

RESEARCH ARTICLE

Eco-mechanics of lamellar autotomy in larval damselflies

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ABSTRACT

In larval damselflies, the self-amputation (autotomy) of the caudal lamellae permits escape from predatory larval dragonflies. Lamellar joint size declines among populations with increasing risk of dragonfly predation, but the breaking force required for autotomy and the biomechanical factors that influence breaking force are unknown. If autotomy enhances survival in larval damselflies, then predation by larval dragonflies should select for joints that require less force to break. We test this adaptive hypothesis by evaluating whether breaking force is negatively related to local predation risk from larval dragonflies. We also test a cuticle structure hypothesis, which predicts that breaking force is positively related to joint size and to joint cuticle thickness because of a structural support relationship between joint and lamella. The peak force necessary for lamellar autotomy was assessed on individual larval *Enallagma* damselflies collected from populations that varied in risk of predation. Easier lamellar autotomy occurred in larvae from sites with higher predation risk because damselflies from fishless ponds (where predatory larval dragonflies are likely more abundant) had lower breaking forces than those from ponds with fish (where larval dragonfly predation is likely reduced). Furthermore, breaking force was a positive function of joint size and also of total cuticle cross-sectional area after controlling for joint size. This suggests that autotomy may evolve in larval damselflies under selection from small grasping predators such as larval dragonflies by favouring smaller joint size or reduced cuticle area of lamellar joints.

KEY WORDS: Self-amputation, *Enallagma*, Lamellae, Functional morphology, Adaptive diversity, Predation risk

INTRODUCTION

The repeated evolution of autotomy, or self-amputation, across diverse vertebrate and invertebrate taxa suggests that predation regularly favours the evolution of appendage detachment in prey (Fleming et al., 2007). Autotomy may be advantageous if it provides a last-ditch escape strategy by a prey organism. Appendage loss must also be costly (Robinson et al., 1991a; Gyssels and Stoks, 2005), but the immediate survival benefits of predator escape must sometimes outweigh such disadvantages for autotomy to evolve (Robinson et al., 1991b; Stoks, 1998; Fleming et al., 2007). Appendages that are not essential for survival, but that are frequently a target of attack, may reduce the costs of autotomy (Fleming et al., 2007). Adaptive autotomy is expected to be a consequence of underlying functional morphological traits that permit self-amputation at a fracture plane, such as a joint, when an external force is applied (Wilkie, 2001; Fleming et al., 2007). However, the relationship between form and function is generally incompletely

understood for many morphological traits (Arnold, 1983; Wainwright, 1994; Ferry-Graham et al., 2002), not the least of which are those that mediate autotomy.

Damselfly larvae (Order: Odonata, Family: Zygoptera) possess three caudal lamellae that are thin leaf-like structures attached to the posterior end of the abdomen by lamellar joints. Lamellae serve multiple functions, such as generating thrust for swimming as the larva undulates its abdomen (McPeck et al., 1996; McPeck, 1997), contributing to gas exchange (Eriksen, 1986; Burnside and Robinson, 1995) and perhaps in agonistic signalling between conspecifics (Johnson, 1991). Larval damselfly face two classes of predators that can generate diversifying selection on lamellae and other traits: large predatory fish, and large predatory invertebrates such as larval dragonflies that predominate in the absence of fish (McPeck, 1990a; McPeck, 1990b; McPeck et al., 1996; Strobbe et al., 2011). Autotomy of caudal lamellae enhances escape and survival against grasping predators such as larval dragonflies (Baker and Dixon, 1986; Stoks, 1998; McPeck, 1990b), but is ineffective against larger fish predators that swallow larvae whole (Baker and Dixon, 1986; McPeck, 1990b). However, evasive swimming is also more effective against attacking dragonfly larvae than against fish predators (Gyssels and Stoks, 2005), and so larger lamellae that enhance escape swimming performance can be favoured in ponds with high dragonfly predation risk (McPeck, 1997). Thus, the lamellar joint may mediate a trade-off under dragonfly predation between structural support for larger lamellae that enhances evasive swimming and structural support for smaller lamellae that enhances lamellar autotomy (Bose and Robinson, 2013). Losing lamellae can have additional costs related to survival, such as decreased swimming performance (Robinson et al., 1991a; Burnside and Robinson, 1995; McPeck et al., 1996; Stoks, 1999b; Gyssels and Stoks, 2005), which can impair future escape attempts (Gyssels and Stoks, 2005); reduced attempts at escape under predation threat (Robinson et al., 1991a; Gyssels and Stoks, 2005); greater risk of being cannibalised by conspecifics (Stoks, 1998); and reduced oxygen consumption (Eriksen, 1986; Robinson et al., 1991b), foraging (Stoks, 1999a) and growth (Stoks, 2001). These costs may be lessened by regenerating missing lamellae in as little as two moults (Robinson et al., 1991b), but they also strongly suggest substantial survival benefits of lamellar autotomy.

In larval damselflies, autotomy occurs at a joint that connects each lamella to the abdomen, but relatively little is known about how joint features influence autotomy performance, which we define here as the ease with which lamellae can be amputated. Burnside and Robinson (Burnside and Robinson, 1995) proposed a biomechanical structural hypothesis that the amount of cuticle attaching the lamellae to the abdomen determines the amount of force required to remove lamellae during autotomy, and so predicted a positive relationship between joint diameter and breaking force that has not been tested. An extension of this hypothesis also predicts that breaking force should increase in similar sized joints with proportionally thicker cuticles. Lamellar joint size varies positively with body size and also among damselfly taxa and populations (Burnside and Robinson, 1995; Bose

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and Robinson, 2013). In larvae from the genus *Lestes*, the rate of growth of lamellar joint size with lamellar size declines with increasing predation risk from dragonflies, suggesting that smaller joints that facilitate autotomy are favoured in high-predation environments (Bose and Robinson, 2013). Joint size and predation risk were not so related in *Enallagma* damselflies, although their smaller joints and larger lamellae compared with *Lestes* may limit reductions in joint size (Bose and Robinson, 2013).

Here we test the relationships between breaking force and lamellar joint size, breaking force and cuticle thickness, and breaking force and predation risk in order to better understand the biomechanics of lamellar autotomy, and how joint morphology may respond to selection from larval dragonfly predators that favour autotomy. We predict that larger joints and thicker cuticles require greater breaking forces and also that lamellar breaking forces will decline in larvae from populations under increased risk of larval dragonfly predation.

RESULTS

Effect of joint size on breaking force

There was a significant positive relationship between breaking force and lamellar joint diameter (slope=0.632, s.e.m.=0.075, $t=8.47$, $P<0.0001$; Fig. 1), although an additional 65% of variation in breaking force was not accounted for by joint diameter, particularly with larger joints. The relationship between breaking force and joint diameter was positive although not always significant for each population. Significant evidence of positive slopes were present in three populations (Pond 5, slope=0.684, s.e.m.=0.190, $P=0.001$; Pond 9, slope=0.671, s.e.m.=0.124, $P<0.0001$; and Pond 45, slope=0.69, s.e.m.=0.234, $P=0.008$), and weak or absent for two populations (Pond 44, slope=0.814, s.e.m.=0.407, $P=0.068$; and Pond 1, slope=0.402, s.e.m.=0.299, $P=0.19$). Larvae were rare in Ponds 44 and 45 (Table 1), and the variation in joint diameter among larvae sampled from Pond 1 (s.d.=0.039) was less than half that of the most variable pond, Pond 9 (s.d.=0.082). We next explore which factors may contribute to explaining the residual variation in breaking force that is unrelated to joint diameter.

Effects of other factors on breaking force

Source pond had a significant effect on mean breaking force of lamellar joints (ANCOVA, pond, $F_{4,129}=4.85$, $P=0.001$) after accounting for the effect of joint size (ANCOVA, joint diameter,

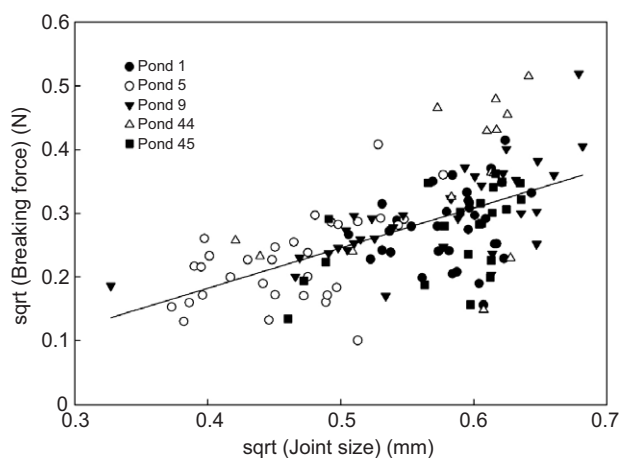


Fig. 1. Positive relationship between breaking force and lamellar joint size among *Enallagma* larvae sampled from five pond populations. Results from linear regression: $R^2=0.35$, $N=135$, slope=0.632, s.e.m.=0.075, $t=8.47$, $P<0.0001$.

Table 1. Proportions of re-grown lamellae for each of the five populations estimated in the present study (2011) and in 2010 by Bose and Robinson (Bose and Robinson, 2013)

Pond	Fish status	2011 estimate	N	2010 estimate	N
44	Fish	0.38±0.10	24	0.204±0.039	108
5	Fish	0.20±0.067	55	0.217±0.086	23
1	No fish	0.29±0.057	78	0.340±0.049	94
9	No fish	0.17±0.062	65	0.348±0.059	66
45	No fish	0.090±0.11	21	0.403±0.056	77

Data are means ± s.e.m.

$F_{1,129}=52.2$, $P<0.0001$). However, there was no evidence that the relationship between joint diameter and breaking force varied among the five pond populations (ANCOVA, pond × joint diameter interaction, $F_{4,125}=0.26$, $P=0.90$). Including a main effect of pond in the model explained 43% of the variation in breaking force. Multiple comparisons revealed that the adjusted mean breaking force of lamellar joints of larvae from Pond 44, which contained fish predators (0.111 N), was significantly larger than the mean breaking force for larvae from the fishless Ponds 1 and 45 (respective means=0.066 and 0.063 N).

Variation in mean lamellar joint breaking force among ponds was related to the status of fish in the local pond. We combined pond data into two groups, one composed of the two ponds containing fish and the other composed of the three fishless ponds, because breaking force did not significantly differ among ponds in either grouping (ANCOVA, fish Ponds 5 and 44, pond, $F_{1,43}=1.75$, $P=0.19$; no-fish Ponds 1, 9, 45, pond, $F_{2,86}=2.86$, $P=0.06$). Fish status had a significant effect on breaking force when comparing larvae from fish versus non-fish sites (ANCOVA, fish status, $F_{1,132}=8.87$, $P=0.003$; Fig. 2) after accounting for joint size (ANCOVA, joint diameter, $F_{2,132}=81.64$, $P<0.0001$). At a mean joint diameter of 0.309 mm, the mean breaking force of larval damselflies from fishless ponds (0.069 N) was less than that of larvae from ponds containing fish (0.092 N). There was little evidence that fish status influenced the relationship between breaking force and joint size (ANCOVA, fish status × joint diameter interaction, $F_{1,131}=3.11$, $P=0.08$). The effect of including fish status after accounting for joint size explained 39% of variation in lamellar joint breaking force.

The two species groups of *Enallagma* evaluated here (*E. hageni*/*E. ebrium* and *E. boreale*/*E. cyathigerum*) did not differ in the breaking force required for lamellar autotomy. Neither the relationship between joint size and breaking force (ANCOVA, species × joint diameter interaction, $F_{1,131}=1.65$, $P=0.20$) nor the species main effect accounted for significant variation in breaking force (ANCOVA, species, $F_{1,132}=0.04$, $P=0.85$) after accounting for the effects of joint size (ANCOVA, joint diameter, $F_{2,132}=27.52$, $P<0.0001$).

Breaking force increased with total cuticle area at the joint fracture plane measured from scanning electron microscopy (SEM) images (Fig. 3). The greatest variation in breaking force occurred in a subsample of joints between 0.355 and 0.403 mm in diameter (0.60–0.64 units; Fig. 1, horizontal axis). After statistically removing the effects of joint size on breaking force, residual breaking force was strongly positively related to total cuticle area ($N=30$, slope= $4.41E^{-5}$, s.e.m.= $8.0E^{-6}$, $t=5.31$, $P<0.0001$; Fig. 4). Cuticle area explained 50% of the variation in breaking force in this sample.

Lamellar re-growth frequency and breaking force

The per capita frequency of independently re-grown lamellae varied among the five ponds in 2011 from a low of 9% of lamellae in larvae from Pond 45 to a high of 38% in Pond 44 (Table 1), but

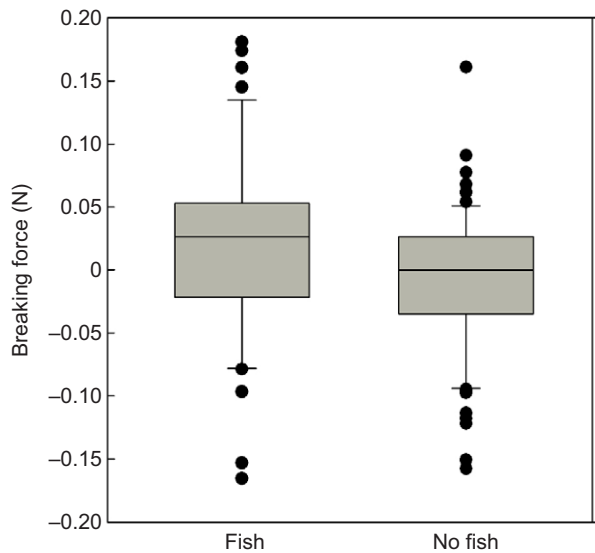


Fig. 2. Boxplots expressing the residual variation in breaking force of lamellar joints of larvae from ponds with fish ($N=46$) and without fish ($N=89$) after accounting for joint size using linear regression (see Fig. 2). Mean breaking forces adjusted to a joint size of 0.309 mm are 0.092 N with fish present and 0.069 N with fish absent. The horizontal line within a box identifies the median breaking force value, boxes represent middle quartiles and whiskers represent outer quartile ranges with likely outliers indicated as single points.

these did not correspond to estimates from 2010 (Bose and Robinson, 2013). Breaking force was unrelated to variation in the proportions of regenerating lamella among populations in 2011 (one-sided $P=0.44$); however, breaking force was negatively related to the 2010 estimates of lamellar re-growth frequencies (slope = -0.298 , s.e.m. = 0.11 , $t = -2.66$, $P = 0.038$).

DISCUSSION

Our results provide insights into how lamellar autotomy is achieved in larval damselflies of the genus *Enallagma* by testing a biomechanical explanation for variation in lamellar autotomy among populations faced with different risks of larval dragonfly predation. Burnside and Robinson (Burnside and Robinson, 1995) proposed that the cuticle was the main load-bearing structure of the joint and that the force required for lamellae to autotomize would be a function of the size of the lamellar joint because joint diameter is positively related to the amount of cuticle. Breaking force was positively related to lamellar joint diameter as predicted, but we also discovered that 65% of the variation in breaking force remained unexplained by joint size. Cross-sectional area of the joint cuticle at the fracture plane explained 50% of this residual variability in breaking force independent of joint size. Thus, increased ease of autotomy may be achieved either by reducing joint size or by reducing cuticle area at the joint. We also found that lamellar breaking strength was reduced in larvae from ponds without fish, where larval dragonfly predation is likely high, compared with ponds with fish, where dragonfly predation is reduced, consistent with adaptive variation in autotomy in response to variation in predation risk. We evaluate what factors may influence breaking force before discussing the evolution of lamellar autotomy.

Taxonomic influences

Taxonomic differences could contribute to variation in breaking force should taxa differ with respect to traits other than cuticle

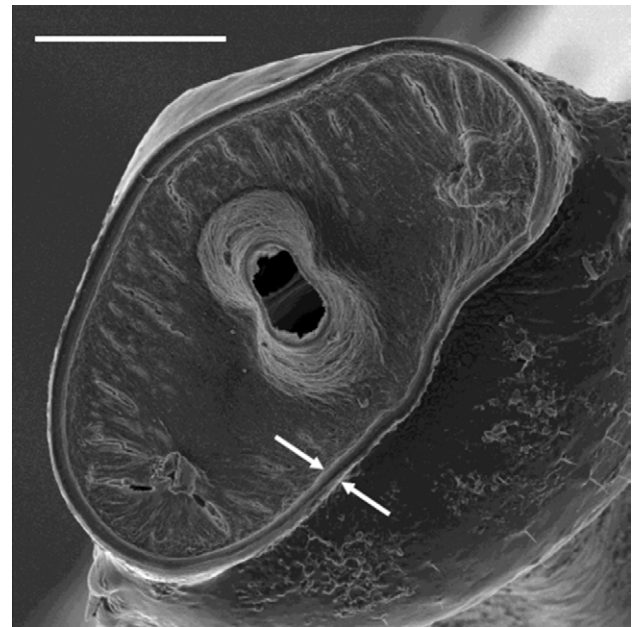


Fig. 3. Scanning electron microscopy image of the fracture face of a detached lateral lamella from a larval *Enallagma* damselfly. The joint cuticle lies between the arrowheads and surrounds the entire joint. The opening at the joint centre shows the trachea inside the lamella. Scale bar, 120 μm .

thickness that affect lamellar joint structure and function. Joint shape may directly influence breaking force depending on how joints break (e.g. tearing versus popping), or indirectly depending on how joint shape influences the amount of cuticle present. We found no evidence that variation in breaking force was related to the *E. ebrium*/*E. hageni* versus *E. boreale*/*E. cyathigerum* species-pairs here, which underscores the close taxonomic affinity of most *Enallagma* species (Turgeon and McPeck, 2002) and likely functional similarities of their lamellar joints. However, such functional similarity may not hold among other taxa because the cross-sectional shape of the lamellar joints in these *Enallagma* species is oval (Fig. 3), whereas the lamellar joints of *Lestes* are less symmetric and more angular (Bose and Robinson, 2013). Thus, the absence of species effects here cannot be generalised beyond *Enallagma* damselflies until other studies test for the effects of joint shape on the force required for autotomy.

Joint morphology

Almost two-thirds of the variation in breaking force was not explained by joint size, suggesting that other factors affect joint breaking force. SEM imaging of a subset of joints of similar size with nonetheless large variation in breaking force revealed considerable variation in the thickness of the joint cuticle. In this subset, breaking force was positively related to total cuticle cross-sectional area, confirming Burnside and Robinson's (Burnside and Robinson, 1995) functional cuticle hypothesis, even though they did not explicitly consider the effects of cuticular thickness on the amount of force required for autotomy. Thus, our results expand the functional cuticle hypothesis by demonstrating that at least two morphological properties of the lamellar joint functionally affect autotomy: joint size and joint cuticle thickness.

Selection should favour an optimal lamellar breaking force that balances the survival benefits of autotomy against the costs of

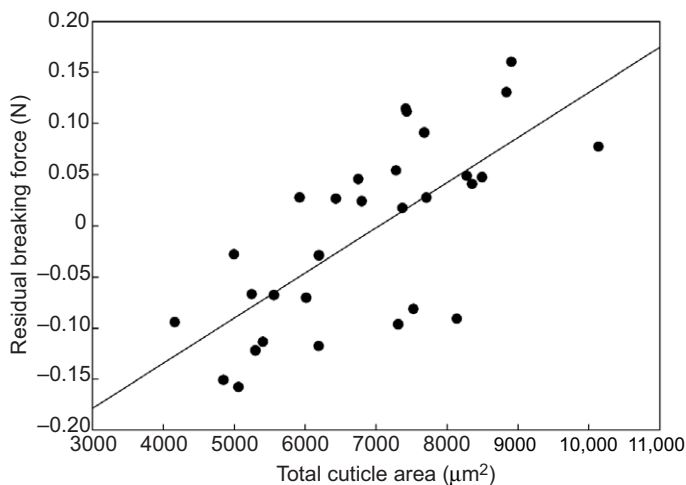


Fig. 4. Positive relationship between the residual breaking force and cuticle area of lamellar joints. Positive and negative values of residual breaking force reflect greater and lesser mean breaking force, respectively, after adjusting for joint diameter (see Fig. 2). Results from linear regression: $R^2=0.50$, $N=30$, slope= $4.41E^{-5}$, s.e.m.= $8.0E^{-6}$, $t=5.31$, $P<0.0001$.

lamellar loss and re-growth (Robinson et al., 1991b; Stoks, 1998; Fleming et al., 2007). However, there may be limits to variation in joint size (Bose and Robinson, 2013), in which case selection may favour changes in cuticle thickness. The SEM images of lamellar fracture planes (Fig. 3) suggest that additional morphological features may influence ease of lamellar autotomy, such as the area of the tracheal holes in the joint plane. Additional key biomechanical uncertainties exist about the breaking stress – the breaking force per unit of load-bearing cross-sectional area – of the cuticle. We have to assume that the material properties of the cuticle (and of other joint tissues) remained constant, even though changes in proteins may also influence how tissues respond to mechanical stress (Hopkins and Kramer, 1992) and perhaps the ease of autotomy. If breaking stress differs between damselfly larvae from ponds with and without fish, then this would suggest that the material of the cuticle itself may also vary in response to predation selection.

Another uncertainty is the strain rate that the lamellae experience during predatory strikes. While dragonfly larvae are capable of extremely fast predatory movements, it is not clear whether autotomy occurs on the same time scale as the strike itself, or whether it happens a second or two later after a lamella has been grasped, and the larva struggles to swim away. In simulated predatory strikes in the laboratory conducted with forceps, we often see the latter. This question could be answered by observing predatory strikes by dragonfly larvae on damselfly larvae using a high-speed video camera. Regardless, the strain rates we used in the laboratory were almost certainly slower than those experienced in the wild. We used a relatively slow strain rate to increase the precision and accuracy of our force measurements and to avoid inertial artefacts that occur at large cross-head accelerations. While it is possible that higher strain rates would have yielded different results, we view this as unlikely.

Our study does not address whether the variation in breaking strength and cuticle thickness found here reflects evolutionary (genetic) divergence of populations, the effects of ongoing selection via survival under predation that varies among ponds, or plastic developmental responses by individual larvae to local cues of predation risk (e.g. Arnqvist and Johansson, 1998). Rearing damselfly larvae collected from a fishless site with versus without dragonfly

predator cues could evaluate the degree to which breaking forces, cuticle width and joint size may express adaptive plastic responses to local predation risk, while longitudinal (cohort) methods in the wild could address selection (McAdam and Boutin, 2003).

Relationships between fish status, invertebrate predation risk and autotomy

We expect that autotomy at a lower breaking force is advantageous in ponds without fish because escape swimming and autotomy are not effective against fish predators (McPeck, 1990b) but are effective against odonate predators (McPeck, 1997; Gyssels and Stoks, 2005). Damselfly larvae from ponds without fish had lower mean lamellar joint breaking forces (easier autotomy) than larvae that coexisted with fish. If predation risk by larval dragonflies and other larger invertebrate grasping predators is inversely related to fish predation risk (see below), then this is consistent with the hypothesis that variation in ease of autotomy enhances survival under strong odonate predation and so may be adaptive.

Because of their size and speed advantage, large predatory fish can have strong effects on odonate community structure by regulating the distribution and abundance of their prey, such as larval dragonflies and damselflies (McPeck, 1990b). In the absence of fish, larval dragonflies emerge as a top predator of invertebrates such as larval damselflies (McPeck, 1990b). Consistent with Sih's (Sih, 1987) divergent lifestyle hypothesis, fish predation also generates selection that favours less active larval damselflies (McPeck, 1990a; McPeck, 1990b; McPeck et al., 1996; Stoks et al., 2003; Strobbe et al., 2011), while dragonfly predation selects for greater behavioural activity, and any morphological traits such as larger lamellae that functionally increase escape swimming speed or enhance autotomy (McPeck, 1995). The reduction in mean breaking force in larvae from fishless compared with fish ponds suggests that selection may act on traits that affect the ease of autotomy because this enhances survival when attacked by grasping predators. Ease of lamellar autotomy limits the time available to the predator to grasp more vital parts of the body, such as the abdomen, that would dramatically increase the risk of mortality.

However, our evidence that breaking force responds to invertebrate predation risk is less clear. Breaking strength is negatively related to the 2010 estimates of relative frequency of re-grown lamellae (Bose and Robinson, 2013) but not to our 2011 estimates. The frequency of re-grown lamellae varied among the five populations in 2011, but were inconsistent with prior estimates from 2010. The discrepancy primarily arose from Ponds 44 (fish) and 45 (fishless), where estimates of lamellar re-growth frequencies reversed between successive years. We note that in 2010, the frequency of re-grown lamellae was generally higher in fishless compared with fish ponds, as expected if fish predation negatively affected larger invertebrate predators such as larval dragonflies (Table 1), while our current (2011) estimates show no such relationship. The estimates of larval dragonfly predation based on the earlier 2010 study may be more reliable for two reasons. First, the small sample sizes and correspondingly high standard errors of estimates from Ponds 44 and 45 in 2011 compared with other ponds (Table 1) and compared with 2010 studies of the same ponds suggest that these two 2011 estimates have much lower precision. Second, Bose and Robinson (Bose and Robinson, 2013) found that the 2010 lamellar re-growth frequencies were positively correlated with the relative abundance of larval dragonflies to total odonate larvae, consistent with a direct effect of predation risk. Interestingly, this effect was stronger for *Lestes* than for *Enallagma*, suggesting that larval dragonfly predation risk may be more reliably estimated from

Lestes, while we only evaluated *Enallagma* here. Fish predation risk has not changed in Ponds 44 and 45 between 2010 and 2011, and so likely continues to exert some predatory control on the larval odonate community. Thus, we feel that the lamellar re-growth estimates from 2010 (Bose and Robinson, 2013) are more reliable than the 2011 estimates reported here. In summary, our finding that lamellar joint breaking force is lower in damselfly larvae from fishless compared with fish-containing ponds along with the negative relationship between lamellar joint breaking force and 2010 estimates of re-growth frequency (Bose and Robinson, 2013) suggest that breaking force may adaptively respond to larval dragonfly predation risk.

Autotomy mechanisms

Hypothesised morphological adaptations assume that form sets upper limits on functional performance, which in turn influences organismal fitness (Arnold, 1983; Wainwright, 1994). Our work reveals some of the biomechanical features and mechanisms that influence appendage loss in larval damselflies and so helps to establish the first link in this causal chain. However, various uncertainties about the forces required for autotomy and the biomechanics of lamellar loss remain, such as the functional significance of other features present on the fracture plane of the lamellar joint revealed by SEM images. The influence of functional morphology on performance may also change over development (Husak, 2006). If predators grasp the body in small damselfly larvae, then lamellar autotomy may be ineffective and so reduced in small versus large larvae (e.g. proportionally higher breaking force required after accounting for joint size). The opposite occurs in the sea star *Asterias rubens*, where the tensile strength of the arms increases with body size, and smaller individuals autotomize limbs more quickly than larger individuals under a constant force (Marrs et al., 2000).

The relationship between performance and fitness is also uncertain because survival and reproductive success integrate performance over multiple traits in a particular environment (Wainwright, 1994; Ghalambor et al., 2003), and behaviour may modify how selection acts on functional morphology in nature (Irschick and Garland, 2001; Swallow et al., 2009). For example, Bateman and Fleming (Bateman and Fleming, 2008) examined the relationship between body size and autotomy in grasshoppers (Orthoptera) in order to evaluate whether time to autotomy of a hind limb was proportional to body size. Autotomy was unrelated to body size, suggesting that autotomy may be partially under individual conscious control. Such effects may generate performance mismatches between laboratory and field studies, limiting inferences from laboratory studies alone (Irschick, 2003; Irschick et al., 2008; Swallow et al., 2009). Nonetheless, by testing the relationship between morphology and performance, laboratory studies can generate useful ecological hypotheses about how morphology may subsequently affect fitness in nature (e.g. Ferry-Graham et al., 2002; Ghalambor et al., 2003; Carroll et al., 2004).

Conclusions

Our work is an important first step in understanding the functional morphology and evolution of lamellar joint breakage in larval damselflies because we demonstrate that cuticle area influences breaking force (Burnside and Robinson, 1995). Variation in breaking force during lamellar autotomy is affected by both the size of the joint and features within the joint that change the relationship between joint size and breaking force – such as cuticle area – and vary independent of joint size. Thus, the force required for autotomy

potentially can be modified in multiple ways in response to selection from invertebrate predators. Constraints on the evolution of lamellar joints may be revealed should either of these features respond more to variation in predation risk. It will also be interesting to evaluate what other features (joint shape, cuticle proteins) may affect breaking force and test for plastic responses to predator cues. Lamellar autotomy may also be an adaptive response to dragonfly predation risk because the breaking force of lamellar joints is lower in damselfly larvae from fishless compared with fish-containing ponds, and is negatively related to estimates of regrown lamellae from 2010 (Bose and Robinson, 2013). Thus, approaching autotomy from morphological, developmental and ecological perspectives can provide insights into how morphology affects performance and relative fitness as we attempt to understand the evolution of organismal morphology.

MATERIALS AND METHODS

Specimen collection

Larvae of *Enallagma* damselflies were sampled near Guelph, ON, from five ponds known to vary in odonate predation risk (Bose and Robinson, 2013) (Table 1). Sampling occurred between September and December 2011 in order to collect a wide size range. Larvae were collected by sweeping a D-net through submersed vegetation and along the benthic surface, and were returned live to the laboratory, where they were kept individually in plastic cups filled with well water in order to control for possible effects of water condition on lamellar properties. Fish status was determined for each pond by standardized trapping effort with at least four minnow traps. Ponds were classified as fishless in the absence of captured fish after 100 trap hours.

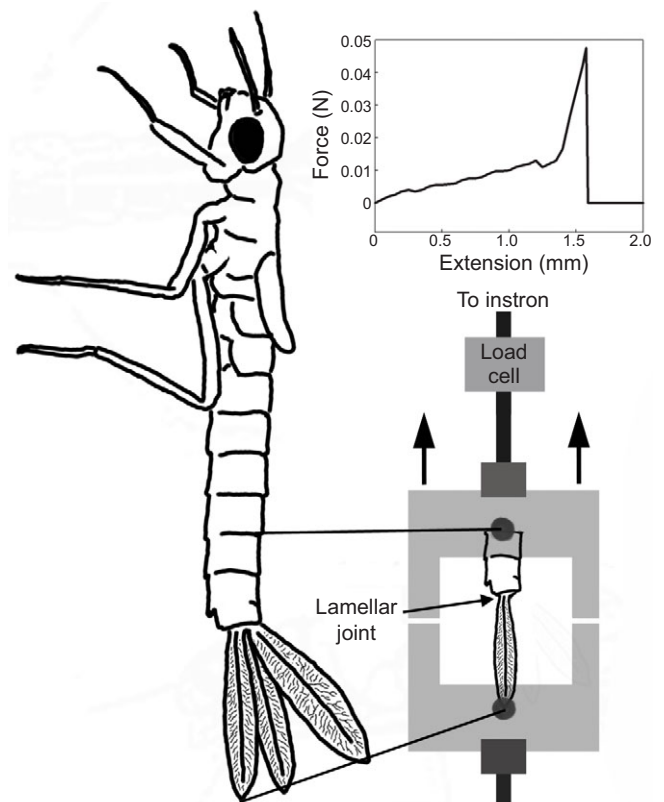


Fig. 5. A lateral view line drawing of an *Enallagma* damselfly larva displaying the three lamellae, which are each connected to the abdomen by a lamellar joint. The figure also demonstrates a dissected abdomen with attached lamella in the Instron set-up, as well as an example of a typical extension and force graph creating during tensile testing. The peak of the graph represents the moment autotomy occurred.

Two ponds contained predatory centrarchid fish (Ponds 5 and 44) while three ponds contained no fish (Ponds 1, 9 and 45).

Specimen identification

Specimens were identified to the *Enallagma* genus using the Odonata Larvae of Michigan key (Bright and O'Brien, 1999). *Enallagma* represented the largest proportion of genera present in the ponds over the sampling period (followed by *Lestes* and *Ichnura*, which were not used). *Enallagma* larvae were further classified to one of two species-pairs – *E. ebrium*/*E. hageni* and *E. boreale*/*E. cyathigerum* – because larvae of each species-pair are morphologically indistinguishable, and were therefore pooled following prior studies (McPeck, 1990a).

Specimen preparation

Lamellar joint breaking force was measured on a single normal non-regenerated lateral lamella on each specimen (Fig. 5). Before tensile testing, the medial and opposite lateral lamellae were removed by grasping them with forceps while the larva was immersed in water. In most cases, this induced larval swimming that resulted in lamellar autotomy. If this did not work, then the abdomen was lightly squeezed with forceps to induce escape swimming while simultaneously grasping the lamella. Removed lamellae were preserved individually in 95% ethanol.

Lamellar re-growth frequencies

Relative invertebrate predation risk among ponds was indirectly estimated from the frequency of re-grown lamellae in *Enallagma* samples taken from each population following Bose and Robinson (Bose and Robinson, 2013). Larval dragonflies, damselflies and other invertebrates all contribute to lamellar loss and regeneration in natural populations (Baker and Dixon, 1986). Specimens with multiple regenerating lamellae of the same size were scored as a single failed predation event. Multiple regenerating lamellae differing in size on an individual, however, were each scored as independent predation attempts. Lamellae are lost during sampling and handling, and so missing lamellae were not used to assess predation risk, nor were these individuals counted towards the total number of specimens in samples. The total count of independently regenerated lamellae per sample was divided by the total number of sampled specimens, and used as a relative estimate of the per capita predation risk from larval dragonflies and other grasping invertebrates among ponds.

Tensile testing

Lamellar joint breaking force was measured using a benchtop Instron Model 3343 Universal Testing Machine (Illinois Tool Works, Glenview, IL, USA). Immediately before mounting, the head and thorax were dissected off (preserved in 95% ethanol) and the abdomen with attached lamella was glued across a diamond-shaped hole punched through the middle of a 1×3 cm piece of card stock paper (Fig. 5). The abdomen and its lamella were secured on each side of the hole with fast-curing Super Glue so that the joint was centred over the hole. The card with attached specimen was mounted on the Instron using pneumatic grips, taking care not to tear the lamella or joint. The paper on either side of the hole was then carefully burnt away using a cauterizer so that the lamellar joint was unsupported. Force was measured using a calibrated 5 N load cell. Specimens were strained at a rate of 10 mm min⁻¹ until autotomy occurred, with time, extension and force data collected at 10 Hz (Fig. 5). Tensile testing generally lasted for 3 s and joint failure typically occurred at small extensions on the order of <1 mm. Samples remained hydrated during testing by moistening the joint prior to extension. Bluehill (Instron Corp. v2.9, Norwood, MA, USA) was used to determine the peak force, which we refer to as the breaking force here. After autotomy, the specimen was removed from the Instron grips, the card stock paper was cut away from the abdomen and the detached lamella, and these were preserved.

Imaging and joint morphometrics

Digital photographs were taken of each post-tensile tested lamella (above) using a Leica MZ8 microscope (Allendale, NJ, USA) fitted with a Nikon Coolpix 950 camera (Mississauga, ON, Canada). Each lamella was laid flat

against a 0.5 mm grid for scale and maximum joint diameter was estimated from digital photographs using ImageJ software (version 1.45s, National Institutes of Health, Bethesda, MD, USA). Joint size was measured as the widest linear distance across the diameter of the lamellar joint at the fracture plane (Bose and Robinson, 2013).

In order to evaluate whether cuticle area influenced breaking force independent of joint size, we subsampled 30 lamellae over a narrow interval of joint sizes (0.355–0.403 mm) but with a wide range of breaking forces. Individual lamellae were mounted on stubs, coated in gold palladium, and the joints were imaged along with a scale using a Hitachi S-570 scanning electron microscope (Tokyo, Japan) at 10 kV. Total area of each cuticle at the breaking plane was measured from the SEM images using ImageJ by tracing a line around the inside and outside of the cuticle, and subtracting the difference in areas.

Statistical analysis

Linear regression was used to evaluate the relationship between breaking force and joint size for larvae sampled from each of the five source populations separately and for all populations combined. Joint diameter and breaking force were transformed by square roots rather than by logarithm to reflect the expected functional effect of joint area on breaking force (the linear relationship between joint diameter and breaking force was stronger with square root, $R^2=0.35$, than with logarithm, $R^2=0.33$). Interaction and main effect ANCOVAs were then used to separately test the effect on breaking force of three factors: taxon group, pond and fish status. The relationship between breaking force and cuticle area was tested after removing the effects of joint size. 'Size-free' breaking strength was estimated as residual breaking strength from the combined population analysis above for the subsample of 30 lamellae measured for total cuticle area. Linear regression was then used to test the relationship between cuticle area and residual breaking strength. The relationship between the mean breaking force of each pond population (adjusted for lamellar size) and lamellar re-growth frequency was also evaluated by linear regression. We applied Tukey's multiple comparisons at $\alpha=0.05$ where necessary. All statistical tests are two-sided except where noted, and were performed with JMP v. 4.0.2 (SAS Institute, Cary, NC, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.E.G., B.W.R. and D.S.F. conceived of the experiments and wrote the paper; J.E.G. collected and analyzed the data.

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