements) was a body rotation of  $332 \pm 22$  deg (mean  $\pm$  s.d.) that lasted  $63 \pm 19$  ms and reached a peak velocity of  $0.07 \pm 0.02$  m s<sup>-1</sup>. If the head unfurled earlier in the cycle, the rotation was smaller and the larva dived downwards. A distinct category of single rotations of approximately 180 deg (8%) resulted in a larva finishing with its head pointing in the opposite direction. A sequence of rotational movements (22%) resulted in more extensive displacements. The area of the tail fan was reduced by folding during part of a cycle. It was made of a row of 26 radiating filaments with interlacing hairs between adjacent filaments and resilin at their ventral midline articulations with the body. The fan sprang back passively to its splayed position after being forcibly folded. Reducing the area of the fan by 80% decreased angular rotation and impaired stability so that 33% of movements ended with the body upside down. A 6 mm long pupa also moved by curling and unfurling motions of the head and tail that lasted  $215 \pm 19$  ms and generated slower velocities of  $0.03 \pm 0.01$  m s<sup>-1</sup>. The pupal tail fan was membranous, oriented differently, had resilin at its articulations and its area could be changed.

**KEY WORDS:** Kinematics, Resilin, Locomotion**INTRODUCTION**

Many insects from at least 13 different orders that live on land as adults have aquatic larval and pupal stages. Such early developmental stages therefore have to use very different locomotory mechanisms in water to those of the adults moving on land or flying in air. Their locomotion may also be driven by the demands of ventilation. Some use gills but others either exchange gases with the water through the cuticle or carry an accessible supply of air with them underwater. The latter strategies require movements through the column of water or even periodic replenishment at the water surface. In turn, this means exposure to aerial predators and those living on the water surface, in addition to a whole range of predators with which they share their aquatic environment. The need to move rapidly and thus either avoid or escape from many predators becomes imperative. Many solutions to these locomotory problems are shown by aquatic larvae and pupae of insects. Larval caddis flies (Trichoptera) and dragonflies (Odonata) have legs so that they can move around on the bottom of ponds or streams or on submerged vegetation. Dragonfly larvae also use a jet propulsion mechanism in which a fine jet of water is

forcibly expelled through the anus (Hughes, 1958). Others, such as the larvae of beetles and hemipteran bugs, have legs that are adapted as paddles to aid swimming. Damselfly larvae (Odonata) swim slowly by undulations of the body accompanied by sequential paddling movements of the legs of first one side then the other (Brackenbury, 2002; Brackenbury, 2003a). During faster swimming, the legs are held along the body in a streamlined position. The body can also be rapidly flexed away from a mechanical stimulus or twisted in an escape response. Mayflies (Ephemeroptera) swim by rapid dorso-ventral undulations of the body with the legs trailing (Brackenbury, 2004).

Still other types of larvae have few, if any, appendages that could contribute to locomotion. Among these are the aquatic larvae of the mosquitoes *Culex* or *Aedes* (Diptera, family Culicidae), which move by side to side flexions of the abdomen with the head and tail describing a circular motion (Brackenbury, 2001b; Nachtigall, 1961; Nachtigall, 1963). A tail fan of bristles with many overlapping hairs, which together act as a continuous surface (Cheer, 1987), aids the production of thrust (Brackenbury, 2001b). The larvae can also glide more slowly through the water, propelled by movements of hair brushes on the mouth (Brackenbury, 2001a). *Culex* pupae also dive at a velocity of 15 cm s<sup>-1</sup> from their resting position at the surface of the water by an upwards and downwards motion of the body (Awasthi et al., 2012; Brackenbury, 1999; Nachtigall, 1962). As the body flexes, first in one direction and then in the other, two paired tongues of stiff cuticle that protrude from adjacent abdominal segments are stressed and act together as a click-like mechanism that contributes to the changing velocities during the two phases of movement (Brackenbury, 1999). The larvae of *Chironomus*, a non-biting midge (Diptera, family Chironomidae), swim by a wave of side to side bending that passes backwards along the body or crawl in a looping fashion similar to some caterpillars on land (Brackenbury, 2000; Brackenbury, 2003b; Nachtigall, 1961).

The phantom midge *Chaoborus* (Diptera, family Chaoboridae) spends most of its life cycle underwater; the aquatic larvae are able to overwinter in contrast to the aquatic pupae, which live only a few days, and the aerial adults, which live 1–2 weeks. The biology of the larvae (Akehurst, 1922) has been described extensively with emphasis, for example, on how live prey is captured by the prehensile antennae (Swift and Fedorenko, 1975) and the function of two pairs of air sacs (Krogh, 1911). The volume of air in these sacs can be adjusted by changes in the sac walls to maintain neutral buoyancy, thus enabling a larva to float horizontally at particular levels within the water column (Damant, 1924; Krogh, 1911; Teraguchi, 1975). The air sacs have no respiratory function and no other respiratory organs are present, meaning that gaseous exchange must take place with the water through the cuticle. Typically, larvae are close to the surface at night but deeper during the day time. Most of the time, the larvae remain still, which, coupled with their transparency, may be their first line of defence against predators. Larvae move only occasionally when, for example, a 'prey comes

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near they dart upon it like an arrow' (Krogh, 1911). These movements are effected movements, variously described as 'lashing' (Harper, 1907) or a 'blitzschnelle Sprungbewegung' (lightning-fast jumping movement) (Nachtigall, 1963). The movements involve rotation of the body, probably assisted by a tail fan described as being made of a row of bristles (Harper, 1907), or of a few sparse hairs (Nachtigall, 1974). These movements may help to maintain the level of the larvae in the water column but they also occur more frequently in water that has previously contained prey (Berendonk and O'Brien, 1996), implying a role in predation. A few of these movements may be linked into a sequence that produces swimming movements away from a mechanical disturbance, implying a role in escape from predators.

This paper analyses further the movements of *Chaoborus crystallinus* (De Geer 1776) (formerly *Corethra plumicornis* Fabricius) larvae and pupae, as revealed by high-speed imaging in an environment enabling movements in any direction. Previous studies of larval movement were made in chamber that had a depth of water of 7 mm (Nachtigall, 1963), which is less than the body length of the larva itself. Movement would therefore have been severely restricted and probably involved frequent contact with the walls of the chamber. The present study also investigates whether altering the area of the larval fan can influence locomotion and searches for the presence of resilin in the tail fans of both larvae and pupae.

## RESULTS

### Body shape

The body length of larvae (Fig. 1A) was  $10.4 \pm 1.3$  mm (mean  $\pm$  s.d.,  $N=21$ ) and that of pupae (Fig. 1B) was  $6.1 \pm 0.3$  mm ( $N=8$ ). Both have a transparent cuticle through which internal organs and muscles can be seen. Larvae had prehensile antennae (Fig. 1C), whereas the heads of pupae were occupied by the developing legs and wings of the adult. The tracheal system of larvae consisted of an anterior and a posterior pair of pigmented air-filled sacs (floats) (Fig. 1A,D). By contrast, pupae had an anterior pair of air sacs that

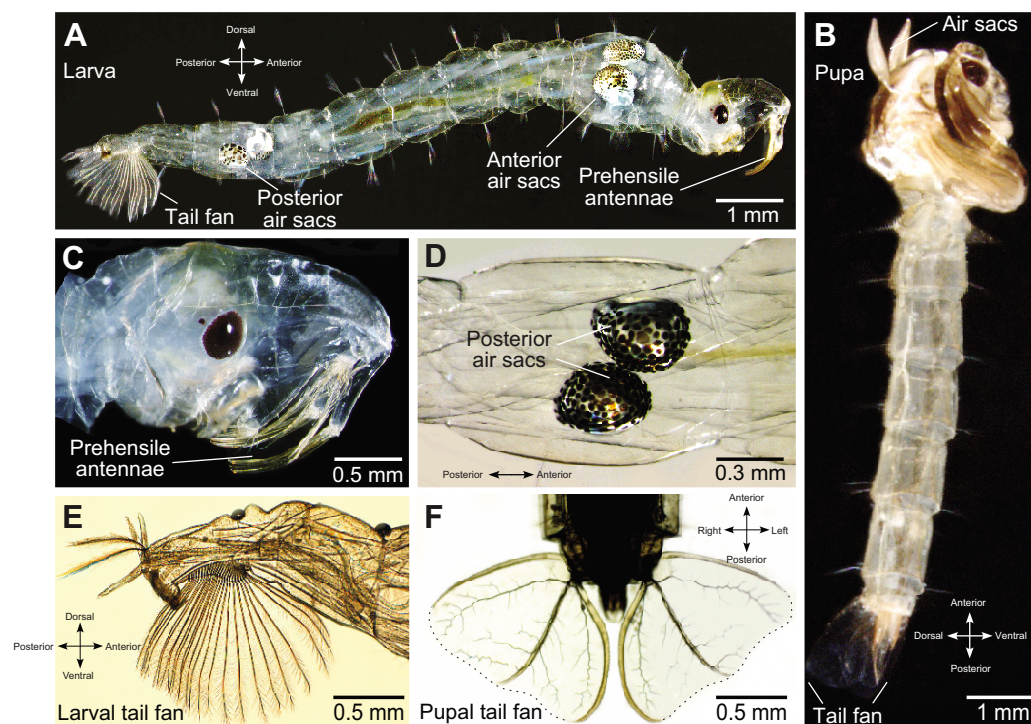
projected from the dorsal surface of the head and were each attached by a thin trachea to a more extensive tracheal system (Fig. 1B).

Larvae floated with the longitudinal axis of their body at a mean angle of  $-1 \pm 20$  deg (two measurements of position for each of 50 larvae) to the horizontal (Fig. 1A). By contrast, pupae normally floated with their longitudinal axis vertical and with their head up and their external air sacs above the head (Fig. 1B). No consistent variations were found from this vertical orientation. Larvae had a complex fan that projected ventrally from the last body segment, which could be splayed fully open (Fig. 1E) or folded. By contrast, the tail fans of pupae were membranous structures that projected posteriorly and could be partially folded (Fig. 1F).

### Larval swimming and escape movements

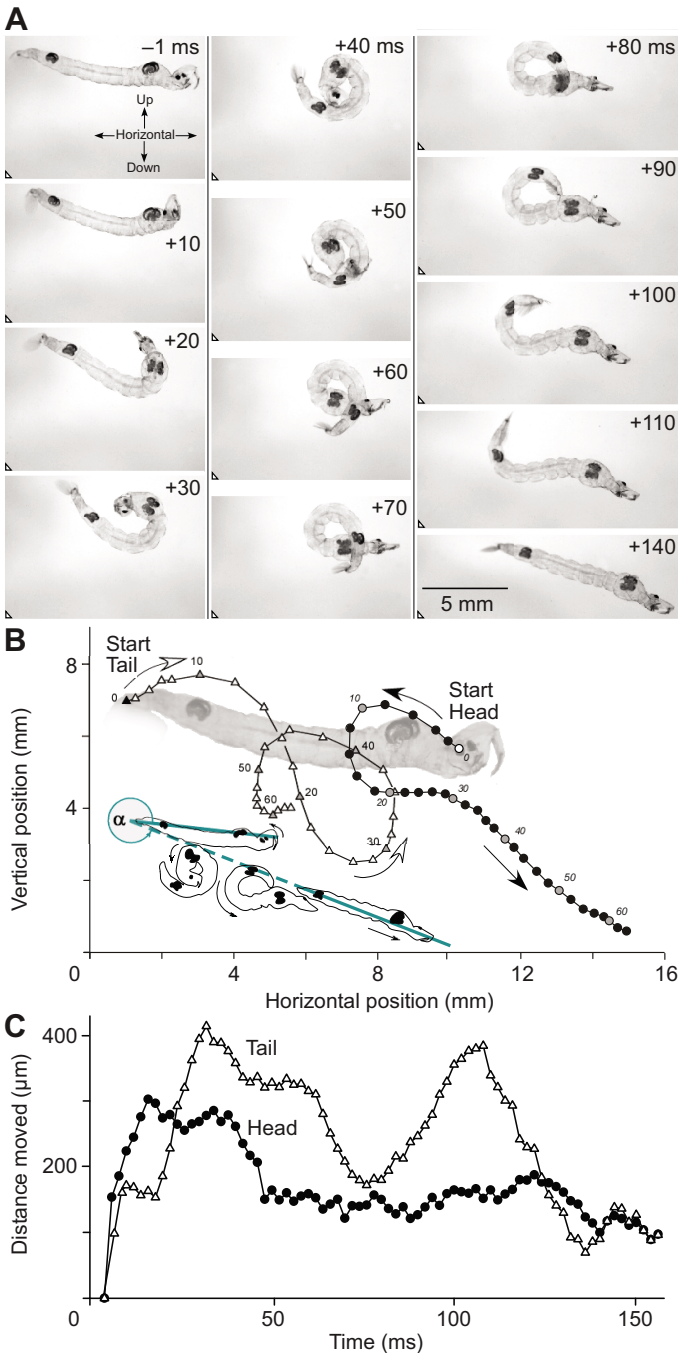
The quiescent floating of larvae was interrupted by sudden rapid movements in which the body curled quickly into a tight circle with the head tucked alongside the tail (Figs 2, 3) and then unfurled so that it was once again straight. These movements could be elicited by mechanical disturbance of the water surface and by the proximity of creatures in the water or their contact with a larva. At other times, there appeared to be no overt stimulus that elicited a movement. Two broad categories of movement through the water were recognised.

The first category was single cycles of movement in which the body curled into a tight circle and then unfurled so that a larva moved away from its starting position (Fig. 2A–C; supplementary material Movie 1). These were the most common movements and comprised 70% of all observations. The average duration of one cycle was  $63 \pm 19$  ms (mean of 33 movements by seven larvae), the mean angle of body rotation was  $332 \pm 22$  deg and the mean peak velocity measured as the tail was unfurling towards the end of the movement was  $0.07 \pm 0.02$  m s $^{-1}$  (Table 1). The smaller the angle of rotation ( $\alpha$  in Fig. 2B) then the earlier the head emerged from the cycle with the consequence that the larva dived downwards in a direction determined by this angle. The displacement of the body



**Fig. 1. Structure of a larva and pupa of *Chaoborus*.** (A) Side view of a larva in its natural horizontal orientation. The gut and individual muscle fibres are visible through the transparent exoskeleton. (B) Body of a pupa in its natural vertical orientation. (C) Head of a larva to show the prehensile antennae. (D) The two posterior larval air sacs viewed dorsally. (E) Side view of the larval tail fan in its fully spread position. (F) Ventral view of the pupal tail fan. The posterior edges are outlined by dotted lines.





**Fig. 2. Spontaneous rapid movement cycle of a larva.** (A) Selected images captured at 1000 images s<sup>-1</sup> at the times indicated are arranged in three columns. The open triangle at the bottom left corner of each image represents a constant point in space. The first detectable movement in the sequence began at time 0 ms. (B) Positions of a point on the head (filled circles) and another on the tail (open triangles) plotted every 2 ms. The starting position of the head is indicated by an open circle and of the tail by a filled triangle, and these points are superimposed on a image of the body captured at this time. Points every 10 ms are shown in grey and with a number representing the time from the start of the movement. The green line defines the angle through which the body rotated. The direction of movement is indicated by the curved arrows (black filled arrowheads for the head and white ones for the tail). The cartoons on the left show four views of the body and the angle  $\alpha$  through which it has turned in one swim cycle. The same conventions are also used in Figs 4, 8 and 9. (C) Plot of distance moved every 2 ms (expressed as rolling five-point averages, also in Fig 4B and Fig. 9C) of the head and tail. The slope of the line represents velocity.



**Fig. 3. Rapid escape movement by a larva.** Images at the times indicated and captured at 1000 images s<sup>-1</sup> are arranged in one column as an object approached from the right of the picture.

ranged from 10 to 30 mm, equivalent to one or, at most, a few body lengths. If the angle of body rotation was large then the body remained at approximately the same vertical level in the column of

Table 1. Swimming performance of *C. crystallinus* larvae

Larva	Peak swimming velocity (m s <sup>-1</sup> )	Angle of rotation during a swim (α in Fig. 2B and Fig. 8) (deg)	Time for one cycle of movement (ms)
Intact tail fan			
Average (N=7; n=33)	0.07±0.02	332±22	63±19
	0.19	333	99
Clipped tail fan			
Average (N=7; n=30)	0.03±0.01	267±33	54±11
	0.08	326	35

Tail fans were intact (N=7) or clipped (N=7). The mean of means for the performance of each individual ±s.d. is given for both groups. The performance by a particular individual with the highest swimming velocity is given in the second row. N, the number of animals; n, the total number of swims analysed for that test group.

water, and the head ended up pointing in the same direction as at the start. Each cycle of movement began with a turn of the head to either the right or the left (Fig. 2A and Fig. 3). This was followed by contractions of muscles in the anterior segments so that the body began to curl or fold upon itself (Fig. 2A) and with the head then turning further to face towards the tail (Fig. 3). The anterior curling of the body continued with the head either passing above or below the posterior air sacs so that the whole body now formed a tight circle with a diameter of approximately 4 mm and with the tail curved so that it was near the anterior air sacs (Fig. 2A). The head then emerged from this circle and the tail swept outwards so that the curvature of the circle was reduced, and eventually the body was straightened. The progression of this wave of contraction starting at the head passes down the body at a velocity of ~0.16 m s<sup>-1</sup>, suggesting that the rate is limited by muscular contraction rather than conduction of nerve impulses.

The common features of all these sudden movements were revealed further when the vertical and horizontal displacements of the body were plotted (Fig. 2B). The relative timing and velocities of these movements also became apparent when the displacement of a point on the head and another on the tail were plotted every 2 ms (Fig. 2C). The head moved first and was followed after a delay by an increase in the velocity of the tail. While the body was curled, the velocity of the head fell quickly, whereas that of the tail declined over a longer period. The subsequent unfolding of the tail was represented by a second rise in its velocity, which was not mirrored by that of the head. Overall therefore, there was an initial peak in the velocity of the head but two peaks in the velocity of tail movements. In two of the 21 larvae analysed, the body only rotated through approximately 180 deg and finished at almost the same place and level in the water but with the head pointing in the opposite direction. Such movements comprised a further 8% of all movements. Together, the single cycles of movements showed a bimodal distribution for the angles of rotation; the small number of 180 deg turns indicates that they were not just outliers of a broad distribution of angles.

The second category of movement was a series of cycles of movement that were linked together in a sequence (22% of all movements) and which resulted in a more extensive and, usually, downwards displacement of a larva (Fig. 4; supplementary material Movie 2). In such a sequence, the first cycle differed only from that described above in that the body did not straighten completely but instead was thrown into a second rotation, typically in the opposite direction (Fig. 4A and Fig. 5). For example, the first turn by the head in Fig. 4 was anti-clockwise but the first turn by a different larva in Fig. 5A was clockwise. In both larvae, the second turn was in the opposite direction to that of the first (Fig. 4A and Fig. 5B). The transition between successive turns was such that the head began to move in the opposite direction before the tail had unfurled from the

preceding turn. Plotting the distance moved every 2 ms by the head and tail against time showed a characteristic sequence of peaks in velocity of both (Fig. 4B). The head moved fastest on its first cycle, whereas the tail velocity tended to increase on subsequent cycles. Only at the end of the swimming sequence did the tail straighten fully so that the larva glided in the direction determined by the overall angle of rotation measured from its original starting position. Once the straightening movement stopped, the forward movement slowed, indicating the viscosity of the water was eating up momentum. The overall trajectory of the both single and multiple cycles of movement can be inferred from the general displacement of the body. There was no single point on the body that followed a smooth line, indicating that the rotations were not about a single pivot.

Larval tail fan

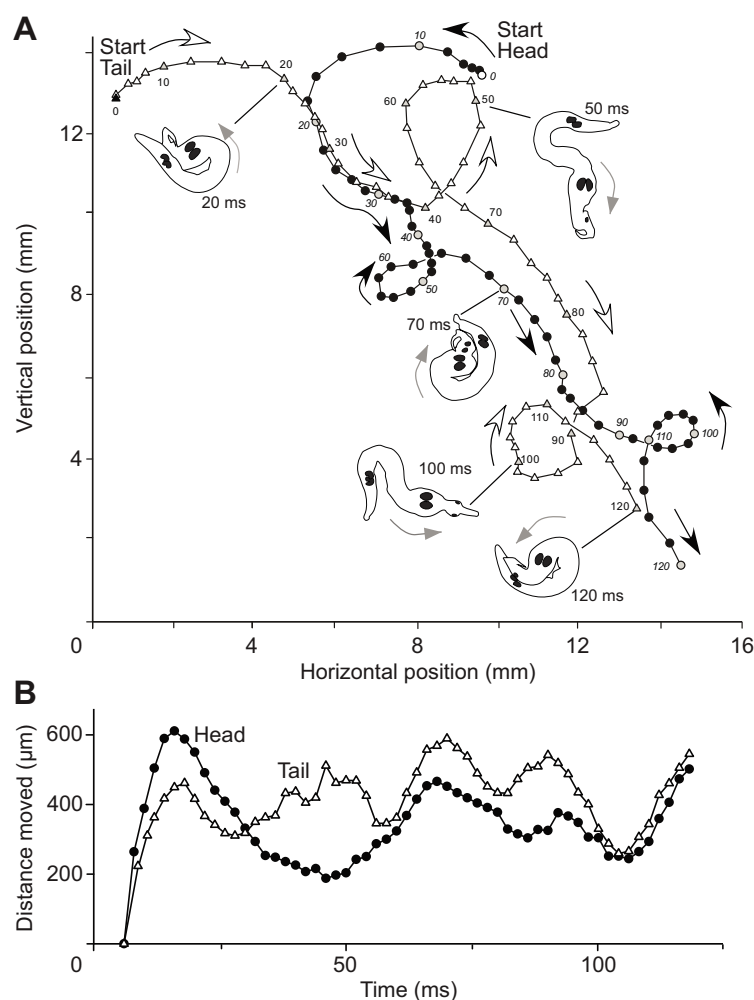
To understand what contribution the fan might make to locomotion, its anatomy and movements were analysed and then the effects of reducing its area were tested.

Structure

The larval tail fan consisted of a single row of twenty-six (mode, range 24–26, N=7 fans) 0.7 to 0.9 mm long tapered struts or filaments that radiated ventrally from the midline of the last segment of the body (Fig. 1E and Fig. 6). At their bases, the filaments had a diameter of 11.5±1.2 µm (mean ± s.d. of all filaments in one fan) and were separated from each other by 10 µm when the fan was fully spread (Fig. 6A–C). The thinner tips of these filaments were some 100–150 µm apart when the fan was fully splayed. Two rows of hairs, 50 µm long and spaced 5 µm apart arose at an angle of 35 deg from both anterior and posterior sides of each filament (Fig. 6A,C,E). Hairs from adjacent filaments were therefore interlaced, except at the tips of the filaments. Each of the fan filaments articulated with the same number of elements at the ventral midline of the body (Fig. 6A). These body elements formed a herring-bone pattern with two rows of arms pointing laterally, anteriorly and dorsally from the midline (Fig. 6C,D). Each arm had a diameter of 8.4±1.3 µm close to their midline articulation with the fan filaments but then tapered laterally. They ranged in length from 130 µm at the anterior end of the fan to 50 µm posteriorly. The articulations of alternate filaments were offset from the midline (Fig. 6D), thus allowing greater compaction when the fan was folded.

Presence of resilin

Both the fan filaments and the arms of the body elements fluoresced bright blue when illuminated with UV light of a particular wavelength, indicating the probable presence of the elastic protein resilin (Fig. 6B–D). In intact larvae, the blue fluorescence in the filaments was most intense close to their bases, which could indicate



**Fig. 4. Three rapid rotations propel a larva downwards.**

(A) Positions of the head (filled circles) and tail (open triangles) are plotted every 2 ms. The changing body shape at given times is represented by tracings from the video that are also linked by lines to the graph. The direction of head rotation of each cartoon is shown by a grey arrow; the black arrows show the direction of movement of the head in the graph and the open arrows show that of the tail. (B) Distance moved by the head (filled circles) and tail (open triangles) plotted every 2 ms against time to show the changing velocities. See Fig. 2 legend for the description of conventions.

an uneven distribution of resilin along their length or that the fluorescence was simply reflected internally within the filaments from the body elements. To distinguish between these two possibilities, individual filaments were cut close to their articulations and removed for examination on their own under a microscope (Fig. 6E,F). Fluorescence was then observed to occur along the whole length of a filament (Fig. 6E) with brighter spherical patches at the articulation of each lateral hair (Fig. 6F).

#### Contribution of the fan to swimming movements

In natural swimming, the larval tail fan changed shape from its fully expanded form to one where it was folded flat and pointing posteriorly along the ventral surface of the abdomen. In the former position it would offer its greatest surface area and thus its greatest resistance to the water. When the body was stationary and its longitudinal axis was horizontal, the fan was in its most expanded state (Fig. 7A). It was difficult to follow the shape of the fan during the turning and twisting movements of locomotion, but in the most favourable circumstances clear changes in the area of the fan could be observed (Fig. 7A). As the tail was curved and came to lie close to the anterior air sacs, the fan was almost fully folded so that its area was reduced by 81% from its fully expanded state (Fig. 7A, frame 71 ms). As the tail was then straightened, the fan began to unfurl rapidly (Fig. 7A, frame 78 ms) and reached its fully expanded form in a further 12 ms (Fig. 7A, frame 90 ms).

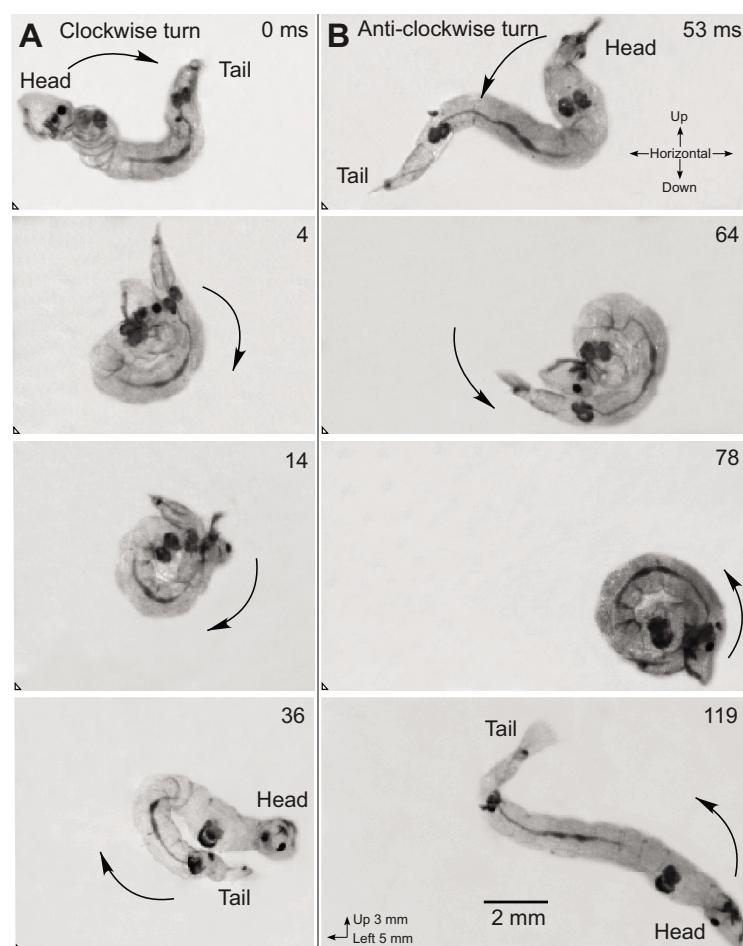
If the blue fluorescence indicates the presence of resilin, then it would be expected that the fan itself would have elastic mechanical

properties. To test this, the elasticity of fans in recently dead larvae was analysed (Fig. 7B–E). The fan in dead larvae always assumed its fully splayed position. From this position, the fan was then moved forcibly into its folded position (Fig. 7B) by a pin held in a micromanipulator, and images of the movements were captured at a rate of 125 images  $\text{s}^{-1}$ . When the pin was quickly removed, the fan sprang back to its fully splayed position (Fig. 7C–E) in a mean time of  $114 \pm 15$  ms (range 96–152 ms, 11 movements of fans in three larvae).

To analyse the contribution of the fan to swimming performance and to determine whether it acts like a rudder to stabilise the body, or whether by increasing the surface area of the tail it increases propulsion (it might do both), 14 larvae that were caught in the same location and on the same day were divided into two groups of seven. The first control group did not undergo any surgery (intact fan, Table 1). The area of an intact tail fan when all the filaments were fully splayed was calculated by treating it as a segment of a circle with a radius that included the entire length of an individual filament ( $r_3$  in Fig. 8). The insertions of the filaments with the body elements ( $r_1$  in Fig. 8) were treated as a smaller segment, which was then excluded. The total surface area of the fully expanded fan was  $1.6 \text{ mm}^2$  ( $N=7$ ). In the second group, the filaments of fans were cut (clipped fan, radius  $r_2$  in Fig. 8; supplementary material Movie 3), reducing the area to  $0.3 \text{ mm}^2$  (an 80% reduction). A minimum of four swims were recorded for each individual from the two groups.

Larvae with reduced fans frequently adopted postures that were different from intact larvae; the head would point downwards at a





**Fig. 5. Sequential rotations in opposite directions by the head of a larva during a swimming sequence.** (A) A clockwise rotation. (B) An anti-clockwise rotation. Selected images from a swim are shown at the times indicated and were captured at a rate of 1000 images  $s^{-1}$ . The frame at 119 ms has been shifted by the amount indicated in the bottom left-hand corner.

steeper angle or the larvae would even float upside down. Its stability and ability to control its body orientation was also impaired, particularly after swimming movements. For example, 10 of the 30 (33%) recorded swimming movements of larvae with clipped fans ended with the body upside down. This contrasts with intact larvae, which were not observed to assume this upside down posture.

In swimming movements, the angle ( $\alpha$  in Figs 2, 8) through which the body rotated in one cycle was smaller in larvae with a clipped fan ( $267 \pm 33$  deg) than in an intact larva ( $332 \pm 22$  deg). These values are significantly different (Student's *t*-test:  $t_{56} = 6.91$ ,  $P = 0.0001$ ; Shapiro–Wilk test of normality: intact 0.315, clipped 0.585). The average velocity of movements and the average time taken to execute one cycle of movement in larvae with clipped fans were not significantly different from those in normal larvae.

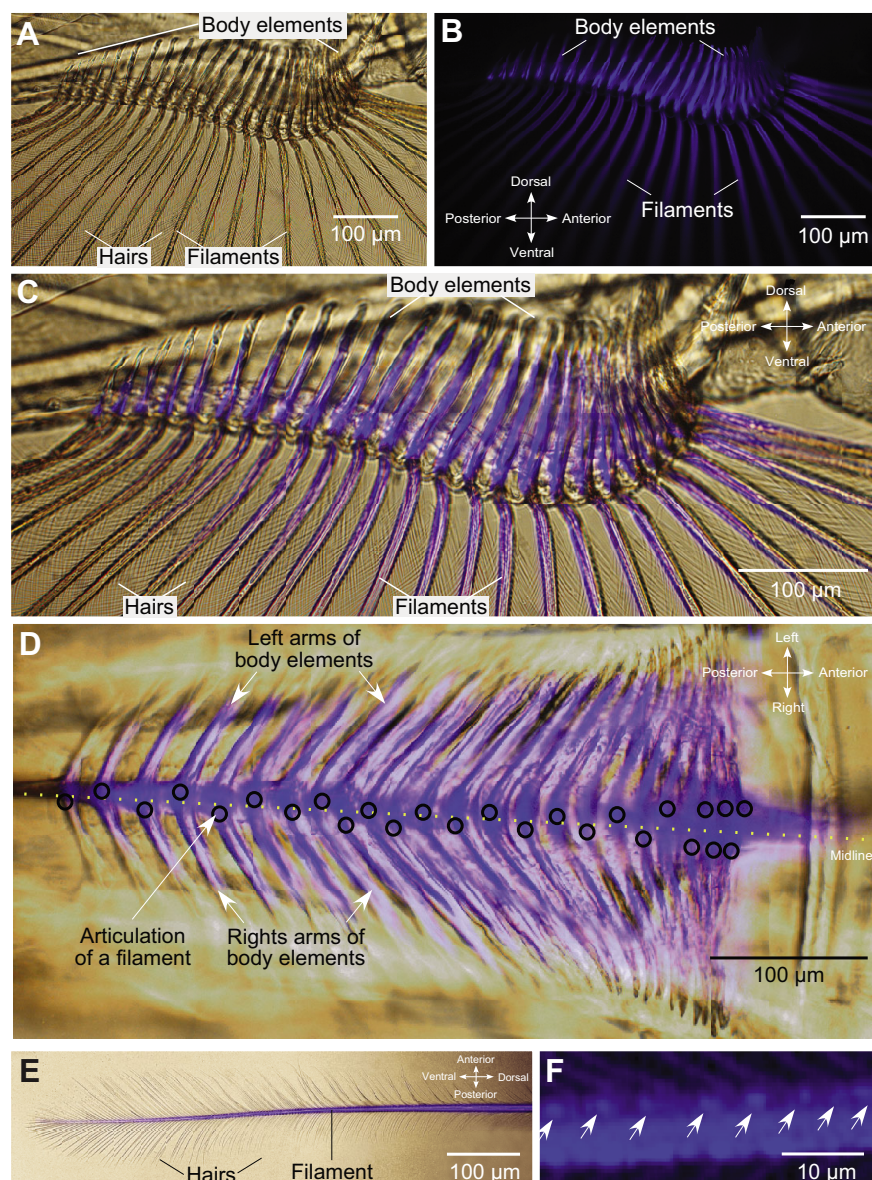
To test whether the shape of the fan could be altered by the flow of water as the body moved, dead larvae were dragged head first through the water at velocities experienced during natural swimming. The external forces would therefore act along the antero-posterior axis of the body and fan in the same plane as it would normally fold. When moved in this way, the fan was folded and its area was reduced by  $34 \pm 1.2\%$  (mean of means  $\pm$  s.d. for four larvae with three movements each). At the end of the imposed movement and as the velocity decreased, the fan returned to its original splayed position.

#### Pupal swimming and escape movements

Pupae, like larvae, spent much of their time stationary but in a vertical orientation with the head up. Movements were, again, sporadic and involved rapid rotations of the body, some of which

were single rotations and some a concatenation of these rotations into a sequence. In the most common form of movement (49% of all pupal movements), the head was initially moved backwards and then the anterior part of the abdomen also curved backwards (Fig. 9A; supplementary material Movie 4). This resulted in the body assuming a circular shape with a diameter of 2.2 mm in which the head and tail were at the same vertical level within the water but with the main loop of the body above. The tail then straightened and the head reversed direction so that it now moved out of the curl and the pupa was now upside down in the water (Fig. 9A, first row). The head continued to move upwards and sideways and the tail curled so that, once again, the body formed a curved shape in the water but with the main loop of the body now below (Fig. 9A, second row). Finally, the head and the body straightened so that the head resumed its original upright position. The whole cycle thus consisted of two distinct phases of movement, in contrast to an individual cycle of movement by larvae. During the backwards movements of the head, the air sacs were passively pushed forward and, during forwards movements of the head, they were passively pushed backwards so that they laid flat against the head during the rapid phases of either movement (Fig. 9A). The whole cycle lasted  $215 \pm 19$  ms (mean  $\pm$  s.d. of 14 swims by six pupae) (Table 2) with the shortest being executed in 177 ms. The overall result of the movement was to displace the body downwards.

The other common movement (44% of all pupal movements) resulted from joining a series of individual cycles into a sequence (Fig. 9B–D; supplementary material Movie 5). The body thus moved



**Fig. 6. Blue fluorescence indicative of resilin in the larval tail fan.** (A) Side view of the fan viewed under white light. (B) Same view under UV light shows blue fluorescence in the filaments and in the repeating body elements. (C) Images viewed in the same orientation and captured under white and UV light are superimposed. (D) Dorsal view of the herring-bone arrangements of the left and right arms of the body elements. Brightfield and UV images are superimposed. Black circles show the filament articulations at the midline (dotted line). (E) A filament dissected from the fan shows blue fluorescence along its length. (F) Short length of a filament viewed at higher magnification. A circle of blue fluorescence occurs at the base of each hair (white arrows, top row).

in a series of curling and unfolding movements with the head alternately turning first in one direction and then in the other (Fig. 9B). Usually, these movements resulted in a pupa diving from the surface of the water. Plotting the distance moved by the head and tail every 2 ms revealed a series of peaks in the velocity of both (Fig. 9C). The initial peak in the velocity of the head preceded that of the tail and reached its highest value on the first cycle. The velocity of the tail increased on subsequent cycles of the movement, reaching a peak of  $0.03 \text{ m s}^{-1}$  (mean of 14 swims by six pupae; Table 2). Even the fastest velocity reached ( $0.04 \text{ m s}^{-1}$ ) was only half of that attained by a larva. The trajectory of the diving movement, as measured by a line projecting from the starting point of the head to its final position (angle  $\beta$  in Fig. 9D), was  $58 \pm 27$  deg.

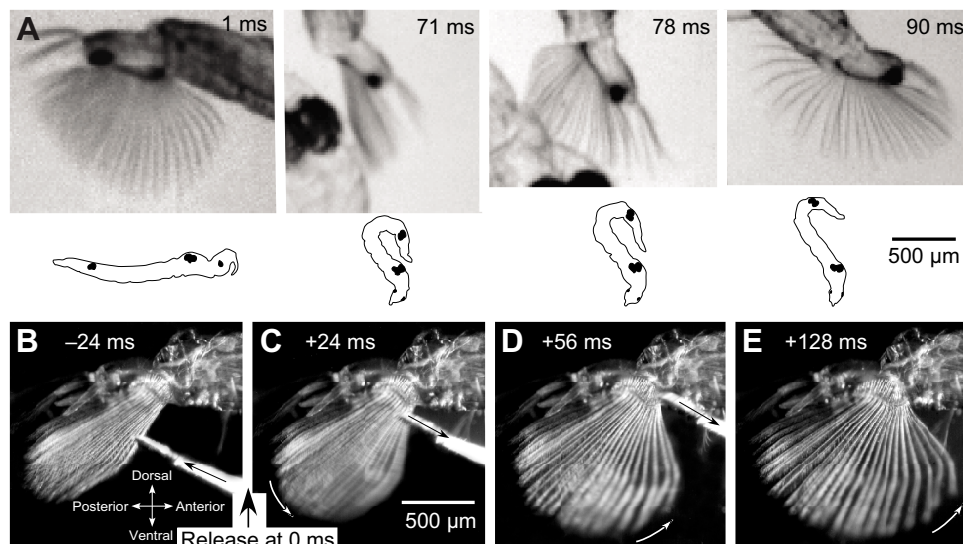
Brief forward nodding movements of the head were also observed in two pupae and represented 7% of all movements. They involved the head initially bending forwards and downwards while the posterior segments of the abdomen curled forwards and upwards. Both the head and the body then straightened with the result that the direction of motion was upwards, but the velocity of the movement was slow and the displacement of the body was small.

### Movements and structure of the pupal tail fan

The tail fans of pupae, like that of larvae, also changed shape during swimming movements (Fig. 10A). In its normal vertical resting posture, the fan was fully flared and offered its maximum surface area to the water. As the head curved upwards during the second phase of a single cycle of movement and the tail pointed upwards, each lobe of the fan moved medially so that the two lobes overlapped and offered their smallest surface area (reduced by 45% from the fully splayed state) to the water (Fig. 10A, frame 88 ms). As the body progressively straightened, the fan gradually returned to its fully flared position (Fig. 10A, frames 146 and 166 ms).

The structure and orientation of the pupal tail fan was different from that in a larva (Fig. 1F and Fig. 10B). The pupal fan consisted of two paddle-like membranous structures that had a combined area of  $1.7 \text{ mm}^2$  and which protruded from the lateral and posterior corners of the last abdominal segment. Both the left and the right fans were bi-lobed and were made of transparent flexible membrane, stiffened by three curved cuticular struts on the lateral and medial edges and in the centre. The posterior trailing edges were not stiffened. The proximal junction of the three struts formed the articulation with the





**Fig. 7. Movements of the larval tail fan.** (A) Changing shape of the fan during a natural swimming movement, which began at time 0 ms (cartoons show the profile of the whole body). The images, at the times indicated, were captured at  $1000 \text{ frames s}^{-1}$ . (B–E) Elastic recoil of the fan from its folded position. (B) The fan of a dead larva was forcibly moved into its folded position. (C–E) Upon release at time=0 ms, the fan progressively sprang back (curved white arrows) to its fully splayed position. The black arrows show the direction of movement of the pin used to manipulate the fan. Images were captured at  $125 \text{ frames s}^{-1}$ .

body. The membrane of each paddle was penetrated by three tracheae, which each gave rise to finer tracheoles. The pupal fan thus extended the length of the body posteriorly and its dorso-ventral flattening increased the width of the body by adding, when fully extended, an area equivalent to 700% of the last abdominal segment. When the posterior of the pupal abdomen was viewed under UV light, blue fluorescence was revealed, extending from the articulation of each paddle to the midline (Fig. 10B).

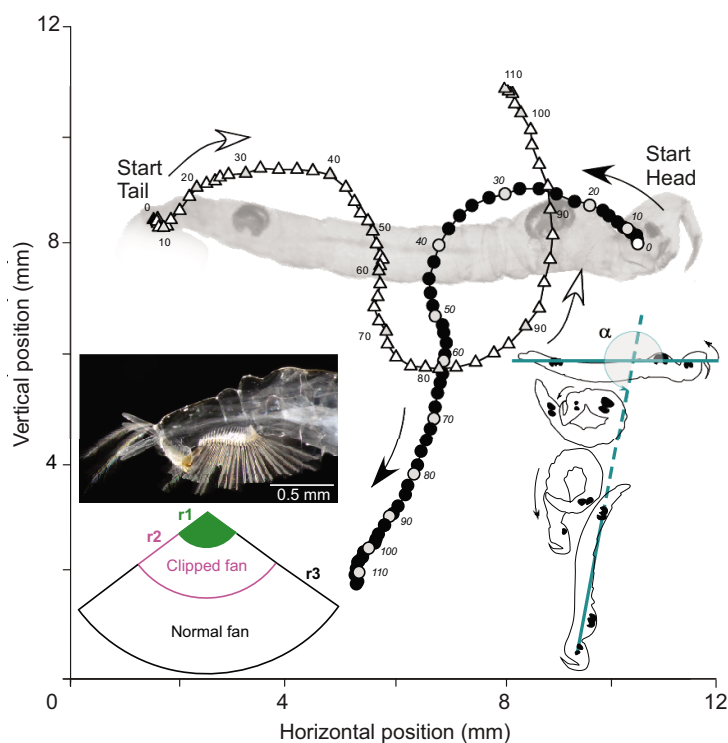
## DISCUSSION

### Larval and pupal movements

Larvae and pupae of *C. crystallinus* spent much of their time motionless in the water; the larvae floated with the longitudinal body axis almost parallel to the horizontal, whereas the pupae floated with the body vertical and the head pointing upwards. These periods of

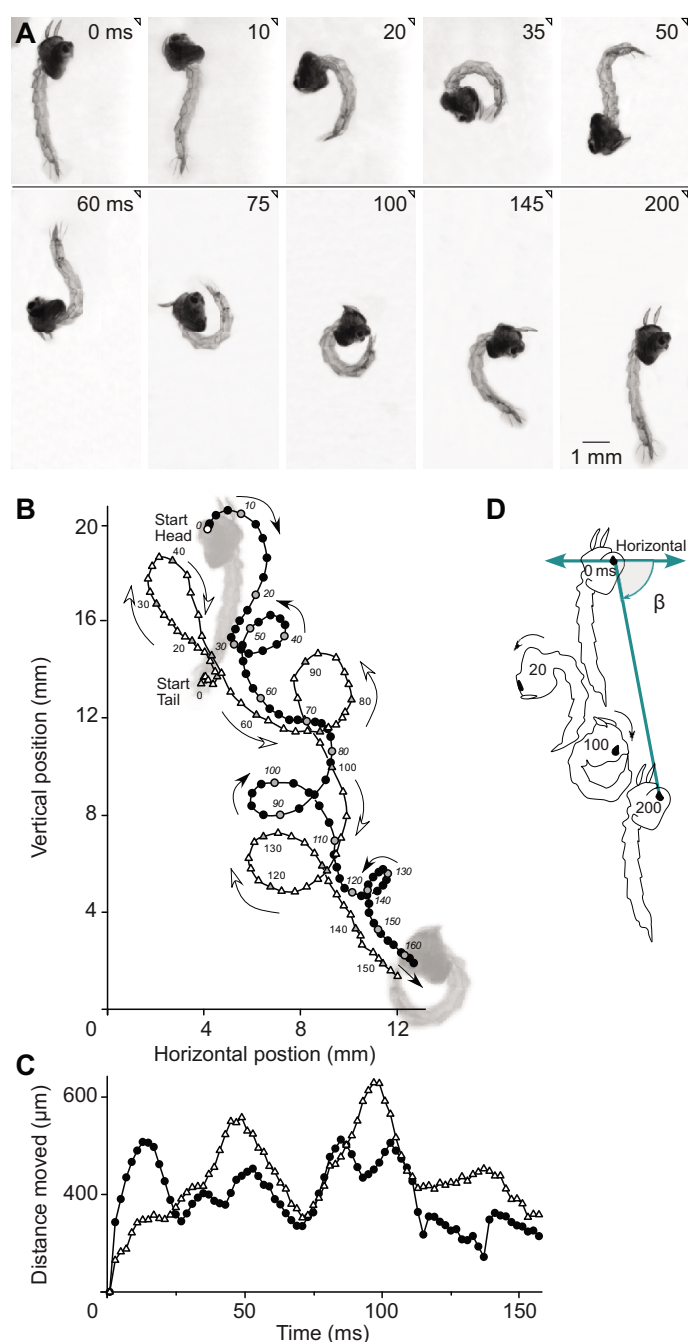
quiescence were punctuated by rapid movements generated, in both larvae and pupae, by rotational movements of their limbless bodies assisted by movements of their very different tail fans. These movements consisted either of single cycles of rotation or a concatenation of a few cycles into a sequence. In the experimental chamber used for capturing high-speed images of the movements, a few cycles would move the body from top to bottom. In normal life, however, larvae living in deep lakes can move diurnally many metres, implying that many cycles of these movements must be executed.

In larvae, two types of single rotational movements were recognised. In the most common (70% of all larval movements recorded), the body rotated through a mean angle of 332 deg so that the head ended up pointing almost in the same direction as at the start. The overall angle varied so that, when it was smaller, the head



**Fig. 8. Movements of a larva with a reduced tail fan.** Positions of the head (filled circles) and tail (open triangles) are plotted every 2 ms. The photograph shows the clipped fan, and the diagram shows the area of the normal fan (radius  $r_3$ ) and the reduction (radius  $r_2$ ) after clipping. The cartoons (right) show four views of the body and the angle  $\alpha$  through which it turned. See Fig. 2 legend for the description of conventions.





**Fig. 9. Pupal swimming movements.** (A) Selected images, at the times indicated, from a swim captured at 1000 images  $s^{-1}$  are arranged in two rows. (B) Plot of head (filled circles) and tail position (open triangles) every 2 ms during three cycles of a downwards swim. (C) Plot of distance moved by the head (filled circles) and tail (open triangles) against time. (D) Cartoons show the trajectory (angle  $\beta$ ) of the diving movement. See Fig. 2 legend for the description of conventions.

would emerge earlier from the rotation and determined the angle of the dive. Infrequently (8% of larval movements analysed), the body would rotate by approximately 180 deg so that the head ended up pointing in the opposite direction from that at the start. The head and body always moved in the same direction throughout these single rotational movements of a larva. By contrast, a single cycle of movement by a pupa consisted of two distinct phases. In the first, the head moved backwards until it was upside down and directly

below the tail. In the second, the direction of rotation was reversed so that the head now moved forwards to regain its original head up and vertical orientation. A single cycle of rotation by a larva was thus more than three times quicker than a rotation by a pupa, and the peak velocity of the tail movement was twice as fast.

In the experimental chamber used here, both larvae and pupae had enough space to move freely in any direction. Movements were spontaneous or were elicited by disturbances to the water surface. No prey or predators were present. In this limited environment, the relative proportions of a large sample of the different types of movements performed by a large number of larvae and pupae were quantified. Single cycles of movement were more common than sequences of cycles in both larvae and pupae. Nachtigall (Nachtigall, 1963) gave no indication of either the number of larvae or the number of movements he studied and analysed. Hence, he did not give any indication of the relative proportions of the different movements he described but still gave equal prominence to 180 and the 360 deg ‘jumping movements’ of larvae. Furthermore, he studied all larval movements in a chamber that was 7 mm deep (Nachtigall, 1963), which must therefore have precluded free movements of a 10 mm long larva in the vertical plane.

The single cycles of movement of larvae have been suggested to maintain its vertical position in the water column (Nachtigall, 1963) by correcting for possible imbalances in the buoyancy provided by the air sacs. A larva is, however, both a predator that needs to detect and catch other aquatic animals, and potential prey for other predators seeking it as food. Assigning a function to a particular movement in the absence of a behavioural context becomes very difficult. Single cycles of movement may indeed adjust the position of the body in a vertical column of water, but they may also orient the body towards a prey and may move the body sufficiently far away from small predators. A sequence of several cycles could also achieve any of these objectives and, in addition, enable a larva to escape from large predators and to undertake more extensive movements.

Four types of movement by pupae have been described (Nachtigall, 1962). Two called ‘Rückzucken’ and ‘Abtauchen’ were distinguished by Nachtigall on the basis of whether the body moved up or down. The first was a rapid backwards movement of the head followed by a straightening of the body which had no locomotory effect (Nachtigall, 1962). It was not observed in our study. The second (Abtauchen) was the movement also found in our study that led to diving movements. The third movement was a rapid sideways movement with either an upwards or a downwards tendency. Many of the diving movements observed in our study had a sideways component because the trajectory had a mean angle of 58 deg from the horizontal. The very similar overall form of these movements led us to lump them together. Nachtigall’s fourth movement was a slow upwards movement towards the surface, but the detail of the movement was not obvious and could not therefore be compared with the movements we observed.

An escape strategy that involves the resting larva first rotating its body and then ending up with the head facing in more or less the same direction after almost a full cycle of rotation seems like one that would invite capture by a predator. It also suggests that when the body is straight and the tail fan is splayed, as it is when resting, the larva is unable to push against the water in a way that would generate forwards momentum. Instead, what the larva is demonstrably able to do is to contract muscles on one side to curl the body, fold the tail fan to increase streamlining and thereby create a rotatory motion. The larva can then generate forwards momentum by converting some of this rotatory motion into linear motion by

Table 2. Swimming performance of *C. crystallinus* pupae

Pupa	Peak swimming velocity (m s <sup>-1</sup> )	Dive trajectory (β in Fig. 9D) (deg)	Distance travelled* (mm)	Time for one cycle of movement (ms)
Average (N=6; n=14)	0.03±0.01	58±27	5.4±1.4	215±19
	0.04	72	6.7	177

The mean of means for the performance of each individual ± s.d. is given. The performance by a particular individual with the highest swimming velocity is given in the second row. \*Distance travelled is defined by the x–y position of the head at the start and the end of one cycle of movement. *N*, the number of animals; *n*, the total number of swims analysed.

straightening the body and extending the tail fan to increase lateral thrust. In addition, the actively retracted tail fan is released and springs back passively, giving an extra kick to the forward momentum. The size of the tail fan coupled with its speed of movement indicates that it is operating at a low Reynolds number of ~10 so that the water would act as if it were sticky honey. To begin the next cycle in a series, at least part of the forwards linear motion can then be converted back to a rotatory motion.

Tail fans

In all of these movements, thrust was generated by the action of the cylindrical, limbless body against the water as it first curled and then unfurled. What contribution does the tail fan make to swimming movements? The tail of a larva had a prominent fan that projected

ventrally from the last abdominal segment and, when fully extended, added an extra 1.6 mm<sup>2</sup> surface area to the tail end. This means that the presence of a fully expanded tail fan increases the lateral surface area of the last abdominal segment by almost 500%. This is the part of the body that moves at its fastest when the body is unfurling. The tail fan changed in area during the course of one cycle of rotation; as the curved tail approached close to the anterior air sacs, the fan was folded, reducing its area by approximately 81%, but then expanded again as the body unfurled. Experimentally clipping the fan to reduce its area by approximately the same amount had two profound effects on movements. First, the angle of body rotation during a single cycle of movement was significantly reduced. Second, stability was impaired because a third of rotatory movements ended with the body upside down, an outcome that was



Fig. 10. Tail fan of a pupa. (A) Four images of the changing shape of the tail fan taken, at the times indicated, during a natural swimming movement, which started at time 0 ms. Images were captured at a rate of 1000 s<sup>-1</sup>. Profiles of the whole pupa are shown below. (B) Superimposed images of ventral views of the fan taken under brightfield and UV illumination.

never observed in a larvae with an intact fan. These results suggest that actively controlling the shape and area of the fan controls the transmission of thrust to the water and helps, through a rudder-like action, to stabilise body movements.

How might the observed changes in the shape of the larval tail fan be controlled? Dragging the body of a larva head first through the water at the velocities measured during natural swimming caused the fan to fold and its area to be reduced by 34%. This is approximately a third of the reduction observed during natural swimming and indicates the external forces acting along the antero-posterior axis of the fan can contribute to the folding. Does the complete folding of the fan depend on an active mechanism involving muscular contractions? The individual muscle fibres that can be readily seen through the transparent cuticle of the body have a diameter that ranges from 25 to 35  $\mu\text{m}$ . The last body segment is  $\sim 600 \mu\text{m}$  long and 300  $\mu\text{m}$  wide, and the whole set of body elements of the fan is only 450  $\mu\text{m}$  long. There is therefore insufficient space for each of the 26 fan filaments to be operated by an individual muscle fibre. Indeed the last segment, like other segments, contains only a few muscle fibres so that an active compression of the fan into its folded position would need to be brought about by the action of a very few fibres pulling on skeletal elements in the last segment, which in turn would compress the body elements of the fan. One such fibre is oriented (Fig. 6A,C) in a way that its contraction might compress the fan into its folded state.

Adjacent radiating filaments that make up the larval tail fan have rows of densely spaced hairs that interlace with each other. The narrow spaces between these hairs suggests that the whole fan acts as a continuous sheet when moved through the water at the observed velocities and at the expected low Reynolds numbers (Cheer, 1987). Another feature of this type of construction is that it enables the fan to be compacted when folded so that its area can be varied over a wide range.

The default position of the fan is fully splayed. In a recently dead larva, the fan can be moved forcibly into its fully folded position but, when the imposed force is released, the fan quickly springs back to its default fully expanded state. Similarly, the fan of a dead larva was compressed when the body was dragged through the water but quickly regained its fully splayed state as the velocity of movement slowed. This implies that the fan must have its own elasticity. When the fan was illuminated with UV light of a specific wavelength, each of the filaments and the body elements with which they articulate showed bright blue fluorescence. This fluorescence is one of the key defining characteristics of the elastic protein resilin (Andersen and Weis-Fogh, 1964; Weis-Fogh, 1960). Thus, the larval fan has a demonstrated elasticity and almost certainly contains the elastic protein resilin. When the fan was folded, three effects on its structures were seen. First, the elements within the body were compressed. Second, the filaments rotated at their articulations with these elements. Third, the filaments themselves were bent, as also occurred when the splayed fan encountered resistance from the water in certain directions during swimming. All these events will result in distortion and bending of the resilin within the body elements, at the articulations of the filaments and along the length of the filaments themselves. Once these forces are reduced, the resilin, acting like a spring, will rapidly resume its original shape and thereby restore all the structures to their original state. The elasticity ensures that the default position of the fan is the fully expanded or splayed position. The presence of resilin along the length of each filament with spherical areas of more intense fluorescence at the articulation of each hair could have two further effects. It will ensure that the long thin filaments remain sufficiently

flexible so that they do not snap when they are distorted and that they can rapidly resume their default shape. The resilin at the base of the interlacing hairs also determines their set position, to which they will return when any resistance encountered during movement of the fan is removed. The integrity of the fan shape will thus be maintained.

In the moult to a pupa, this fan is replaced by an arrangement that is quite different in both orientation and structure to, but with approximately the same area and with what appears to be the same action as, the larval fan. In a pupa, the fan projects posteriorly from the last abdominal segment and is a bilateral structure that has two articulations with the body. Each fan is a membranous structure stiffened by three struts and also supplied by tracheae. Like its larval counterpart, the fan can be altered by folding and its articulations also fluoresce bright blue, suggesting the presence of resilin. During swimming, its area is changed by approximately 40%, indicating an active control of both thrust transference and possibly stability control. In some movements, Nachtigall (Nachtigall, 1962) considered that the pupal fan had no steering function.

### Future experiments

Two issues that warrant more work have emerged from this study. First, which elements of the body movements are under active muscular control and which result passively from the preceding movements? Nachtigall (Nachtigall, 1962; Nachtigall, 1963) considered some movements in both a larva and a pupa to be generated by active muscle contractions and others by passive forces. We still have no information about when the muscles contract relative to the observed movements. We also do not know whether muscle contractions alter the shape of the larval tail fan at different phases of a movement. Monitoring the electrical activity of the motor nerves or the muscles themselves would give this information, but recordings would be challenging to make. Alternatively, information about the flow fields around the animal might help to identify the probable sites and time course of muscular contractions in the body. A second series of experiments would seek to find functional explanations for why the construction and orientation of the tail fan changes so fundamentally in the moult from larva to pupa even though body shape and movements remain similar.

### MATERIALS AND METHODS

Larvae and pupae of *C. crystallinus*, called phantom midges or glassworms, were collected from a fish-free pond in Llandinam, Wales (52.47107, -3.43987; 52°28'15"N, 3°26'23"W), in November and December 2011–2013. The genus has a worldwide distribution and belongs to the order Diptera and family Chaoboridae.

Intact larvae and pupae were photographed in the laboratory with a Nikon DXM1200 digital camera attached to a Leica MZ16 stereo microscope and with a MicroPublisher 5 camera (Q-Imaging, Marlow, Buckinghamshire, UK) attached to an Olympus BX51WI compound microscope.

Sequential images of movements were captured at a rate of 1000 s<sup>-1</sup> and an exposure time of 0.1 or 0.2 ms with a single Photron SA3 camera [Photron (Europe), High Wycombe, Buckinghamshire, UK]. The images were fed directly to a computer for storage and later analysis. Larvae and pupae were able to move freely in a chamber containing 80 ml of pond water and made of optical quality glass [width 55 mm, vertical height 60 mm, depth (front to back) 25 mm]. Movements occurred spontaneously or were elicited by a drop of water pipetted onto the surface of the water. The camera, fitted with a 100-mm micro Tokina lens, pointed at the middle of this chamber. The images were therefore of side views of an animal performing movements in the vertical plane and parallel to the image plane of the camera. Selected image files were analysed with Motionscope camera software (Redlake Imaging, Tucson, AZ, USA) or with Canvas versions 12 and 14 (ACD Systems International, Seattle, WA, USA). Peak velocity was



calculated as the distance moved in a rolling three-point average of measurements taken from successive images. A total of 188 swimming movements by 21 larvae (a minimum of five movements by each), and 85 by eight pupae (a minimum of 10 by each) were recorded and analysed. Measurements of the orientation of the longitudinal body axis of resting larvae were made from a series of still photographs of five groups of 10 larvae in the chamber at one time. They were allowed to equilibrate in the chamber for 30 min after removal from holding chambers containing the same depth of water maintained at 4°C. Temperatures in the chamber ranged from 22 to 25°C. Data are given as means  $\pm$  s.d.

To analyse the role of the tail fan in swimming movements, larvae were first restrained in a cavity slide with the tail protruding from underneath a coverslip. The fan was then cut with a sliver of razor blade. A larva was then placed in the chamber, and free swimming movements were recorded after a 30 min recovery period. A minimum of five swimming movements by each of the seven larvae that had been operated on were then compared with the same number of movements by seven intact larvae. Photographs of a fan taken before and after the operation allowed the reduction in its area to be measured.

To search for the possible presence of the rubber-like protein resilin, live and intact larvae and pupae were placed in a cavity slide and restrained with a coverslip before being viewed through MPlan  $\times 10/0.25$  NA and LUCPlanFLN  $\times 20/0.45$  NA objective lenses, under UV or white epillumination on the Olympus BX51WI compound microscope. UV light from an X-cite series 120 metal halide light source was conditioned by a Semrock DAPI-5060B Brightline series UV filter set (Semrock, Rochester, NY, USA) with a sharp-edged (1% transmission limits) band from 350 to 407 nm. The resulting blue fluorescence emission was collected at wavelengths from 413 to 483 nm through a similarly sharp-edged bandpass filter and dichroic beam splitter. Brightfield and UV images of the same area were superimposed with Canvas 14.

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#### Author contributions

Both authors contributed equally toward all aspects of this paper.

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#### Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.102483/-/DC1>

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