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Hydrotimetes natans as a suitable biological control agent for the invasive weed *Cabomba caroliniana*

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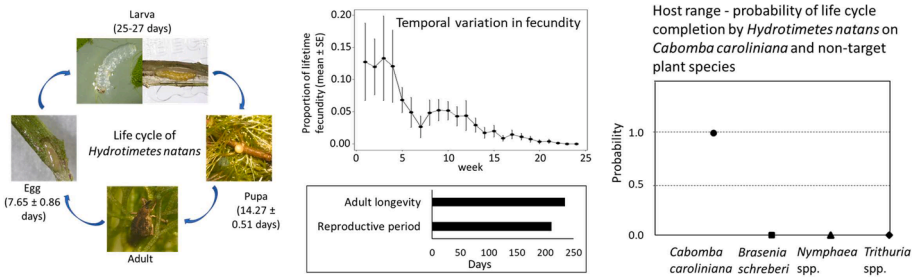
HIGHLIGHTS

- *Cabomba caroliniana* is a major invasive weed in several countries.
- *Hydrotimetes natans* was identified as a biocontrol agent for *C. caroliniana.*
- *H. natans* female laid 123.13 ± 23.03 eggs in 24 weeks.
- Adult survival and reproductive periods were 235 ± 21 and 211 ± 35 days, respectively.
- *H. natans* is adequately host-specific and it was approved for release in Australia.

GRAPHICAL ABSTRACT



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ABSTRACT

The aquatic macrophyte *Cabomba caroliniana* A. Gray is a major invasive weed in Australia and several other countries. A classical biological control program was initiated in Australia in 2003 and native range explorations in Argentina that year led to the discovery of the aquatic weevil *Hydrotimetes natans* Kolbe feeding on *C. caroliniana*, making it the first, and so far, only potential biological control agent for the weed. However, the program was discontinued because the largely unknown biology had made rearing of *H. natans* difficult under quarantine laboratory conditions. We report here key aspects of the biology and reproductive behaviour of *H. natans* that provided significant insights to successfully establish a laboratory colony when the program was restarted in 2016. In addition, we studied the physiological host range of *H. natans* and determined its risks to 15 non-target plant species. The preoviposition period of *H. natans* was 6.50 ± 0.85 days, and the development times of eggs and pupae were 7.65 ± 0.86 and 14.27 ± 0.51 days, respectively. Egg to adult development time was 46.52 ± 0.82 days with a larval development time of 25–27 days. A single female laid 123.13 ± 23.03 eggs in 24 weeks under non-limiting laboratory conditions. Oviposition was intermittent and age-dependent with 75% eggs oviposited within 8.54 weeks after adult eclosion; percent viability of these eggs was 55.62 ± 4.61. Females oviposited mostly on the apical tips followed by on the first few nodes from the tip. Adults survived a maximum of 521 days with a mean longevity of 235.16 ± 21.16 days and females remained reproductive for 211.00 ± 35.05 days. Field surveys and laboratory host-specificity studies demonstrated *H. natans* is adequately host-specific to *C. caroliniana*. No non-target effects were observed on *Nymphaea, Victoria* and *Trithuria* species. *Brasenia schreberi* indicated the possibility of lifecycle completion by *H. natans* in choice and no-choice trials but

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did not sustain a population in continuation trials. The risks to *B. schreberi* were deemed negligible and *H. natans* was approved for release in Australia.

1. Introduction

Freshwater systems provide a wide range of ecosystem services including habitat provision, cycling of nutrients, flood regulation and climate regulation (Green et al., 2015; Biggs et al., 2017). These systems have been experiencing severe ecological changes at an unprecedented scale worldwide, and invasion by aquatic weeds is one of the main stressors driving these changes (Adams et al., 2015; Havel et al. 2015; Vári et al., 2021). Aquatic weeds negatively affect freshwater systems through biodiversity loss and altering nutrient dynamics, water quality, and habitat ecology, and consequently affect the broader ecosystem functioning and productivity (Bunn et al., 1998; Sidorkewicj et al., 2004).

Cabomba caroliniana A. Gray is a submerged macrophyte invasive in several parts of the world (Ørgaard, 1991). Native to the temperate and tropical regions of southern Brazil, Uruguay, Paraguay and central to north-eastern Argentina, *C. caroliniana* was introduced as an aquarium plant into other geographic regions including Canada, the Netherlands, Belgium, Malaysia, Japan, India, China and Australia (Wilson et al., 2007; Jacobs and Macisaac, 2009). It was introduced into freshwater systems in the non-native range through disposal of aquarium materials and has become a serious pest because of its propensity to form dense monotypic populations (Mackey and Swarbrick, 1997). Negative impacts resulting from this weed include obstructed waterways, affecting recreational activities, and alteration of aquatic community dynamics including displacement of native species (Mackey and Swarbrick, 1997; Schultz and Dibble, 2012). Being an opportunistic macrophyte that grows well in nutrient-rich water (Bickel and Schooler, 2015), *C. caroliniana* has been reported to alter the nutrient dynamics of the habitat (Mackey and Swarbrick, 1997; Zhang et al., 2003).

A classical biological control program was initiated in Australia in 2003 to develop sustainable management options for *C. caroliniana* (Schooler et al., 2012). Native range surveys were conducted in South America in collaboration with United States Department of Agriculture-Agricultural Research Service South American Biological Control Laboratory (currently, Fundación para el Estudio de Especies Invasivas [FuEDEI]) Hurlingham, Argentina. Three candidate agents, the cabomba weevil *Hydrotimetes natans* Kolbe (Coleoptera: Curculionidae), and the moths *Paracles burmeisteri* Berg (Lepidoptera: Erebidae) and *Paraponyx diminutalis* (Snellen) (Lepidoptera: Crambidae) were prioritized for further investigation. Moth species were rejected however because of their non-target feeding on other aquatic plants in laboratory no-choice host-specificity tests (Schooler et al., 2012). In the case of *H. natans*, preliminary no-choice tests found no feeding or oviposition on *Egeria densa* Planch., *Ceratophyllum demersum* L. and *Myriophyllum aquaticum* (Vell.) Verdc. under laboratory conditions in Argentina (Cabrera-Walsh et al., 2011). In addition, *H. natans* was recorded only on *C. caroliniana* and not on other co-occurring species (*E. densa, E. najas* Planch., *Najas* sp., *C. haynesii* Wiersema, *Nymphoides indica* (L.) Kuntze, *Potamogeton illinoensis* Morong, *Po. gayi* A. Benn., *Ce. demersum, Urticularia platensis* Speg. and *U. foliosa* L.) in field surveys (Cabrera-Walsh et al., 2011). These native range studies suggested *H. natans* was likely specialised on *C. caroliniana*.

Hydrotimetes natans is an aquatic weevil belongs to the Erirhininae subfamily. Members of Erirhininae are restricted to aquatic plants or are endophagous in roots, stems, leaves, and fruits of terrestrial plants (Kuschel, 1971). Adult *H. natans* are dark brown dorsally with light brown-cream venters and are approximately 4.6 mm in length. They feed on the tips of the plant and cause considerable damage when in large numbers. Larvae are endophagous in stems and cause substantial damage to the main stem through tissue necrosis. Previous attempts at establishing a colony of *H. natans* in Argentina found that adults survive for 5–9 months under experimental garden conditions and that full development from egg to adult takes approximately 40 days at 27 °C.

Hydrotimetes natans was imported into quarantine in Australia to conduct host-specificity testing in 2004. However, it could not be reared since the biology and life history was largely unknown apart from the preliminary laboratory and field observation on a few aspects of its biology in Argentina. These challenges impeded the continuation of the biological control research and led to discontinuation of the project in 2006 (Cabrera-Walsh et al., 2011).

The biological control program was restarted in 2016 in Australia. In collaboration with FuEDEI, additional field surveys were made in Argentina and detailed biology studies were conducted under quarantine conditions in Australia. We report here various key aspects of the biology and behaviour such as development time of all life stages, oviposition behaviour, fecundity, reproductive period and adult longevity of *H. natans* that can aid establishment of a colony and to subsequently develop biological control options for *C. caroliniana*. We also systematically quantify the risks to non-target plants in Australia from *H. natans*. Based on our results, we infer the suitability of *H. natans* as a biological control agent for *C. caroliniana*.

2. Materials and methods

2.1. Insect source and insect colony maintenance

Hydrotimetes natans were collected from the Iberá wetlands (−28.53789; −57.18650) in the province of Corrientes, Argentina. Larvae, pupae and adults were collected along with *C. caroliniana* plants and extracted by air-drying the plant material in Berlese funnels in the laboratory at FuEDEI following methods described in Cabrera-Walsh et al. (2011). Adult insects were imported during 2018, 2019 and 2020 under appropriate regulatory permits (Import permit 0001766948) into a quarantine facility at the Ecosciences precinct, Dutton park, Queensland, Australia. For insect colony maintenance, 6 to 8 sprigs (~30 cm length) of *C. caroliniana* with growing tips were submerged in a food-grade plastic tub (23H × 21.5W × 21.5L cm) with eight litres of water. The sprigs were bundled and bound at the base using a stainless-steel nut to keep them anchored and upright once submerged.

Insect colony maintenance and the quarantine-based experiments described below were conducted in a quarantine glasshouse set at 27 ± 4 °C, 40–60 ± 5% relative humidity and 12:12 (light: dark) photoperiod. Laboratory studies in Argentina described below were conducted in a glass house set at 27 ± 4 °C, 40–60% relative humidity and 12:12 (light: dark) photoperiod during winter, and solar photoperiod the rest of the year.

2.2. Plant source and maintenance

Cabomba caroliniana plants used in insect colony maintenance and all experiments were sourced from Lake MacDonald (-26.402848, 152.947338) and Burringbar Creek (-28.439028, 153.489248) in Australia. These plants were maintained as submerged sprigs in 160L aquarium tanks illuminated using fluorescent lights with 8:16 (light: dark) photoperiod in a laboratory set at 23 ± 2 °C. To adjust the nutrients in the water, the following were added to 100 L of reverse osmosis water: 5 mL of Prime® conditioner (Seachem Laboratories, Madison, USA), 6 g carbonate hardness generator (Aquasonic private limited, Wauchope, Australia), 25 mL of fertilizer mix containing $(NH_4)_2SO_4$, KNO_3, Chelated Fe, $MgSO_4$ and KH_2PO_4 (Gilbert's Brew), 8.5 mL Rexolin APN solution (Duralite, Heatherton, Australia). The pH of

the water was adjusted to 6.2 to 6.5 using acid and alkaline buffers (Seachem Laboratories, Madison, USA) based on the water quality parameters recorded in the Iberá wetlands. This water mix was used in aquarium tanks and all experimental arenas. Constituents of commercial aquarium chemicals are provided in the supplementary file (Table S1).

2.3. Biology of Hydrotimetes natans

Cabomba caroliniana sprigs of ~ 30 cm size maintained in plastic tubs (23H × 21.5 W × 21.5L cm) filled with water mix were exposed to adult *H. natans* for 24 h for oviposition. This was repeated seven times with different cohorts of *H. natans* (4 to 11 individuals per cohort) to obtain adequate number of eggs, larvae and pupae required for the various life history observations described below. Because *H. natans* adults are sexually monomorphic, pairs *in copula* were used.

To locate the oviposition site and eggs, a subset of exposed sprigs of *C. caroliniana* was dried on paper towel briefly and then submerged for 10 min in a 10% food dye ("Pillar Box Red", Dr Oetker Queen, Alderley, Australia) diluted with tap water (Thies et al., 2002). The excess dye was removed by gently rinsing the sprigs in tap water. The dye did not directly stain the eggs but stained the plant material and gave the eggs a distinct and discernible red-ringed appearance (Fig. S1). Scanning under a microscope, a section of leaves with eggs was excised and the length and width of eggs (n = 52) were measured using NIS Elements imaging software v5.01 (Nikon Instruments Inc., New York, USA) on a Nikon SMZ800N microscope at 7x magnification. The leaf sections with eggs were then placed individually in a petri dish lined with moist filter paper and monitored daily to record the time from oviposition to emergence of neonates. The remainder of the exposed sprigs were maintained in plastic tubs (23H × 21.5 W × 21.5L cm) and monitored daily to record pupation and adult eclosion. From these arenas, data on egg to pupal development time and pupa to adult development time were recorded. Larval development time could not be recorded as the endophagous larvae tunnel into the main stem as they mature. Therefore, the larval development time was estimated using the data on egg development time and egg to pupa development time.

For head capsule measurements, larvae (n = 100) were extracted periodically from the exposed sprigs from day 5 through to day 32 of exposure. If immediate extractions were not possible, the plant material with larvae was placed in 80% ethanol on the desired day of larval development and the larvae were extracted later. Head capsule width (across the widest point) was measured using a microscope as above at 8x magnification and number of larval instars in *H. natans* was statistically determined (see Statistical analyses, below).

To estimate the fecundity of *H. natans*, three pairs of newly eclosed adults *in copula* were introduced into a plastic container (25.5H × 10.9 W × 16.4L cm) filled with 2L of water mix along with a ~ 30 cm sprig of *C. caroliniana* kept upright. Fresh sprigs were exposed to *H. natans* for 48 to 72 h every other day for 24 weeks and number of eggs laid on flower tip, apical tips and each of the first 20 nodes from the apical tip was counted. This trial was repeated six times using different cohorts of *H. natans*. The data on number of eggs laid are presented as cumulative weekly fecundity.

Observations on the preoviposition period, adult longevity and duration of the reproductive period were made in additional trials. This trial was repeated six times using different cohorts of *H. natans*, with 6 or 7 individuals per replicate. In these trials, *C. caroliniana* sprigs maintained in plastic tubs filled with water mix were exposed to *H. natans* from the day of adult eclosion until all adults died (up to 551 days). Sprigs were initially examined daily to determine the pre-oviposition period, then three times a week to check the viability of the oviposited eggs. To confirm their viability, eggs were either monitored for hatching of neonate larvae or the exposed plant material was maintained until late instar larvae or pupae were noticed. Adult mortality was noted throughout the trial.

2.4. Host-specificity of Hydrotimetes natans

Host-specificity studies included field surveys in Argentina, laboratory studies on *H. natans* feeding on cut leaves/plant material in Argentina and Australia, and feeding and development trials on live plants of the target and each of the selected non-target species in Australia. Feeding and development trials on live plants were setup as no-choice, choice, and continuation trials.

2.4.1. Field surveys

Field surveys were conducted between April 2017 and December 2018 in six sites in the Iberá wetlands (Argentina) and three sites in Paraguay (Encarnación, San Ignacio and Pilar) where *C. caroliniana* was present. At these sites three people spent 90 to 255 min each day for three days in Argentina and 60 to 120 min per site in Paraguay to verify the presence of *H. natans*, adult feeding damage and larval tunneling. Field collections of *H. natans* were made in Galarza, Iberá wetlands, Argentina, and San Ignacio, Paraguay. Several species of non-target plants coexisting with *C. caroliniana* (*E. najas, Ny. indica, Nymphaea prolifera* Wiersema, *Salvinia minima* Baker and *Ludwigia grandiflora* (Michx.) Greuter & Burdet) were air-dried in Berlese funnels in the laboratory at FuEDEI to verify the presence of *H. natans* adults and larvae in them.

2.4.2. Laboratory feeding trials on cut leaf discs

For laboratory host-specificity studies, non-target test plants were selected based on their phylogenetic proximity to *C. caroliniana* following the centrifugal phylogenetic approach (Wapshere, 1974; Briese, 2004; Gilbert et al., 2012). *Cabomba caroliniana* belongs to the family Cabombaceae within the order Nymphaeales, a basal angiosperm order (Stevens, 2001). There are only two species in Cabombaceae in Australia; one being *C. caroliniana*, the other in another genus is *Brasenia schreberi* J.F.Gmel. (watershield). *Brasenia* is a monotypic genus and is native to Africa, Asia, east Australia, and the Americas from Canada to northern Venezuela and Guyana (WCSP, 2021). The only other families in the Nymphaeales are Nymphaeaceae (the water lilies) and Hydatellaceae (Stevens, 2001).

Cut leaf trials in Australia included leaf discs (*B. schreberi*, *N. caerulea, N. gigantea* Hook. and *N. mexicana* Zucc.) or whole leaves (*N. nouchali* Burm. f) or small stem sections (*C. caroliniana*) of plants depending on the size or structure of the leaves. Leaf tissues were kept in a round plastic container (12 cm dia, 700 mL) with water mix and a pair of *H. natans* was released into each container. After 10 days, leaves were observed under a microscope and number of feeding lesions was recorded. The study was replicated six times.

The genus *Cabomba* has no related species in Cabombaceae in Argentina, so the test plants were selected from among other Nymphaeales that coexist with *Cabomba*: *C. caroliniana* var. *caroliniana, C. caroliniana* var. *flavida, N. prolifera, N. caerulea* Saligny and *Victoria cruziana* Orbign. Adult feeding was assessed on leaf discs floating in 2 cm of rainwater in plastic containers (12 cm dia, 700 mL) with ventilated lids. Larval specificity was evaluated by placing second and third instar larvae on 20-cm stem segments, and recording larvae entering stems and resuming feeding. Five replications were performed with each test species, each paired with a container of *C. caroliniana* var. *caroliniana* as control, using between 3 and 5 adults, and 3 and 7 larvae per replication, depending on their availability at the time of the test. Since *Cabomba* has finely dissected, flabellate leaves that cannot be cut into discs, sprigs with buds and two leaves were used. Tests were kept for two weeks for adults, and until all the larvae died in the test plants.

2.4.3. No-choice feeding and development on live plants

A total of 13 representative species from all three families of Nymphaeales, *viz*. Cabombaceae (*B. schreberi*), Nymphaeaceae (*N. alba* L., *N. caerulea*, *N. gigantea*, *N. immutabilis* SWL Jacobs, *N. mexicana*, *N. nouchali*, *N. pubescens* Willd. and *N. violacea* Lehm.) and

Hydatellaceae (*Trithuria austinensis* D.D.Sokoloff, *T. fitzgeraldii* D.D. Sokoloff, I.Marques, T.Macfarlane, Rudall & S.W.Graham, *T. lanterna* D. A.Cooke and *T. submersa* Hook.f.) present in Australia were included in no-choice trials to assess the risks of *H. natans*. Selection of representative non-target species emphasized the endemicity, economic importance and biogeographic overlap with *C. caroliniana* where possible (Table S2).

For no-choice trials, potted plants were setup either in a 68L food grade polypropylene crate (*B. schreberi* and *Nymphaea* species) (41.3H × 39.7 W × 64.5L cm) or 8L plastic tub (*Trithuria* spp.) (23H × 21.5 W × 21.5L cm) filled with water mix depending on the size/structure of the plant species. Our laboratory colonies of *H. natans* were maintained on *C. caroliniana* in both 68L crates and in 8L tubs used in these trials, and the biology, behaviour and life cycle of *H. natans* were unaffected by any abiotic differences between these volumes of water. Three pairs of sexually mature *H. natans* adults were introduced into the testing arena and maintained for the duration of the full lifecycle of *H. natans* (46 to 50 days). Observations on oviposition were made three times a week, and at the end of the trial, number of pupae and adults eclosed were recorded. Plant material was microscopically examined and observation on number of adult feeding lesions was recorded. Multiple trials were run from 2017 to 2019 and a control containing *C. caroliniana* was used in each trial. Most plant species were tested at least six times, where possible, using geographically isolated accessions (i.e., plants collected from distinct populations). *Trithuria fitzgeraldii* and *T. austinensis* were replicated four and three times, respectively, because of the difficulty with sourcing plants.

2.4.4. Choice and continuation trials

Only *B. schreberi* was used in choice and continuation trails based on the results from the no-choice trials. For the choice trial, potted plants of both *C. caroliniana* and *B. schreberi* were maintained in a single polypropylene crate and three pairs of *H. natans* were introduced. The trial was replicated five times and was run for the duration of full lifecycle of *H. natans*. For continuation trials, *B. schreberi* plants were maintained in a no-choice setting in a polypropylene crate with three pairs of *H. natans* adults. Additional plants were provided as required to ensure that plant availability was not a limiting factor for lifecycle completion and population sustenance. The continuation trial was replicated eight times with adult *H. natans* from different cohorts and was run for 150 days to correspond to the duration of three generations of *H. natans*. Observations on oviposition, larval development, pupation and adult eclosion were made from both choice and continuation trials.

2.5. Statistical analyses

For descriptive statistics means ± 1 SE are provided. Head capsule data were subjected to Hartigan's Dip test of modality to determine the number of larval instars (Hartigan and Hartigan, 1985). One-way ANOVA with test species as a fixed effect was used to compare the data on number of adult feeding lesions, eggs laid on different oviposition sites, age-dependent fecundity and viability at different age (Zar, 1999). Posthoc pairwise comparisons were made using Tukey's HSD test. A non-linear regression was fitted to the cumulative weekly fecundity data with age of *H. natans* to characterise the reproductive period. Data from the no-choice host-specificity trials were analysed as a binary response (yes or no), and a logistic regression model was fitted to calculate the likelihood of oviposition, larval feeding and development, pupation and lifecycle completion by *H. natans* on the non-target plant species. Data from choice trials with *C. caroliniana* and *B. schreberi* (n = 5) were subjected to a one-tailed binomial test (Zar, 1999) and the observed proportion of successful oviposition, larval development, pupation and lifecycle completion (against hypothesised probability of success of 100%) was calculated. Number of pupae recorded from *C. caroliniana* and *B. schreberi* in choice trial was subjected to a Welch's *t*-test (Zar, 1999). All analyses were performed in R3.6.2 (R Core Team, 2020) via the RStudio interface (v 1.2.5033) (R Studio Team, 2020) using the packages brglm2 (logistic regression; Kosmidis, 2017) and ggplot2 (for graphs; Wickham, 2016). For ANOVA, binomial tests and Welch's *t*-test, R's base functions were used (R Core Team, 2020).

3. Results

3.1. Biology of Hydrotimetes natans

Hydrotimetes natans oviposited eggs singly in a small divot (presumably formed by adult feeding) with part of the egg exposed near the apical tip of the stem predominantly on the first division of leaves from the petiole, but occasionally also oviposited in the petiole itself (Fig. S1). More exposed eggs (i.e. in a shallower divot) and those inserted within a thin layer of plant tissue in a deeper divot were also observed (Fig. S1a&b). The preoviposition period recorded was 6.50 ± 0.85 days (mean ± 1SE). Eggs were elongate capsules and creamy in colour, with a length and width of 855.34 ± 50.17 μm and 292.24 ± 25.51 μm, respectively; neonate larvae emerged from eggs in 7.65 ± 0.86 days (Fig. 1).

Larvae were translucent immediately after hatching from the egg and transitioned into creamy white colour as they developed (Fig. S1c). Mature larvae were yellow or green and became more scarabaeiform as they approached pupation (Fig. S1d). A frequency distribution of larval head capsule widths showed three distinct sizes, indicating three larval instars, and the Hartigan's dip test confirmed the non-unimodal distribution of the data ($D = 0.11$; $p \ll 0.01$). Head capsule width of first-, second- and third-instar larvae was 190.31 ± 15.68 μm, 293.52 ± 16.07 μm and 448.98 ± 25 μm, respectively (Fig. 2). First-instar larvae were found feeding in tunnels in the foliage and petioles while second- and third-instar larvae were found tunnelling the main stem.

Pre-pupae exited the stem after approximately 27 days of larval development and pupated at a node near the apical tip of the plant (Fig. S1e). Oviposition to pupation time was 34.05 ± 1.11 days, and adults eclosed from pupae in 14.27 ± 0.51 days. Development from eggs to adult was 46.52 ± 0.82 days at 27 ± 4 °C, 40–60 ± 5% relative humidity and 12:12 (light: dark) photoperiod (Fig. 1). Survival rates of eggs, larvae and pupae were 55.62 ± 4.61%, 50.70 ± 5.19% and 58.49 ± 5.28%, respectively.

Adult feeding was usually focused on the petioles at the apical tip of the plant. Necrosis was observed around these feeding lesions, but adults

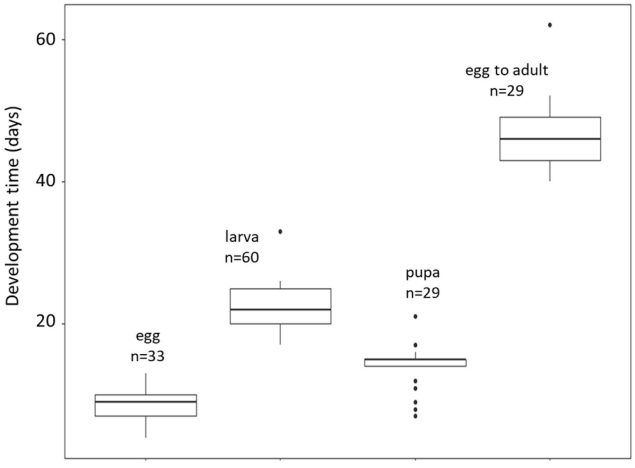

Fig. 1. Boxplot of development time of eggs, larvae, pupae and total development time of *Hydrotimetes natans* under laboratory condition of 27 °C ± 4 °C temperature, 60 ± 5% relative humidity and 12:12 (light: dark) photoperiod. The box plot comprises the median line, interquartile range from 25th to 75th percentile (the bounding box), the minimum (25th percentile − 1.5*interquartile range) and maximum whiskers (75th percentile + 1.5*interquartile range) and outliers (the circles beyond the whiskers).

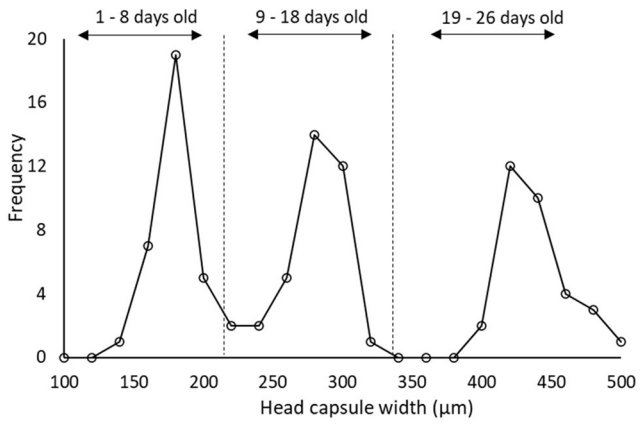

Fig. 2. Frequency distribution of *Hydrotimetes natans* larval head capsule width measurements (µm) and age of the larvae. The distinct nodes confirmed three larval instars, and Hartigan's dip test confirmed the non-unimodal distribution and tri-modality.

seem to otherwise inflict little damage to the plant, unless large numbers were exposed to plant material for a long period of time. Adults were able to survive for several weeks with little to no feeding. Adults survived a maximum of 521 days with a mean longevity of 235.16 ± 21.16 days, and females remained reproductive (i.e. laid viable eggs) for 211.00 ± 35.05 days with a maximum of 342 days.

The total number of eggs laid by a single female was 123.13 ± 23.03 in 24 weeks and fecundity was dependent on age with greater number of eggs laid when the adults were younger ($F_{23,\ 143} = 42.69$, $p \ll 0.01$). Females laid 50% of their eggs in 4.92 weeks and 75% of eggs in 8.54 weeks of adult eclosion (Fig. 3). Per cent viability of eggs was 55.62 ± 4.61, and egg viability did not differ with age ($F_{23,\ 143} = 1.23$, $p = 0.27$). Of the total eggs laid, the greatest number of eggs was laid on apical tips ($F_{21,\ 131} = 23.26$, $p \ll 0.01$), followed by on the first few nodes from the tip (Fig. 4).

3.2. Host-specificity of Hydrotimetes natans

3.2.1. Field surveys

Hydrotimetes natans adults were primarily found on *C. caroliniana* in field surveys, except for a few casual occurrences on *U. platensis, N. prolifera* and *E. najas* that are mixed among *C. caroliniana* patches in the field. *Hydrotimetes natans* larvae were only extracted from *C. caroliniana* var. *caroliniana.* Other aquatic plants co-occurring with *C. caroliniana* at the Iberá wetlands during the survey were *Nymphaea jamesoniana* Planch., *N. prolifera, N. caerulea, Ludwigia peploides* (Kunth) P.H. Raven, *Utricularia* spp., *S. adnata* Desv., *Eichhornia azurea* (Sw.) Kunth, *E. crassipes* (Mart.) Solms, *Hydrocotyle ranunculoides* L. f., and *Potamogeton* spp, and *H. natans* was not found on any of these species.

3.2.2. Laboratory cut leaf discs studies

Cut leaf trials in Australia showed adult feeding lesions on *C. caroliniana, B. schreberi, N. caerulea, N. nouchali* and *N. gigantea*, but not on *N. mexicana*. Number of feeding lesions (mean ± 1SE) recorded on *C. caroliniana, B. schreberi, N. caerulea, N. nouchali* and *N. gigantea* were 4.50 ± 1.34, 8.00 ± 3.61, 1.00 ± 1.00, 4.80 ± 1.32 and 2.60 ± 1.47 respectively, and the differences were not significantly different ($F_{5,\ 24} = 2.04$; $p = 0.11$).

Trials in Argentina revealed that *H. natans* larvae failed to enter stems of *N. prolifera, N. caerulea* and *V. cruziana*, and all the larvae died within 5 days. In contrast, a significant proportion of the larvae (n = 93) re-entered stem sections of *C. caroliniana* var. *caroliniana* (43%) and *C. caroliniana* var. *flavida* (36%), and resumed feeding. Exploratory adult feeding lesions were observed on *N. prolifera* and *V. cruziana,* but the feeding damage was more extensive on *C. caroliniana*. Number of feeding lesions recorded on *C. caroliniana, V. cruziana,* and *N. prolifera* were 12.40 ± 2.94, 0.20 ± 0.20, and 3.29 ± 1.47 respectively.

3.2.3. No-choice feeding and development on live plants

In no-choice trials, adult feeding was observed on *N. gigantea, N. immutabilis, N. pubescens* and *N. violacea*. However, the feeding intensity on *C. caroliniana* was far greater than the *Nymphaea* species ($F_{12,61} = 5.72$, $p \ll 0.01$) (Fig. 5). Oviposition by *H. natans* was not observed on any of these *Nymphaea* species. Neither adult feeding nor oviposition

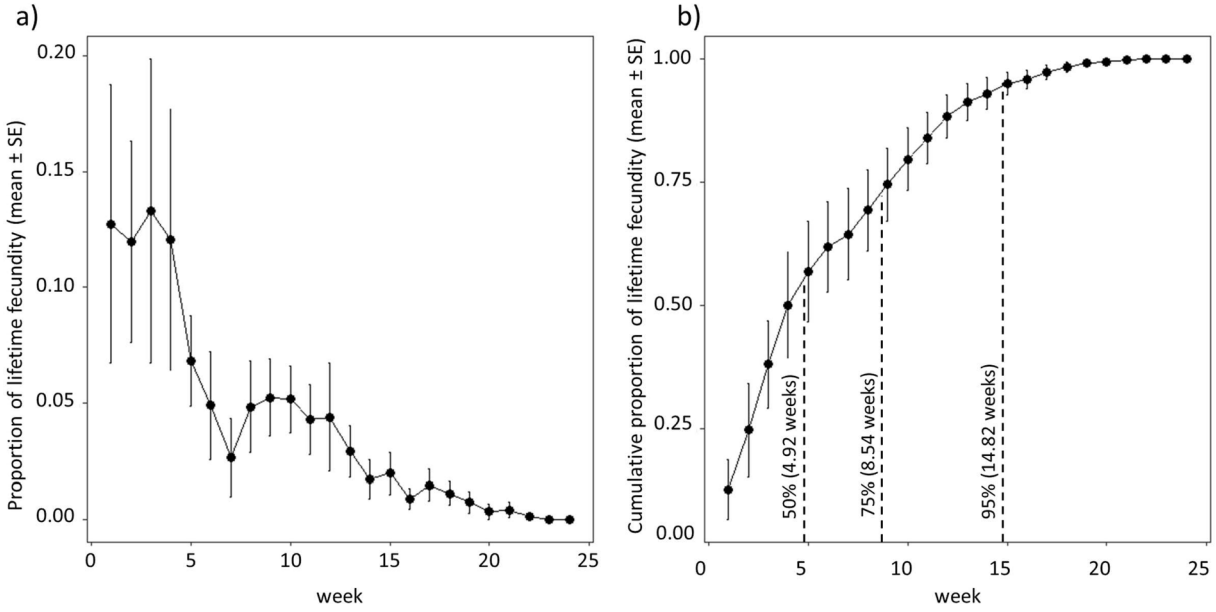

Fig. 3. Age-dependent oviposition in *Hydrotimetes natans* in a non-limiting environment at 27 °C ± 4 °C temperature, 40% relative humidity and 12:12 (light: dark) photoperiod. (A) proportion of lifetime fecundity over 24 weeks; (B) cumulative proportion of lifetime fecundity from three pairs over 24 weeks. Means and SE are indicated, with a non-linear regression fitted to (B) to estimate age thresholds to 50%, 75% and 95% of lifetime reproductive output (drop lines from fitted regression).

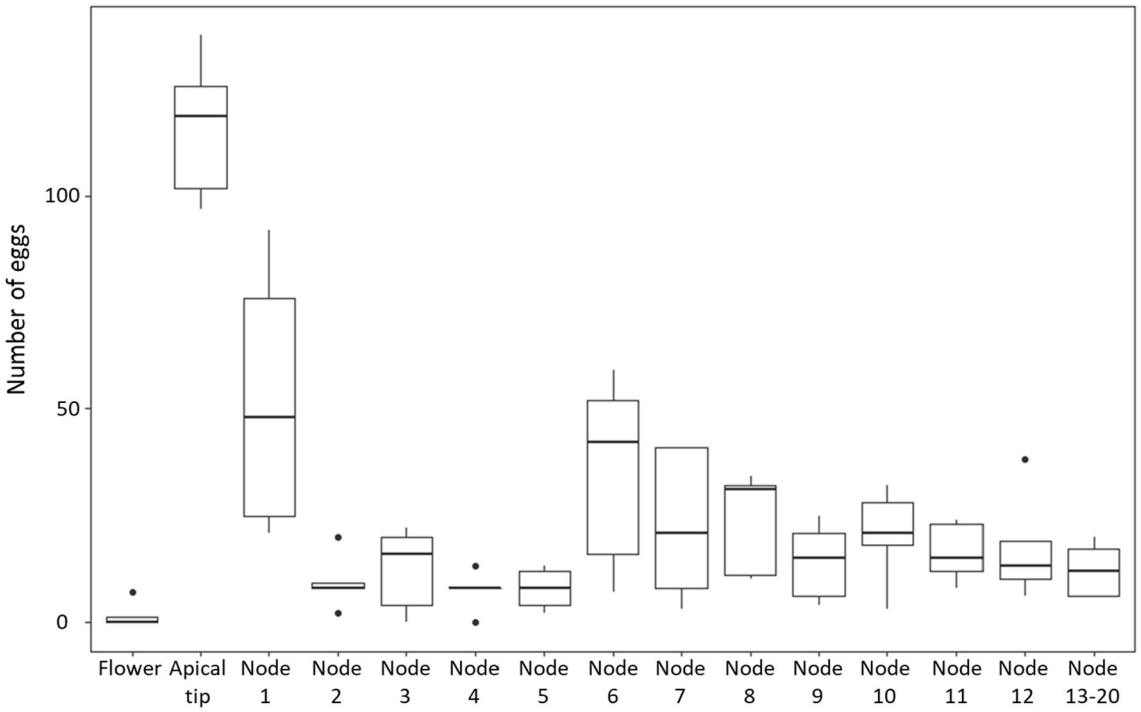

Fig. 4. Boxplot of the oviposition site of *H. natans* on *Cabomba caroliniana*. Nodes are numbered based on their increasing distance from the apical tip, with Node 1 being closest to the apical tip. Node 13–20 represents total number of eggs laid from Node 13 through to Node 20. The box plot comprises the median line, interquartile range from 25th to 75th percentile (the bounding box), the minimum (25th percentile − 1.5*interquartile range) and maximum whiskers (75th percentile + 1.5*interquartile range) and outliers (the circles beyond the whiskers).

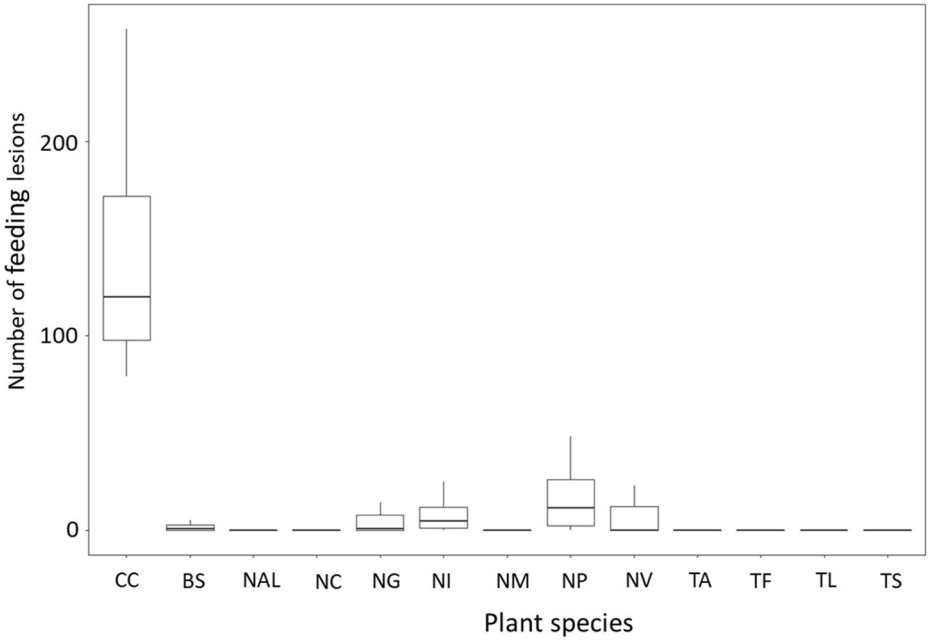

Fig. 5. *Hydrotimetes natans* adult feeding damage on *Cabomba caroliniana* (CC) compared with that on non-target plant species in no-choice trials (BS – *Brasenia schreberi*, NAL – *Nymphaea alba*, NC – *N. caerulea*, NG – N. *gigantea*, NI – *N. immutabilis*, NM – *N. mexicana*, NP – *N. pubescens*, NV – *N. violacea*, TA – *Trithuria austinensis*, TF – *T. fitzgeraldii*, TL – *T. lanterna*, TS – *T. submersa*). The box plot comprises the median line, interquartile range from 25th to 75th percentile (the bounding box), the minimum (25th percentile − 1.5*interquartile range) and maximum whiskers (75th percentile + 1.5*interquartile range) and outliers (the circles beyond the whiskers).

were observed on *N. caerulea, N. alba* and *N. mexicana, T. lanterna, T. austinensis, T. submersa* and *T. fitzgeraldii*. The logistic regression analyses revealed that *H. natans* is far less likely to lay eggs and complete its lifecycle on *Nymphaea* and *Trithuria* species than on *C. caroliniana* (Table 1). Probability of oviposition and lifecycle completion by *H. natans* on *Nymphaea* and *Trithuria* is 0% compared to 100% in *C. caroliniana*.

Adult feeding, oviposition and lifecycle completion by *H. natans* were observed on *B. schreberi*. The intensity of adult feeding was significantly lower on *B. schreberi* than on *C. caroliniana* ($F_{12,61} = 5.72$, $p \ll 0.01$) (Fig. 5). Oviposition was observed on four of the six replicates tested. Larval feeding was observed in three replicates, but development of larvae through to pupation and lifecycle completion was only recorded on one of these replicates. The logistic regression model fitted suggested that the likelihood of oviposition by *H. natans* on *B. schreberi* was not significantly different to that on *C. caroliniana*, but the odds for

Table 1
Summary statistics of logistic regression analyses of the no-choice trials with *Cabomba caroliniana* as a reference species. Statistical significance comparing the response on test plant species relative to *C. caroliniana* as the reference species was evaluated by a *t*-test of the estimates (the difference in regression coefficient between the test plant and the reference). (Abbreviations for test plant species: BS – *Brasenia schreberi*, NAL – *Nymphaea alba*, NC – *N. caerulea*, NG – N. *gigantea*, NI – *N. immutabilis*, NM – *N. mexicana*, NP – *N. pubescens*, NV – *N. violacea*, TA – *Trithuria austinensis*, TF – *T. fitzgeraldii*, TL – *T. lanterna*, TS – *T. submersa*).

Test plant species	Oviposition		Larval development		Pupation		Lifecycle completion	
	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value
BS	−2.120	0.233	−4.007	<0.05	−4.007	<0.05	−4.007	<0.05
NAL	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NC	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NG	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NI	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NM	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NP	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
NV	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
TA	−4.654	<0.05	−4.654	<0.05	−4.654	<0.05	−4.654	<0.05
TF	−4.905	<0.05	−4.905	<0.05	−4.905	<0.05	−4.905	<0.05
TL	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05
TS	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05	−5.273	<0.05

larval development, pupation and lifecycle completion were significantly lower, 0.57%, 0.14% and 0.14% respectively, relative to 1.0% in *C. caroliniana* (Table 1).

3.2.4. Choice and continuation trials using live plants

In choice trials, oviposition and some larval development by *H. natans* was recorded on *B. schreberi* but lifecycle completion was not. The results of a binomial test showed that the probability of lifecycle completion by *H. natans* on *B. schreberi* is 0% compared to 100% in *C. caroliniana*. There was a decrease in the probability from oviposition (40%) through to pupation (20%) and lifecycle completion (0%) in *B. schreberi* (Fig. 6). The number of pupae recorded from *B. schreberi* was 0.40 ± 0.40 (mean ± SE), which is significantly lower (t = -3.93; p < 0.05; 95% conf. interval = -16.24, −2.96) than that recorded from *C. caroliniana* (10.00 ± 2.41).

Continuation trials revealed that *B. schreberi* could not sustain a population of *H. natans*. Oviposition was observed on three replicates and larval development and pupation was observed on one replicate; a single pupa was recorded on this replicate and an adult emerged from this pupa in continuation trials.

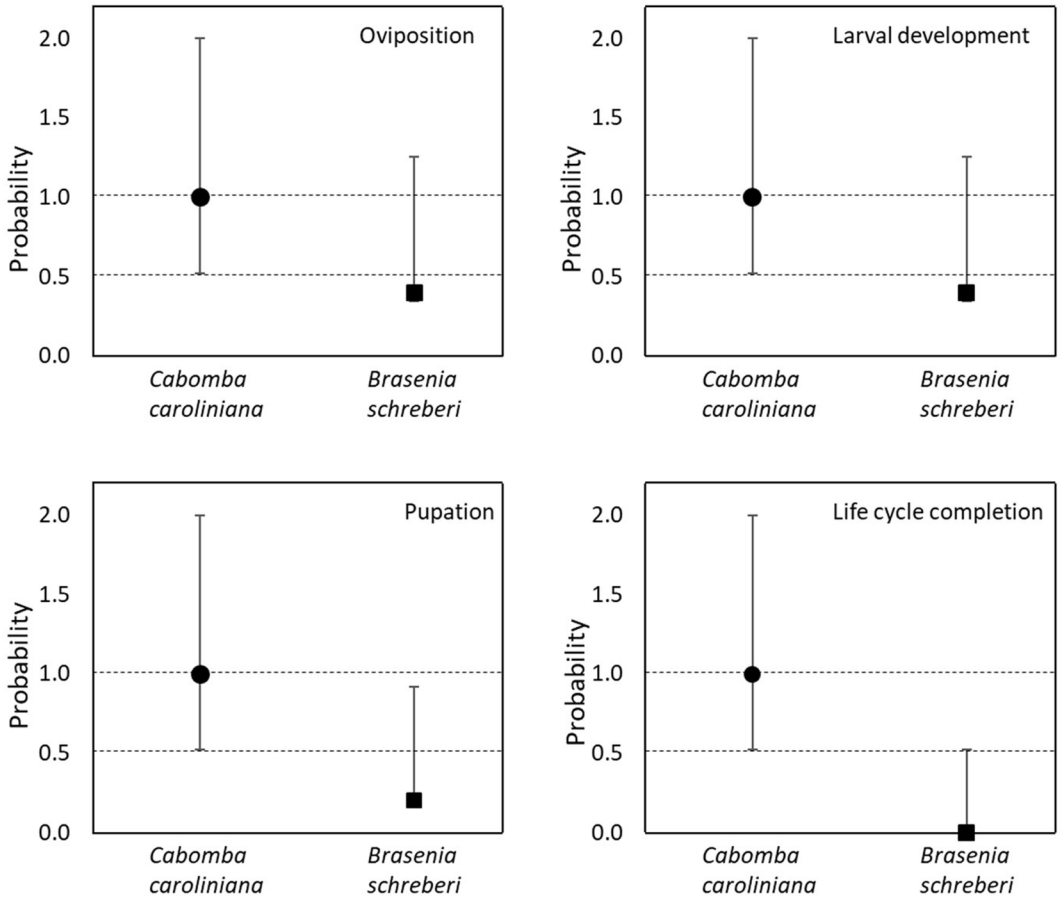

Fig. 6. Probability of successful oviposition, larval development, pupation and lifecycle completion by *Hydrotimetes natans* on *Cabomba caroliniana* and *Brasenia schreberi* under choice conditions. Error bars represent upper and lower 95% confidence intervals.

4. Discussion

4.1. Biology of Hydrotimetes natans

The results from the field surveys and a suite of laboratory trials revealed previously unknown lifecycle parameters of *H. natans* such as preoviposition period, oviposition site, egg viability, fecundity, feeding behaviour, development time and adult longevity. These details supported preliminary observations on longevity and total development time (Cabrera-Walsh et al., 2011). The development time of *H. natans* was relatively similar to the Salvinia weevil *Cyrtobagous salviniae* Calder & Sands (36 days) and shorter than the Water Hyacinth weevil, *Neochetina bruchi* Hustache (70 days) (DeLoach and Cordo, 1976; Sands et al., 1986).

The observations on lifecycle, fecundity and reproductive behaviour of *H. natans* offered significant insights to establish a laboratory colony, and will also assist in developing mass-rearing, release and field evaluation protocols. Adults lived for ~ 8 months and remained reproductively active for ~ 7 months. Nonetheless, their oviposition pattern was intermittent and age-dependent as in other weevil species (Forno et al., 1983; Sands et al., 1986; Zou et al., 2004; Eisenberg et al., 2018). Therefore, adults were frequently exposed to *C. caroliniana* to maximise the oviposition for laboratory rearing and colony establishment. Although the lifetime fecundity of *H. natans* is almost identical to *C. salviniae* (Sands et al., 1986), the reproductive potential seems to be low. The low survival rate of immature stages of *H. natans* (egg: 56%, larvae: 51%, pupae: 58%) compared to *C. salviniae* (egg: 72%, larvae: 77%, pupae: 75%) possibly impacted its reproductive potential in laboratory conditions (Sands et al., 1986). Additional studies are needed to shed light on the effect of abiotic factors (e.g. temperature) and host plant quality on survival of immature stages.

Hydrotimetes natans larvae inflicted greater damage than adults on *C. caroliniana*. First instar larvae fed through the leaves and the petiole and tunnelled through the main stem as they develop into later instars. Tunnelling caused heavy damage to the foliage and stems, and tissue necrosis was apparent as a result. Multiple larvae of different instars have been observed feeding in the same section of a stem; behaviour analogous to *Neochetina* spp. (DeLoach and Cordo, 1976). Under intense larval feeding, stems disintegrated and detached from plants, and subsequently decayed. As *C. caroliniana* primarily reproduces through healthy viable stems fragmenting from the parent plant (Mackey and Swarbrick, 1997; Xiaofeng et al., 2005), larval damage may negatively affect the recruitment and spread of *C. caroliniana*. Adult feeding does not appear to inflict major damage except localised necrosis around the feeding lesions.

4.2. Host-specificity of Hydrotimetes natans

Quarantine-based host-specificity studies predict the physiological host range of biological control agents by evaluating the relative suitability (i.e. in comparison to the target weed) of a related set of non-target plant species, for feeding, oviposition, development and life cycle completion (Schaffner, 2001; Sheppard et al., 2005). These studies are designed to conservatively evaluate the risks under worst case scenarios (e.g. no-choice trials) (Briese, 2005). In our studies, *H. natans* neither fed nor oviposited on *Trithuria* species. Only exploratory feeding by adults was observed on *Nymphaea* species; no oviposition was ever recorded on these species. These non-target species perhaps lack chemicals that serve as olfactory stimulants eliciting oviposition by *H. natans* (Städler et al., 2002).

Brasenia schreberi was the only other test plant species that *H. natans* accepted for oviposition and development. However, given the significantly lower odds for elicitation of oviposition and development than on *C. caroliniana*, we predict *B. schreberi* is a physiologically unsuitable host. We considered the risks posed by *H. natans* to *B. schreberi* to be negligible or low in an Australian context for the following reasons. First, *B. schreberi* did not sustain a population of *H. natans* in continuation trials. Second, *B. schreberi* is primarily a high latitude species (Lloyd and Kershaw, 1997), whereas bioclimatic models predict a more subtropical and tropical distribution for *H. natans* (Kriticos et al., 2021). Therefore, only negligible risks are predicted to *B. schreberi* populations that tend to be more abundant in temperate freshwater bodies in south-eastern Australia. Third, *B. schreberi* is of minor importance as an aquarium or ornamental species. Finally, in a global context, *B. schreberi* is not endemic to Australia and has a native range spanning the Americas and the Old World (Kim et al., 2008).

The high degree of host-specificity of *H. natans* is not surprising given its phylogeny and the characteristics it shares with other weevils in the Erirhininae (marsh weevils). Weevils, both pests and biological control agents in this subfamily are either monophagous or oligophagous (Legalov, 2020). Notable examples include the biological control agents *N. eichhorniae* Warner and *N. bruchi* (on Water Hyacinth agents), *Euhrychiopsis lecontei* Dietz (Watermilfoil agent), and *C. salviniae* (Salvinia agent), and monophagous pests such as the Rice Water weevils *Lissorhoptrus oryzophilus* Kuschel. and *Afroryzophilus* spp. The biological control agents in the Erirhininae have proven to be highly host-specific and extremely effective in managing aquatic weeds (McFadyen, 1998; Hinz et al., 2020).

Based on the host-specificity results, permission to release *H. natans* into Australian aquatic systems was granted in 2021 by the Australian Department of Agriculture, Water and the Environment. There has not been a successful use of a weevil on submerged weeds thus far even though it has been considered for *Hydrilla verticillata* (L.f.) Royle and *M. aquaticum* (Oberholzer et al., 2007; Center et al., 2013). Using *H. natans* for the biological control of *C. caroliniana* marks one of a very few attempts of submerged weed control using natural enemies.

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Ethical approval

There are no ethical concerns regarding the organisms and the topic of the research.

Availability of data and material (data transparency)

Not applicable.

Code availability (software application or custom code)

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.104894.

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