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NOVEL LIGATURE METHODS FOR STUDYING SUBLETHAL EFFECTS OF SIT-AND-WAIT PREDATORS: TEST USING *CORDULEGASTER BOLTONII* (DONOVAN, 1807) LARVAE (ANISOPTERA: CORDULEGASTRIDAE)

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A novel method of labial palp ligature was tested as a substitute for palp ablation for studying sublethal effects of larvae of *C. boltonii* on prey populations and their consequences for ecosystem functioning. Two alternative types of ligature were designed to test for neutral or aggressive, but non-lethal, predator-prey interaction effects. Ligature efficiency in preventing prey capture was very high and the effects on larval survival and emergence success were negligible. Potential advantages and drawbacks, compared to other methods, are discussed. The results indicate that this fully reversible method should be applied whenever possible, especially for naturally rare or endangered odon. spp.

INTRODUCTION

Predators can have indirect consequences on prey communities that extend beyond direct predator-induced mortality, although very few studies have compared the relative importance of lethal and sublethal effects of predators (LIMA, 1998; PREISSER et al., 2005). Thus, investigation of the sublethal consequences of prey responses to predators is essential to fully understand predator impacts on prey populations and ecosystem functioning (PECKARSKY et al., 1993; ANHOLT & WERNER, 1998; LIMA, 1998). Until recently, very few studies have

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considered or focused on these "indirect" (i.e. sublethal) predator effects (PECKARSKY et al., 2008). For example, sublethal effects of odonate larvae on prey populations are often not measured (JOHNSON et al., 1987), or are assumed to be negligible compared with direct prey mortality through predation and consumption (WOODWARD & HILDREW, 2002a). However, predator avoidance can induce significant sublethal costs for aquatic invertebrates (PECKARSKY et al., 1993).

Studies considering sublethal effects of odonate larvae have often used caged animals that could not interact physically with their prey (TOUCHON & WARKENTIN, 2008; STAMPER et al., 2009). However, by segregating prey and predator, this method prevents physical encounter and accounts mostly for chemosensory interactions, excluding or interfering with other important cues (tactile or visual for example) through which prey can detect predators (BARBOSA & CASTELLANOS, 2005). Some experimental results have clearly highlighted the importance of investigating freely interacting predators and prey when studying potential effects of predator-prey physical interactions (HAMMOND et al., 2007).

Some studies have assessed the effects of sublethal interactions between larval odonates and their prey by removing the labial palps (VAN BURSKIRK, 1989; WISSINGER & McGRADY, 1993). These structures are at the distal end of the mentum, the extendable mouthpart used to capture prey. Individuals with the labial palps ablated are unable to capture large prey (VAN BURSKIRK, 1989; WISSINGER & McGRADY, 1993). Such larvae are thus assumed to interact physically with prey without causing fatality of the latter, thereby allowing sublethal effects to be tested.

However, there are several major issues to labial palp removal. It is ethically questionable and it increases larval mortality significantly (VAN BURSKIRK, 1989; WISSINGER & McGRADY, 1993). Also, it is irreversible and compromises larval growth (moulting) and emergence into the adult (WISSINGER & McGRADY, 1993). This clearly becomes problematic when considering rare or endangered species. Finally, this method is scientifically debatable as larvae with labial palps removed seem to adjust and switch to smaller prey that they are still able to capture, even without their mandibles (VAN BURSKIRK, 1989). This clearly induces a confounding factor when studying the predator's sublethal effects.

Here we propose and describe an alternative method that allows predator-prey physical interactions without prey fatality and thus enables the study of the sublethal effects of odonate larvae on prey populations. This method uses a single ligature to prevent odonate larvae from capturing and killing prey. It is also adjustable to the type of predator-prey interaction under study, either antagonistic (predator aggression toward prey) or neutral (predator presence without aggression).

MATERIAL AND METHODS

STUDY SITE AND MODEL SPECIES – Animals were collected in the Montagne Noire, southwestern France, a 1,450-km^2 highland region covered by a mixed broad leaf forest. It is drained by a high density of structurally similar first and second-order permanent streams (LECERF et al., 2005). Forestry management is the only major anthropogenic disturbance, without however any marked alterations of stream habitats (LAITUNG et al., 2002). These habitats are highly favourable to cordulegastrid dragonflies (ASKEW, 1988; FERRERAS-ROMERO & CORBET, 1999) and contain high densities of larvae of *Cordulegaster boltonii* (Donovan). Larvae used in our experiments were collected from a single 500 m reach in the Peyreblanque stream (43°25'N, 2°13'E; elevation, 750 m a.s.l.). Animals were captured using hand-nets (0.5 mm mesh size) and dredging the fine sediments of depositional zones where *C. boltonii* larvae burrow (FERRERAS-ROMERO & CORBET, 1999). Larvae were brought back to the laboratory and maintained at 14°C in 50 L coolers filled with aerated stream water until required. Two centimetres of sand (1mm < Ø < 2 mm) were added to allow the larvae to burrow.

LIGATURES – Ligatures were done using a fine surgical needle and

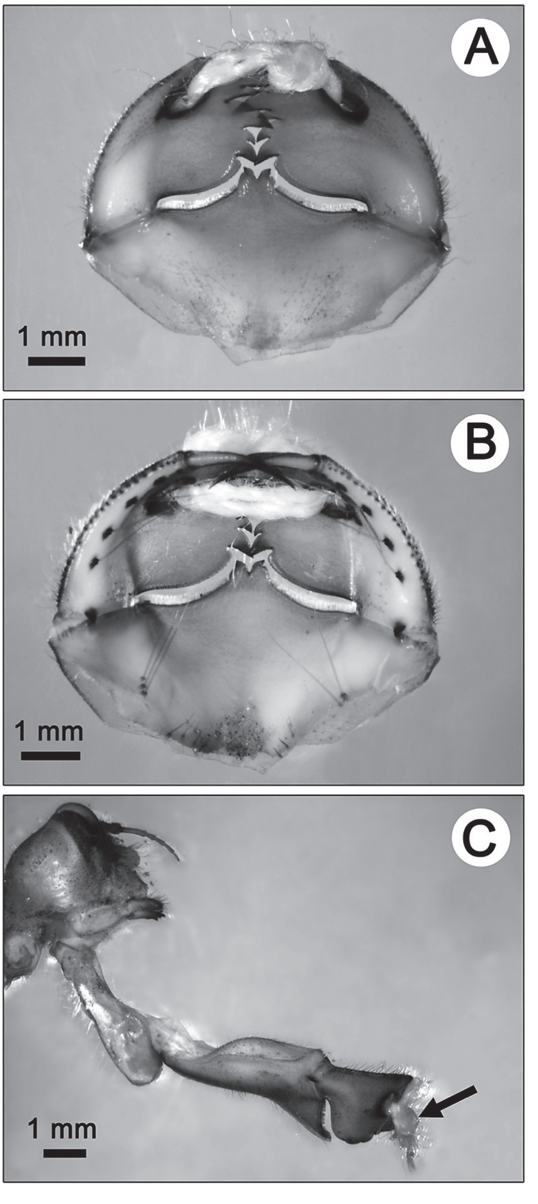

Fig. 1. Pictures of a type I ligature blocking the labial palps in the closed position but allowing full extension of the labium (prementum and submentum): – (A) ventral view of the ligature holding the labial palps closed; – (B) dorsal view of the same ligature; – (C) lateral view of the ligatured odonate larva's head with its labium fully extended and the labial palps maintained closed by the ligature (indicated by the black arrow). – [In the pictures, for visibility purposes, white cotton thread was used for the ligature instead of microbraided black nylon filament]

microbraided black nylon filament (Ø = 0.1 mm). Head-mounted magnifiers were used for precision. During ligature, the labium was gently extended to avoid wounding the larva's head with the needle; no chemical anaesthetic was needed during the operation. The filament was then passed through the first labial palp by piercing the cuticle with the surgical needle and back through the second palp to form a loop with the filament. The loop was loosely tightened to avoid injuries to the larva but still prevent predation. Finally, ligatures were secured with a surgeon's knot. Two alternative ligature types were used. A first method (ligature type I) was designed to block the mandibles (i.e. labial palps) in the closed position (Fig. 1A and 1B) but allow normal extension of the labium (Fig. 1C). The second ligature method (type II) prevented both extension of the labium and opening of the mandibles; the filament going over the head-labium joint thus preventing prementum-submentum joint extension (Fig. 2A and 2B). When completed, both ligature types gave a superficially similar aspect to *C. boltonii* larvae (Fig. 2C).

SURVIVAL AND FEEDING TESTS − To test for ligature durability and efficiency in preventing

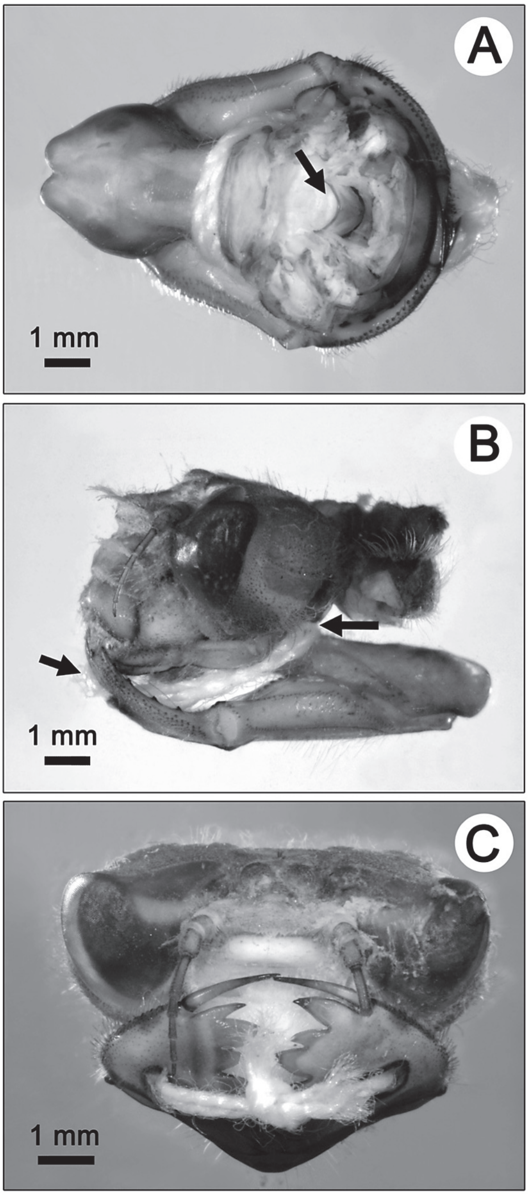

Fig. 2. Pictures of a type II ligature preventing both extension of the labium and opening of the labial palps: (A) dorsal view of the ligature going over the head-labium joint and preventing prementum-submentum joint extension (the black arrow indicates the position of the oesophagus); − (B) lateral view of the odonate larva's head with the labium maintained folded (resting position) and the mandibles closed by the ligature going through each labial palp and around the back of the head-labium joint (ligature indicated by black arrows); − (C) frontal view of the ligatured larva's head. − [Note that both ligature types appear superficially similar when completed. In the pictures, for visibility purposes, white cotton thread was used for the ligature instead of microbraided black nylon filament]

larvae from feeding, as well as possible ligature-induced mortality, control and ligatured *C. boltonii* larvae were kept in enclosures at the study site. For uniformity between treatments, only final instars of *C. boltonii* larvae with no external signs of metamorphosis were used in ligature tests (FERRERAS-ROMERO & CORBET, 1999). Thirty control (i.e. not ligatured) and 54 ligatured (27 with type I and 27 with type II ligatures) individuals were held individually in small 10 mm plastic mesh cages (10 × 10 × 10 cm). Cages were anchored to the stream substrate and contained 2 cm of sediment to allow burrowing. A third of the cages were retrieved after each of 7, 14 and 25 days to assess for temporal ligature effects on *C. boltonii* survival. Survival was compared in a pair-wise manner between treatments (control, type I and type II ligature) and treatment durations (7, 14 and 25 days) using Fisher's exact tests.

At the end of the survival test, *C. boltonii* larvae were returned to the laboratory and ligatures were removed using fine clippers. Larvae were kept individually in 400 mL containers filled with stream water. Animals were checked daily for a week or until faeces were released. Odonate larvae produce compact faeces enclosed in a strong peritrophic membrane, forming a faecal pellet allowing diet examination (LAWTON, 1970). These pellets were collected, fixed in 70% ethanol and examined under a compound microscope. Presence of sclerotized animal parts (mandibles or claws for example) in faecal contents indicated that larvae were able to kill and eat prey. Larvae were then offered four amphipods [*Gammarus fossarum* (Koch); Amphipoda: Gammaridae], which are large and fast moving prey, to test for post-ligature effects on *C. boltonii* predation abilities. Finally, larvae retrieved after 7 and 14 days were released at the study site. Individuals held in cages for 25 days were kept in the laboratory for emergence tests.

EMERGENCE TEST – Twenty-seven larvae held in cages at the study site for 25 days were kept in the laboratory to test for post-ligature effects on *C. boltonii* emergence success. Ten control and 17 previously ligatured (9 type I and 8 type II; one type II ligatured larva died before the cages were retrieved) individuals were kept in three separate 15 L aquaria (one per treatment group) filled with aerated stream water and 2 cm of sand. Emergence cages (5 mm diameter plastic mesh) were anchored to the substrate in the middle of the aquaria, allowing emergence (PURSE & THOMPSON, 2003). Larvae were fed *ad libidum* with live *G. fossarum*. Aquaria were checked daily for a month for newly emerged individuals (tenerals). Tenerals were retained until their body hardened, enabling flight. Emergence was considered successful for animals released alive and capable of flying. Deaths during emergence or deformities preventing flight were recorded. Furthermore, the labium of each newly emerged larva was checked for ligature-induced problems. Proportions of individuals that successfully emerged (i.e. emergence success rates) were compared in a pair-wise manner between treatments (control, type I or type II ligature) using Fisher's exact tests.

RESULTS

SURVIVAL AND FEEDING TESTS

Two individuals (one in type I and one in type II ligature treatments) lost their ligature during the experiment, but whether they actively removed it or it was not properly secured could not be determined.

Overall, mortality was very low and there was no significant ligature-induced mortality in caged animals; only one ligatured (type II ligature) larva died. Survival rates were not significantly different between treatments: control (100%), type I ligatures (100%) and type II ligatures (96.3%) ligatures (Fisher's exact tests in pair-wise comparisons; all $P > 0.05$). Similarly, mortality rates of ligatured individuals were not significantly different between treatment durations (Fisher's exact tests; all $P > 0.05$). Contrary to labial palp ablation (WISSINGER & McGRADY, 1993), ligatures did not cause unsuccessful moulting or metamorphosis. Ligatures induced minimal damage and were effectively fully reversible. Indeed, after ligature removal, all animals resumed feeding and consumed the four *Gammarus fossarum* within 24 hours regardless of treatment origin (control, type I or type II ligatures).

All but one of the 30 larvae from the control treatment released faecal pellets and had thus been feeding hours before being brought back to the laboratory. Accordingly, all 29 pellets contained sclerotized parts of larval insects (Plecoptera, Ephemeroptera, Trichoptera and Coleoptera) or of the crustacean *G. fossarum*. In contrast, a third of ligatured larvae (8 type I and 9 type II ligatured individuals) did not released faecal pellets. Faecal pellets released by the other ligatured larvae did not contain animal remains but constituted an empty peritrophic membrane. Ligatured larvae were thus effectively unable to capture and eat their usual prey or even smaller ones. However, the two larvae that had lost their ligatures had indeed resumed feeding and released faecal pellets containing larval insect parts.

EMERGENCE TEST

After a month, two control, two type I and one type II individuals were still alive but yet to attempt emergence and were thus released at the study site. All remaining larvae (8 control and 7 of each ligature type) had made an attempt at emerging and were included in the analyses. Three individuals, one in each category, died during emergence. In all three cases, ecdysis was incomplete and tenerals were found dead, still partly embedded in the exuviae. Additionally, one individual from the control treatment was deformed, having crumpled wings and a bent abdomen; it was clearly not viable and was not considered as successfully emerged. In pair-wise comparisons, emergence success rates were not significantly different between control (75%), type I (87%) and type II (87%) ligatured *C. boltonii* larvae (Fisher's exact tests; all $P > 0.05$). Furthermore, the labium of newly emerged individuals did not bear any effects, such as scars, from the ligatures.

DISCUSSION

Prey often appear unable to locate odonate larvae and, following encounter, seem physically too slow to evade the typically rapid dragonfly larva attack (<20 ms; PRITCHARD, 1965). However, previous experiments showed that up to 40% of *C. boltonii* attacks were unsuccessful (WOODWARD & HILDREW, 2002b). Odonate larvae can thus interact with their prey without an obligatory fatal output but potentially have sublethal effects (stress or increased emigration rate, for example) on prey populations. Sublethal components of predator effects on prey need to be decoupled from direct prey consumption to fully understand the top-down effects of these predators on aquatic ecosystems.

By permitting predator-prey physical interactions but preventing odonate larvae from actually capturing and eating their prey, ligatures allow testing for predator sublethal effects. This could be done through field and/or laboratory microcosm studies, combining type I (presence with aggression but without prey consumption) and type II (presence without aggression towards prey) ligatures as well as non-ligatured predators and a control treatment without the predator. This novel approach could substantially advance our understanding of the impacts of sit-and-wait invertebrate predators for which sublethal effects have been considered minor so far (WOODWARD & HILDREW, 2002a, 2002b), especially when compared to active predators (PECKARSKY et al., 1993; PECKARSKY & COWAN, 1995; SIH et al., 1998). Furthermore, odonate larvae are comparatively large-bodied invertebrates, potentially influencing smaller invertebrate species independently of predation, through competition for space and/or biotic disturbance, during burrowing phases for example. Overall, type I and type II ligatures should help shed light on the relative importance of predation, sublethal antagonistic interactions (type I ligature) and biotic disturbance (type II ligature) in determining prey distribution.

Compared to labial palp ablation, the method described herein is fully reversible and caused only minor lesions, i.e. a small puncture hole in each labial palp. As a result, ligatures were associated with extremely low mortality in *C. boltonii* larvae and had no effect on metamorphosis and emergence success. Furthermore, preliminary experiments in laboratory microcosms indicate that ligatured animals still behave as sit-and-wait predators, spending long periods of immobility in a buried position interrupted by short periods of active search for new hunting locations. This suggests that ligatures do not greatly affect animal behaviour, thus confirming the validity of our approach to address mechanistic issues in top-down effects of *C. boltonii* in stream food webs. We also believe that contrasting type I and type II ligatures will help shed light on the relative importance of sub-lethal predation due to aggressive behaviour (type I ligature) and biotic disturbance to the substrate (type II ligature) in determining prey distribution.

As animals used in ligature experiments can be released, this novel method is ethically less arguable than palp ablation and is absolutely necessary when using endangered or protected species. However, the method presents some shortcomings. Notably, it is not applicable to all odonate species; for example, larvae with hook-shaped labial palps, such as in the Aeshnidae, are unsuitable. Furthermore, there may be a size limitation in the application of our ligature methods, depending on odonate species and instar classes. Preliminary experiments on *C. boltonii* showed that larvae as small as F-2 instars could be ligatured (see FERRERAS-ROMERO & CORBET, 1999 for instar size classes). In our study, some *C. boltonii* larvae were also able to remove the ligature. This could probably be prevented by using stronger ligatures. Despite these drawbacks, ligature methods should be considered whenever possible as the preferred alternative to labial palp ablation.

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