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New insights into a life in current: do the gill lamellae of *Epeorus assimilis* and *Iron alpicola* larvae (Ephemeroptera: Heptageniidae) function as a sucker or as friction pads?

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Epeorus assimilis and *Iron alpicola* larvae inhabit swift running waters where they scrape algae from the stones. Previous authors suggested that the gill lamellae are modified to a sucker as an adaptation to withstand currents. Video observations show that the gill lamellae stay tilted in strong currents. Larvae can attach to the surface without any problem even if single gill lamellae are missing and therefore no negative pressure can be developed. Consequently, gill lamellae cannot have a sucker function. SEM analysis reveals areas with spike-shaped microtrichia on the abdominal sternites and setose pads ventrally on the gill lamellae. These setose pads look very similar to those described in some terrestrial insects. Setae of *I. alpicola* have a similar size, but a higher density than those of *E. assimilis*, what might cause a greater adhesive strength and could be an adaptation to the swifter currents in which the latter species lives.

Keywords: Ephemeroptera; underwater attachment; attachment devices; sucker; attachment pads; flow velocity

Introduction

Adaptations to currents of aquatic insects living in torrential habitats such as attachment devices have been consistently investigated since the beginning of the last century (e.g. Steinmann 1907; Dodds and Hisaw 1924; Hora 1930; Wesenberg-Lund 1943; Ambühl 1959; Ruttner 1962; Hynes 1970; Smith and Dartnall 1980; Statzner and Holm 1982). By and by, the results and ideas of the observations entered the fundamental literature and limnological textbooks (e.g. Ward 1992; Allan 1995; Merritt and Cummins 1996; Wetzel 2001). However, the function of some structures described as attachment devices has not been observed in detail.

Many larvae of Heptageniidae are typical inhabitants of swift running waters. Heptageniids with sucker-like gill pads are represented among others by *Epeorus assimilis* Eaton, 1885 and *Iron alpicola* (Eaton, 1871). The larvae of both species live in habitats with high flow velocities where they scrape algae and biofilm from surfaces of the stones. Larvae of *E. assimilis* tolerate near bottom velocities up to 0.4 m/s without any problems (Ditsche-Kuru, personal observations in laboratory

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flume). This is a velocity in the upper range of bottom velocities usually measured in mountain streams (Statzner 1988). The larvae dwell on current-exposed places and therefore need morphological adaptations to current. A very well-known adaptation is the dorsoventral flattening of the body which is described for both species (e.g. Haybach and Malzacher 2002).

The flat body was once assumed to use the boundary layer of reduced flow velocity (Ambühl 1959). However, compared with other aquatic macroinvertebrates, the larvae are relatively tall. The height of the boundary layer, on the other hand, mostly measures just fractions of a millimetre (Nachtigall 1982), and it is decreased with increasing current velocity (Vogel 1996). Statzner and Holm (1982) showed with laser Doppler anometry for larvae of the related genus *Ecdyonurus*, which have a similar body shape to *Epeorus*, that they are influenced by flow. Therefore, a strong influence by flow can be expected to affect the larvae of *E. assimilis* and *I. alpicola* who prefer habitats with even higher flow velocities than *Ecdyonurus*. In order to cope with these flow forces *E. assimilis* and *I. alpicola* need further morphological adaptations. For both species strong laterally directed legs and gill lamellae modified to a kind of sucker are described in addition to the dorsoventral flattening (Dodds and Hisaw 1924; Wesenberg-Lund 1943; Ruttner 1962; Uhlmann and Horn 2000; Bauernfeind and Humpesch 2001; Haybach and Malzacher 2002; Staniczek 2003). Ruttner (1962) describes that *I. alpicola* is able to attach to the substrate by using its gill lamellae to form a sucker apparatus. He assumes that for this purpose the gill lamellae are arranged like roofing tiles covering the entire ventral side. However, it has not yet been explained in detail how this "gill-sucker apparatus" works. In contrast, Hora described as early as in 1930 a spinous pad on the gill lamella of *Epeorus sp*. from the Himalaya. He further mentioned that the gill lamellae are arranged in a way that they form complete borders at the sides. But the water can flow in through the gap between the first pair of lamellae and can leave through the posterior gap. Hora was frequently cited in Hynes (1970) who called these mechanisms "friction pads" and "marginal contact". The description in Hynes might have inspired Merritt and Cummins (1996, p. 44) to write "Several aquatic insects have structures that simulate the action of suckers. The enlarged gills of some mayflies (e.g. *Epeorus*) function as a friction pad …". Wichard et al. (1995) show a SEM-picture with specialised attachment structures on the ventral side of the gill lamella of an *Epeorus* larva, but give no further information on their function. The inconsistencies in recent literature lead us to following questions:

(1) Do the gill lamellae of *Epeorus* and *Iron* larvae really function as a "sucker apparatus"?
(2) Are there microstructures on the gill lamellae like those already identified by Hora (1930)?
(3) Are there further attachment structures on the ventral side of the larvae?

Materials and methods

Material

Larvae of *E. assimilis* were collected in the low mountain river Wied near Koblenz (Germany) and transported alive to the laboratory flume in a box cooled by freeze packs. Maximum transport time was about 3 hours. For SEM, specimens were fixed in 70% ethanol according to Wetzel et al. (2005). The total body lengths as well as width and length of all femora of 10 specimens of *E. assimilis* were measured. Larvae of *I. alpicola* fixed in 70% ethanol were kindly provided by the Senckenberg Research Institute and the Natural History Museum, Gelhausen, Germany. For taxonomy of *I. alpicola* we follow here Braasch (2006).

Laboratory flume

E. assimilis larvae were observed in an artificial stream flume made of Plexiglas. A paddle wheel driven by an electric motor forced the water into a circular flow (batch-system). Using a thermostat (5000 W, Phoenix 2, Thermo Haake), a heat exchanger, and a thermal sensor, the experimental water temperature was constantly kept at $10 \pm 0.1^{\circ}$C. The system was fed with a mixture of stream and tap water (1:3) to a water level of 15 cm in height. The larvae were investigated at different bottom velocities up to 0.4 m/s. To reduce differences in flow velocity, a special array of bent plates was installed in the flume to divert water around the corners. By varying the distances between the single plates, in- and outflows were regulated in a way that flow velocity was almost the same over the whole width of the flume. The bottom of the flume was filled with cobbles (slate and red sandstone) of different sizes (range of diameters 10–20 cm). Some stones were covered with periphyton in order to feed the larvae.

Videoscopy

The movement and locomotion of *E. assimilis* larvae were recorded in the flume using a videoscope (Iplex II, Olympus, Hamburg, Germany). Its small diameter of 6 mm allowed moving the videoscope into the gaps between cobbles and even to the lower side of the stones. Selected video sequences taped at 25 fps were evaluated and dismantled in single pictures using SIS picture analysing software EIS (Olympus, Münster, Germany). The magnification of the object depends on its distance to the video camera. Because the distance between object and video camera varies during recording, the magnification could not be directly determined. Therefore, the average length of body or femur of larvae of the same species and larval stage was used for calibration when a scale was needed for reference (Frutiger 1998). Based on this calibration, the near bottom flow velocity was calculated from the length of lines of single particles and bubbles on the pictures.

Scanning electron microscopy (SEM)

For preparation, selected specimens of last instar larvae were dehydrated in an increasing series of ethanol and subsequently placed in a mixture (50:50) of HMDS (1,1,1,3,3,3-hexamethyldisilasan) and isopropanol followed by immersion in pure HMDS, for a time of 10 min each. Due to its vapour pressure, HMDS evaporates so slowly that no surface tension develops to impair the cells. Changes in size and shape lie in a similar range as in the commonly used critical point drying (Jacob 2004). After drying, specimens were attached to a needle using a two-component glue and sputter-coated with gold (Blazer Union SCD 034; Blazer Wiesbaden, Germany). The needle with the larva was clamped in a special sample holder made of aluminium. The latter was built according to Wichard et al. (1995) but in reduced height, with expanded diameter and a laterally positioned screw in order to allow improved flexibility in our SEM. Samples were examined with a LEO 1450 (Leica-Zeiss, Oberkochen, Germany) scanning electron microscope at 15 kV.

Results

Body posture

As observed for several Heptageniidae (e.g. Gonser 1990), the larvae of *E. assimilis* usually arrange their bodies in a specific position to the flow with the head against the current (Figure 1A). The anterior part of the head, the thorax, and the abdomen are in close contact with the substrate (Figure 1B). In this typical resting position, the larvae were observed to remain for longer periods on the same place in the midst of the current. In this resting position, the gill lamellae are tilted. The anterior part of the gill lamellae is in direct contact with the substrate while the lamella itself forms an angle of about 45° to the substrate (Figure 1C). The video recordings show that the gill lamellae remain tilted against the flow even at higher flow velocities (bottom velocities up to 0.4 m/s, $N = 9$). Gill lamellae overlap each other in such a way that

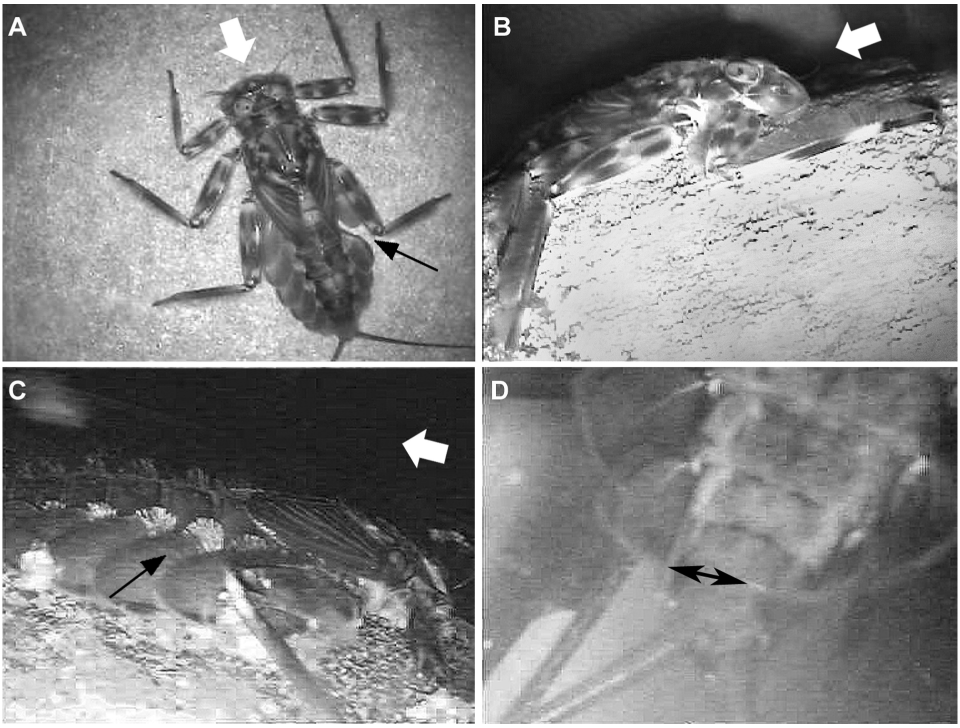

Figure 1. *E. assimilis* larva resting on the surface of a stone in swift currents (A–C) or on Plexiglas (D). White arrows indicate the direction of water flow. (A) Dorsal view, the black arrow shows the gap of a missing gill lamella; (B) lateral view; (C) gill lamellae of *E. assimilis* larvae remain tilted in swift currents. The filamentous part of each gill inserts ventrally of the lamellae and reaches dorsally beyond the lamallae (black arrow); (D) ventral view on a larva recorded through a smooth Plexiglas plane. There is a gap between the last pair of gill lamellae (black arrow).

the front of each gill lamella is covered by the next one (Figure 1A, C). The first gill lamellae are located below the hind femora. Nevertheless, in most cases little gaps can be seen in the video pictures in between the gill lamellae. The observation of gaps had been tested against the theoretical assumption of no gaps and showed a significant difference (Chi-square test: Chi-Sq $= 14.0$, d.f. $= 2$, $P = 0.001$). Anyway, the filamentous part of the gill inserts ventrally of the lamellae and reaches dorsally over the lamellae (Figure 1C) in any observed case ($N = 9$) and therefore avoids a totally tight connection of the gill lamellae to its environment. In a few cases it succeded to videotape the larvae from the ventral side through a normal Plexiglas plane. Two videos show that there is a gap between the last gill lamellae (Figure 1D). Further on, it was accidentally observed in four different cases that larvae with missing single gill lamellae had no problems in staying attached to the substrate during high flow velocities.

In resting position, the legs are directed laterally (Figure 1A) and all femora are tilted against the flow (Figure 1B). Femora and tibiae usually form more or less a right angle (Figure 1A). The forefemora stay anteroventrally in close contact with the substrate surface, while the middle and hind femora usually do not have direct contact with the substrate (Figure 1B). The distances of the femora to the substrate became longer with increasing posterior position of the legs.

Structures on the ventral side of the gill lamellae

SEM shows specialised structures on the ventral side of all gill lamellae of *E. assimilis* and *I. alpicola* (Figure 2A, B). They are located on the thickened rim of each gill lamella. Higher magnification reveals that these devices consist of a large number of tiny protuberances each set within a socket, thus resembling a seta (McIver 1975; Gorb 2001). The single seta is bent at its tip and is about 2 μm wide at the terminal end (Figure 3A, B). While the size of the setae is almost the same in both species, the density of setae is significantly higher in *I. alpicola* (5.2 ± 0.8 setae/100 μm^2, mean $\pm$ SD, $n = 10$) than in *E. assimilis* (1.6 ± 0.2 setae/100 μm^2, mean $\pm$ SD, $n = 10$) (t-test: $t = 14.3$, $P < 0.001$, d.f. $= 10$). Most setae of *E. assimilis* and *I. alpicola* are positioned in such a way that the setal shaft is directed anteroventrally, whereas the tip of the seta is directed more or less in a posteroventral direction. Other setae,

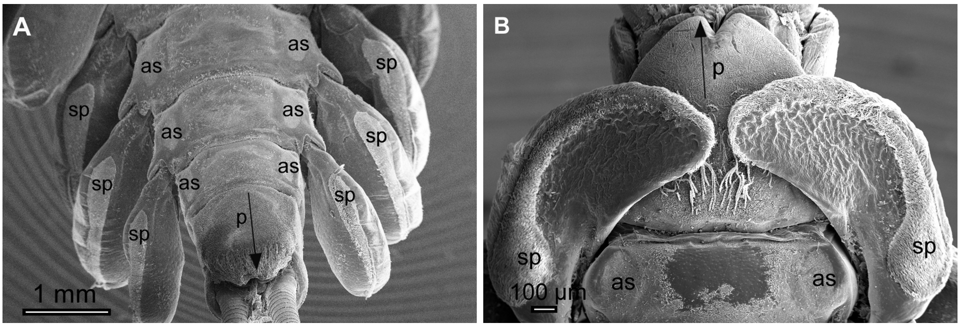

Figure 2. Ventral view on the last abdominal segments and gill lamellae of (A) *E. assimilis* and (B) *I. alpicola*. Abbreviations: sp, setose pads on ventral side of gill lamellae; as, areas with spiky microtrichia; p, posterior.

especially those in the outer parts of the pads, stand in varying directions. In both species, the first gill lamella, which differs in shape and size from the rest, has smaller setose pads.

The distal ends of all pads bear long setae (Figure 3C, D) which have the same diameter like the other setae (about 2 μm). These long setae are positioned all around the first gill lamellae and in the lateral parts of the second to seventh gill lamellae. The lateral parts are not covered by the gill lamellae in front. The number of long setae was also higher in *I. alpicola* (about 30 hairs/100 μm) than in *E. assimilis* (about 19 hairs/100 μm). While these long setae sit in one or two lines around the setae of *E. assimilis* they stand in many lines on the gill lamellae of *I. alpicola*.

Further attachment devices on the abdominal sterna

SEM revealed that larvae of both species possess further specialised structures on the ventral side of the abdomen. These areas are located laterally on the sternites (Figure 2) and consist of microtrichia that are more or less directed in posterodistal direction to the substrate (Figure 4A, B). The tip of each microtrichia is very sharp and measures less than 200 nm in width in both species. The basis (about 2 μm in width) and the length of these spikes (4–5 μm) are also in the same range in both

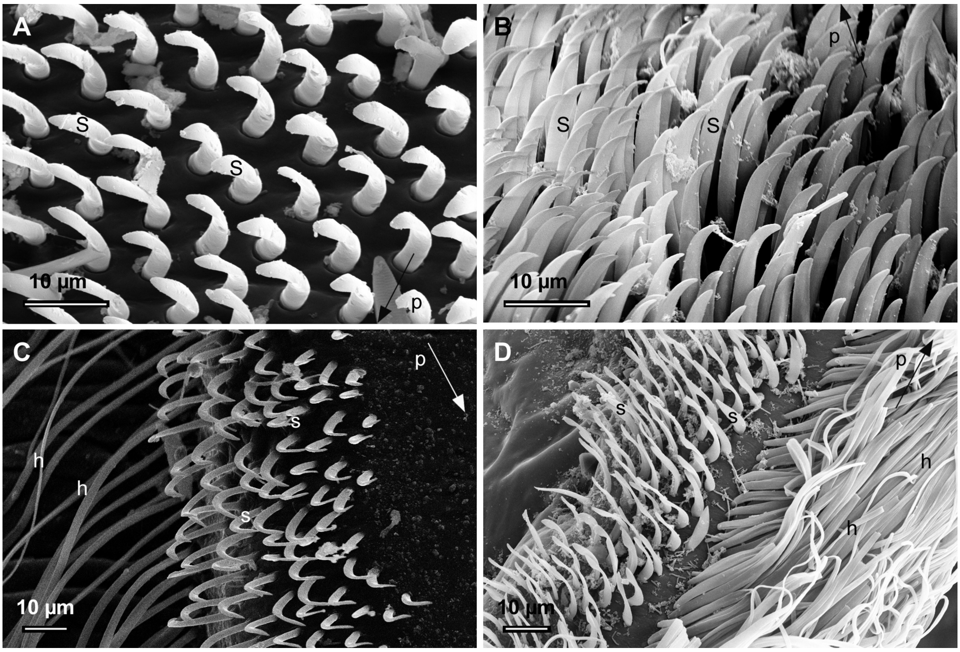

Figure 3. Gill lamellae with ventral setose pads. The density of setae on the pads of (A) *E. assimilis* is lower than on the setose pads of (B) *I. alpicola*. Setae on the lateral part of gill lamellae are of different shape and bordered by a fringe of long setae. (C) In *E. assimilis* this fringe consists of only one or two rows of long setae, whereas (D) in *I. alpicola* it is formed by many rows. Abbreviations: s, setae; ls, long setae; p, posterior.

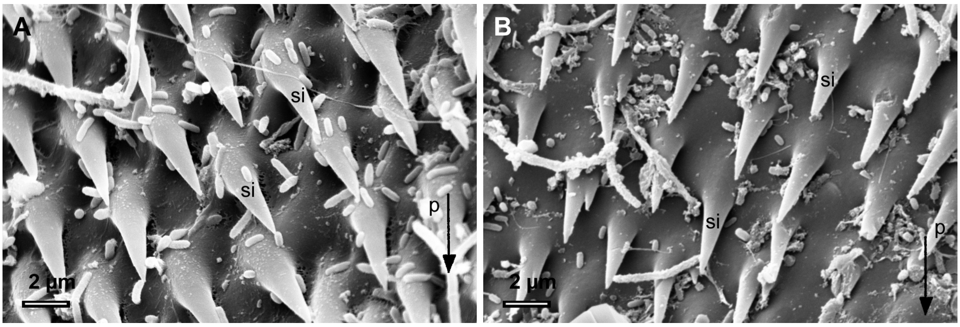

Figure 4. Microtrichia on lateral areas on the abdominal sternites of larval (A) *E. assimilis* and (B) *I. alpicola*. Abbreviations: si, spiky microtrichia; p, posterior.

species. However, the basis of the microtrichia of *E. assimilis* protrudes more than in *I. alpicola*.

The density of microtrichia in both species was similar: 6.8 ± 0.8 spikes/100 μm^2 (mean ± SD, $n = 10$) for *E. assimilis* and 7.1 ± 1.0 spikes/100 μm^2 (mean ± SD, $n = 10$) for *I. alpicola* (*t*-test: $t = 0.67$, $P = 0.514$, d.f. = 18). The spiked areas on the sternites of *E. assimilis* are about 200 μm wide and 500 μm long. The spiked areas of *I. alpicola* are somewhat smaller with 170 μm in width and 350 μm in length.

Discussion

Previous explanations regarding the function of gill lamellae in *Epeorus* and *Iron* contradict each other (sucker, friction pads and marginal contact). Our results derived from SEM and video observation confirm some assumptions while others have been disproved. Nevertheless, they also show that the adaptations to high currents of *Epeorus* sp. and *Iron* sp. are obviously much more complex than assumed before.

Do the gill lamellae have a sucker function?

The assumption that the gill lamellae work as a kind of sucker cannot be upheld. A sucker attaches to the substrate by developing negative pressure under the sucker cup. For the development of negative pressure a tight contact at the borders of the sucker is necessary, otherwise no negative pressure can be built up. Video recordings of *E. assimilis* larvae show that only the anterior part of the gill lamellae is in close contact with the substrate. In addition, little gaps can be seen in between most gill lamellae and the last gill pair show a larger medial gap. Moreover, larvae stay attached on the substrate easily even if single gill lamellae are missing. Subsequently, neither single gill lamellae nor the whole gill apparatus is able to develop a tight contact with the substrate, so the gill lamellae cannot have a sucker function as assumed by previous authors (e.g. Wesenberg-Lund 1943; Ruttner 1962; Bauernfeind and Humpesch 2001; Haybach and Malzacher 2002; Staniczek 2003).

Hora (1930, p. 187–188) noticed that "the thickened portion of the gill lamellae of *Epeorus* larvae is closely applied to the substrate, while the upper free portion is kept in a rapid to-and-fro motion". He hypothesised that the movements of the upper portions of the gill lamellae have the purpose of expelling leakage water. Our results agree with Hora (1930) so far that just the anterior part of the gill lamellae stays in close contact with the substrate while the posterior part does not. However, our video observations did not show an active rapid movement of the upper part of the gill lamellae. A rapid active movement of the gill lamellae further stays in contrast to the described immovability of the gill lamellae of *Epeorus* sp. (e.g. Ambühl 1959; Bäumer et al. 2000). The filamentous part of the gills reaches over the lamellae and Hora might have seen their movements due to the waterflow.

No video observations were possible for *I. alpicola* but its gill lamellae are arranged in a similar way as in *E. assimilis* and might therefore have a similar function. The gill lamellae overlap each other like roof tiles, even if they are somewhat larger than those of *E. assimilis*. The front gill lamella of *I. alpicola* is much larger than that of *E. assimilis* and may contact ventromedially. Morisi et al. (2003) interpreted this as an adaptation of *I. alpicola* to faster currents compared with *E. assimilis*. Referring to Hynes (1970), the authors assume an increased area of marginal contact of the animal with the substrate and thus a reduced possibility of current underneath the larval body. The marginal contact is not complete on the lateral sides due to the observed gaps between the gill lamellae in the case of *E. assimilis*. Nevertheless, the marginal contact, especially in anterior direction, might be an important factor, although we assume that another mechanism is also important. Tilted body parts are pressed to the substrate by the water current. This can be tested in a simple experiment with a tilted panel which stays in its position even at high currents in a flume. A similar effect was already discussed by Steinmann (1907) in connection with dorsoventral flattening of the body. With the exception of the front gills, which are usually covered by the hind legs, all gill lamellae are tilted just like the femora. It was already assumed by Dodds and Hisaw (1924) and Gonser (1990) that in the larvae of Heptageniidae the positioning of the femora plays an important role in stabilising their spatial position.

Setae on the ventral side of the gill lamellae

In both species setose pads have been found on the ventral edge of the gill lamellae. This is the part of the gill lamellae which stays in close contact with the substrate. The density of setae is much higher in the pads of *I. alpicola* than in that of *E. assimilis*.

There are other hairy structures in aquatic insects (e.g. *Dicercomyzon* sp., *Drunella doddsi*) which are assumed to play a role in attachment (Hynes 1970). However, these hairy structures seem to be of a different shape. For example, the hairs of *Drunella doddsi* are very soft and branched (Ditsche-Kuru, personal observation by means of SEM analyses). In contrast to that, the setose pads of *Epeorus* and *Iron* seem to have a high elasticity and look very similar to those known from terrestrial insects. Such setose or ‘‘hairy’’ pads which play an important role in attachment are known from several terrestrial insects as well as from lizards and spiders (Gorb 2001; Arzt et al. 2003; Kesel et al. 2004; Autumn 2006). Terrestrial setose attachment pads are well known for their extraordinary abilities like high attachment force, fast detachment, and directionality. The hairy surfaces guarantee a maximum contact area with diverse substrates regardless of their micro sculpture (Gorb and Beutel 2001), permitting for example attachment to totally smooth surfaces. The mechanism of (terrestrial) attachment is described as a combination of molecular interactions and capillary attractive forces mediated by secretion or purely van der Waals interaction (Autumn et al. 2000; Arzt et al. 2003 according to Stork 1980). The setae of *E. assimilis* show high similarity in shape and size to the tarsal setae of *Forficula auricularia* (Dermaptera) shown in Beutel and Gorb (2001). Nevertheless, there is an important difference regarding the orientation of the setae. In the setose pads of *E. assimilis* and *I. alpicola* most tips are bent in posteroventral direction. This is almost the opposite direction as in terrestrial setose attachment pads in relation to the acting force. Thus the orientation of the setae supports the interpretation that setose gill areas are just friction pads due to interlocking effects as described in Hora (1930). Our investigations of the ventral side of the gill lamellae by means of SEM confirm Hora's observation that *E. assimilis* and *I. alpicola* have pads with tiny protuberances on the ventral side of their gill lamellae. However, not all of these protuberances have a sharp hook-like curved apical portion as described by Hora (1930). Many of them have a blunt tip and are just slightly bent (Figure 3). Furthermore, these protuberances are not spines, but setae. Spines are multicellular processes without differentiation of cells while setae are multicellular processes originating from special differentiated cells (trichogen, tormogen and often also sensory cells) (Gorb 2001 according to different authors). Moreover, the blunt shape of the majority of the seta tips let us doubt that they just function by hooking to the surface irregularities of the substrate, and the investigation of relevant attachment mechanisms of the setae of *E. assimilis* and *I. alpicola* is a matter of further research.

In terrestrial insects setose attachment pads with a higher density of setae as well as with smaller setae tips result in a higher adhesive strength (Arzt et al. 2003). In aquatic environments an animal exposed to higher flow velocities needs to develop stronger attachment forces because higher flow velocities cause higher flow forces. Consequently, we hypothesise that the significantly higher density of the setae of *I. alpicola* is an adaptation to the higher flow velocities of the preferred habitat of *I. alpicola*.

In contrast to the terrestrial setose attachment pads, *E. assimilis* and *I. alpicola* have a fringe of long setae on the distal side of their setose pads. It is known that long hairs (setae) can protect the part lying behind from flow forces, as they do in front of the head plate or mouth parts where they prevent that food is swept away (Gonser 1990). Thus we assume that the long setae around the short setae are important to protect them from being rinsed by the flowing water, which otherwise could be able to interrupt the contact between setae and substrate.

Sternal microtrichia

SEM revealed further specialised devices on the abdominal sterna of the larvae of *E. assimilis* and *I. alpicola*. We assume that these areas are able to increase friction with the substrate. Thus, if the surface of the substrate shows a micro roughness in a corresponding range, the pressure of the animal in caudoventral direction due to tilted body parts will cause an anchoring of the spiky microtrichia to the substrate. Consequently, the areas with microtrichia might bring an advantage for the larvae to attach, especially on substrates with micro roughness.

Cooperation of the different attachment devices

The different attachment devices may offer advantages on substrates with different surface properties.

Besides the described setose pads on the gill lamellae and the areas with spiky microtrichia on the sternites, the larvae have strong claws on their laterally directed legs like all clingers. So far, not much has been said about the question why these mayfly larvae need several attachment devices. There are two possibilities which do not completely exclude each other: (1) the overall attachment force of the animal is increased due to the cumulated resistance of all attachment structures; (2) the different attachment structures just work on corresponding substrate properties. Thus one attachment structure for instance might increase the attachment force on a certain substrate where another attachment structure does not work or has only little effect. One example for the latter can be found in some adult Ephemeroptera, which have one claw and one claw pad (Beutel and Gorb 2001). While claws need a certain surface roughness for attachment, claw pads have the ability to adapt to the profile of a surface and therefore can attach to smooth substrates.

A comparison of the sizes of the different attachment devices of *E. assimilis* larvae indicates that they work best on different surface roughness. The diameter of the claw tip is about 6 μm (Ditsche-Kuru, personal observations). Therefore, in order to hook to the substrate, a corresponding roughness with hollows of clearly more than 6 μm in width is required. In contrast, the tips of the setae measure just 2 μm in width and might work well on smoother substrates with finer roughness. The spikes on the lateral part of the sternites have the finest tips (0.2 μm in width) and therefore might attach best on another micro roughness. In addition to the original surface structure of the hard substrates, the periphyton covers the surface structure. Moreover, other properties of algae and biofilm (e.g. chemical components, elasticity) may have an effect on the functioning of several attachment devices.

We hypothesise that the attachment structures of *E. assimilis* and *I. alpicola* attach on different surface properties (e.g. roughness and structure, elasticity). Nevertheless, there might be overlapping ranges of surface properties for the different attachment devices which offer an additional advantage over special substrates.

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