Marking bark beetle parasitoids within the host plant with rubidium for dispersal studies

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Abstract

A technique using rubidium chloride (RbCl), a trace element, as internal label is proposed for marking hymenopteran parasitoids attacking a concealed host, *Ips typographus* L. (Coleoptera: Scolytidae). RbCl was introduced directly into spruce via the vascular system using glass tubing. RbCl passed through the food chain and was detected at the parasitoid level by electrothermal atomic absorption spectroscopy (EAAS) or flame emission spectroscopy (FES). Mark persistency until day 8 after emergence was tested in labelled *Rhopalicus tutela* (Walker) (Hymenoptera: Pteromalidae). The mark decreased with time only in fed females, probably due to excretion and/or egg resorption. The proportion of marked males did not vary with time or treatment.

Introduction

Insect marking is useful in many types of ecological studies. Marking techniques differ according to whether the identification of individuals or a whole insect population is desired. Based on the intrinsic nature of the marker itself, marking methods can be divided into nine categories: tags, mutilation, paints and inks, dusts, dyes, radioisotopes, pollen marking, genetically engineered marking, and elemental enrichment (Hagler & Jackson, 2001). To be useful, markers must be easy to apply, environmentally safe, persistent, and without consequences for the biology or the behaviour of the insects. Elemental enrichment using a trace element such as rubidium or strontium has been extensively studied because it meets these expectations (see Stimmann, 1991 and Hagler & Jackson, 2001 for a review of the advantages and disadvantages of rubidium). RbCl can be introduced into a host plant by soil treatment or foliar spray (see references below) and could therefore be used to mark a large number of native insects without handling. As the mark is only detectable by the use of special techniques after capture, it does not affect vulnerability to predation (unlike external marking, for example) (Hagler & Jackson, 2001). Finally, because of its close chemical similarity to potassium, RbCl does not present the safety hazard to workers and the environment that radioisotopes do (Akey, 1991).

The use of RbCl as an internal marker was first described by Berry et al. (1972) working on the cabbage looper moth, *Trichoplusia ni* (Hübner). It was subsequently developed for several other lepidopteran species (Stimmann, 1974; Graham & Wolfenbarger, 1977; Graham et al., 1978; Van Steenwyk et al., 1978; McLean & Laks, 1985; Fleischer et al., 1989; Knight et al., 1989; Johnson & Reeves, 1995), and for insect species in other orders such as Coleoptera (Wolfenbarger et al., 1982), Hemiptera (Fleischer et al., 1986), and Homoptera (Frazer & Raworth, 1974). Most often, the label is introduced into the insect via the host plant by foliar sprays (Berry et al., 1972; Stimman, 1974; Graham et al., 1978; Wolfenbarger et al., 1982; McLean & Laks, 1985; Fleischer et al., 1986; Jackson et al., 1988) or via RbCl-enriched artificial diets (Graham & Wolfenbarger, 1977; Van Steenwyk et al., 1978; Jackson et al., 1988; Knight et al., 1989; Johnson & Reeves, 1995). Some studies have been conducted on the systemic introduction of RbCl into the host plant including trees: i.e., Douglas firs,

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pecans, and pin oaks, in order to mark lepidopteran species (see Fleischer et al., 1991 for a review). However, insects living inside host plant tissues, such as phloem feeders, have been less studied regarding this technique: to our knowledge, there have been only two studies undertaken, in Louisiana, on the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae) by Bridges et al. (1989) and Thoeny et al. (1992). Even less studied has been the use of rare elements to label natural enemies. Most studies dealing with the marking of entomophagous insects, mainly predators, have been carried out by externally treating the prey's host plant with RbCl solution sprays, or by adding the markers to an artificial diet (Jackson et al., 1988; Jackson, 1991). Although some data on the transfer of RbCl through tri-trophic levels exist (Payne & Wood, 1984; Thoeny et al., 1992), this is the first study investigating how parasitoids of xylophagous insects can be marked via the first trophic level: the host-tree.

The parasitoid complex associated with *Ips typographus* L. (Coleoptera: Scolytidae), a well-known pest of spruce forests in Eurasia, includes mainly solitary hymenopteran ectoparasitoids attacking the late instar of the bark beetle. Although they are quite widespread, these parasitoids do not often achieve high levels of parasitism on this host, and do not appear to be a factor that determines its population density (Mills, 1983 and references therein; Eck, 1990; Hougardy & Grégoire, 2003). Dispersal, host-finding capacities, reproductive success, niche partitioning, and competition are obviously key elements for explaining this phenomenon, but they are still poorly known. As preparation for further studies of the dispersal abilities of these parasitoids under natural conditions, this study seeks to develop a reliable technique for labelling these insects to be used in future mark-capture experiments.

Using the two most common techniques for RbCl analyses – Electrothermal Atomic Absorption Spectrometry (EAAS) and Flame Emission Spectrometry (FES) (see Akey & Burns, 1991 for a discussion of the available analytical methods for elemental determinations in biological samples), we first tested the reliability of these marking techniques comparing parasitoid wasps emerging from a RbCl-treated tree and an untreated one. Then, as RbCl was eliminated from marked adult insects by excretion, oviposition, and mating (Van Steenwyk, 1991 and references therein), we compared the mark's persistency in labelled parasitoids kept with food or starved for 2, 4, 6, or 8 days after their emergence from the labelled tree. Hymenopteran species considered here are those most frequently found in southern Belgium: *Coeloides bostrichorum* Giraud (Braconidae) and *Rhopalicus tutela* (Walker) (Pteromalidae) (Hougardy and Grégoire, unpubl.). Another natural enemy appears in this study: the adult endoparasitoid *Ropalophorus clavicornis* (Wesmael) (Hymenoptera: Braconidae).

Materials and methods

We tested a technique modified from those of Bridges et al. (1989) and Thoeny et al. (1992). In July 1999, a standing tree (diameter at breast height: 26 cm) was selected in a spruce plantation in southern Belgium. Before any treatment, four circular inner bark tissue samples (1 dm^2) were punched out at breast height around the trunk for phloem analysis. Ten holes (14 mm in diameter and ≈ 3 cm deep) were drilled horizontally around the base of the tree, 30 cm above ground level. A piece of glass tubing (14 mm external diameter), bent at a 90° angle, was pushed into each hole in such a way that one part was horizontal (aligned on the hole) and the other part was vertical. The horizontal part of each tube was 4.5 cm long; the vertical part was 11 cm long. Each tube was filled with 10 ml of an aqueous RbCl (Sigma-Aldrich, Bornem, Belgium) solution, amounting to a total of 45 g RbCl. Transfusion occurred on 12 July 1999. Care was taken to seal the tube hermetically into the holes with modelling clay. Tubes were removed after 3 days, when they were empty. Five weeks later, another group of four circular inner bark tissue samples was punched out at breast height around the trunk. Bark samples were kept in the freezer until analysis for RbCl. The tree was then felled and cut into 50 cm logs.

In order to test whether *I. typographus* and its parasitoids accumulated a significant quantity of RbCl in their bodies, we used the following procedure. Half of the RbCl-treated logs were brought back to the laboratory and exposed to laboratory-reared insects in order to produce RbCl-marked *I. typographus* and *R. tutela.* The remaining logs were moved to an attacked site located in southern Belgium, and attacks by *I. typographus* were induced by stapling a pheromone (Pheroprax®, Cyanamid-Agro, Gembloux, Belgium) dispenser onto the log pile. Once natural parasitism, mainly by *R. clavicornis* and *C. bostrichorum*, had occurred 8 weeks later, field-infested logs were brought back to the laboratory.

Background levels in *I. typographus* and *R. tutela* adults were analysed on insects collected from a culture maintained on untreated spruce logs in the laboratory for ≈ 10 generations. Control *C. bostrichorum* pupae and adults were provided by bark beetle infested bark collected in southern Belgium and stored at 2 °C until needed. No *R. clavicornis* was available for control. All insects, control and treated, were frozen at −18 °C until analysis.

RbCl levels in unlabelled *I. typographus* parents leaving treated logs after oviposition (≈ 20 days after the beginning of gallery construction) were analysed ('Reem' in Table 1)

Table 1 The mean concentration of RbCl (μg/g dry weight ± SD) as determined by Flame Emission Spectroscopy (FES) or Electrothermal Atomic Absorption Spectroscopy (EAAS), present in adults of *Ips typographus*, *Rhopalicus tutela,* and *Coeloides bostrichorum* (ectoparasitoids) as well as *Ropalophorus clavicornis* (endoparasitoid) following their development in spruce logs previously injected with 0 or 45 g of RbCl

Species	Spectroscopy	Test	N	RbCl concentration	Range
Ips typographus	FES	Treated	10	325.9 ± 145.0	128.0–570.2
		Control	10	5.8 ± 1.6	4.5–8.5
	EAAS	Treated	10	604.2 ± 194.5	255.8–896.0
		Control	10	7.0 ± 2.5	3.8–10.3
		Reem	8	342.3 ± 105.3	170.0–493.0
Rhopalicus tutela	FES	Treated	10 f	248.7 ± 88.5	125.0–409.8
		Control	10 f	19.0 ± 9.3	0.0–31.2
	EAAS	Treated	9 f	244.9 ± 141.3	121.3–575.4
		Control	9 f	53.7 ± 42.4	6.9–130.9
		Treated	10 m	251.7 ± 123.9	86.1–484.9
		Control	10 m	4.5 ± 3.5	0.9–13.1
		F1	3 f	33.3 ± 37.0	9.7–76.0
Coeloides bostrichorum	FES	Treated	8 pu	778.4 ± 216.3	408.6–1163.4
		Control	8 pu	17.8 ± 5.5	13.5–28.2
	EAAS	Treated	5 m	349.5 ± 126.0	213.6–490.9
		Control	5 m	11.4 ± 3.5	7.3–15.8
		Treated	1 f	551.9	
		Control	1 f	68.0	
Ropalophorus clavicornis	EAAS	Treated	5 f	19.24 ± 5.90	11.69–25.22
		No control			

N = number of individuals tested; f = females; m = males; pu = pupae; Reem = reemerging bark-beetles; F1 = *R. tutela* produced on untreated spruce by labelled parents (see text).

and compared with both control bark beetles and bark beetles that had developed on treated logs. In *R. tutela*, parasitoid offspring produced on non-treated spruce phloem by labelled parents emerged from the RbCl-treated logs were analysed ('F1' in Table 1). In *C. bostrichorum*, which is a diapausing species, pupae were analysed, in addition to male and female adults, when it appeared that they would not undergo metamorphosis.

The effects of age and feeding on marker retention were tested in *R. tutela*. Males and females that emerged from the lab-infested RbCl-treated logs were divided into two treatments, unfed and fed with a mixture of honey and sucrose. The mean RbCl concentration in fed and unfed labelled *R. tutela* was studied over time, 0, 2, 4, 6 and 8 days after adult emergence. In order to determine accurately the time of emergence, wasps were removed at the pupal stage from the logs and held in Petri dishes until adult emergence. Whenever possible, each group (combination of treatment, time, and sex) comprised five individuals. RbCl content was analysed using EAAS.

The first group of insects that emerged from the treated tree was analysed by FES using a Perkin-Elmer 2380 atomic absorption spectrometer. Subsequent analyses were conducted by EAAS, using a Varian GTA 100 spectrAA-604Z spectrometer. All samples were dried at 60 °C for 24–48 h and weighed. Sample preparation was adapted from Graham & Wolfenbarger (1977). Individual beetles and parasitoids were digested using a mixture (1 : 1) of H_2O_2 30% and HNO_3 65% (H_2SO_4 for FES) (Merck, suprapur quality) with a 10 : 1 (v : w) proportion to sample. Digestions were carried out at 62 °C for 2 h. Solutions were cooled down and, for FES, 0.25 ml of KCl (1%) was added. For EAAS analysis, solutions were filtered on Whatman GF/A glass microfibre filters. Each sample was then diluted to 2.5 ml with milli-Q water (Millipore) just prior to RbCl measurement. Plant tissue samples collected on the same day were ground together and 0.2 g of this homogenized material were prepared for EAAS analysis as described above, except that the digestion was microwave-assisted (Milestone 1200 Mega: 6 min at 250 W, 6 min at 400 W, 6 min at 800 W, 6 min at 300 W, and finally a 5 min ventilation period) and that final volume was set at 25 ml using milli-Q water. Six subsamples were then taken and analysed by EAAS.

Male and female parasitoids were considered separately because of a suspected major difference in dispersal behaviour. Protandric males wait for female emergence on the bark surface (Krüger & Mills, 1990). Mating could thus

occur without any major movement of the males. The mated females have to disperse in order to find susceptible hosts on newly infested trees. A distinction between the sexes was not made for the host *I. typographus* because parasitoids are presumed to develop indiscriminately on males and females. All *Ropalophorus* analysed were females.

Two thresholds for distinguishing labelled from unlabelled insects were compared: Stimmann's threshold (mean ± 3 SD of the control, see Stimmann, 1974) and the maximal value of the control. The data were log-transformed to obtain homoscedasticity among the samples. For each species, differences in treatments (RbCl-treated and control), analytical techniques (FES and EAAS), and sex when relevant were detected by univariate analysis of variance followed when needed by Student-Newman-Keuls multiple comparisons of means tests (significance level set at 0.05): two-way ANOVA (treatment, analytical technique) for *I. typographus*; three-way ANOVA (treatment, analytical technique, sex) for *R. tutela*; one-way ANOVA (treatment) for *C. bostrichorum* adults (sexes were combined, see Results) and for *C. bostrichorum* pupae. The effects of wasp age and feeding on mark disappearance were measured by regression analysis.

Results

Using the glass tubing technique, the infusion of RbCl into the tree was highly successful with the mean level of RbCl present in the phloem 5 weeks after the treatment being three times higher (747.2 ± 41.0 µg RbCl / g dry weight) than that observed in the untreated tree (233.2 ± 30.5 µg RbCl / g dry weight (t-test: $t = 24.6$, 10 d.f., $P < 0.001$). Similarly, a greater level of RbCl was present in *I. typographus* adults emerging from treated rather than untreated logs (Table 1), and the difference was highly significant whether RbCl was analysed by the EAAS or FES methods (two-way ANOVA, 'treatment': $F = 1309.9$, d.f. = 1, 36, $P < 0.001$). However, a closer look at the data revealed that EAAS was more sensitive than FES methods in detecting RbCl (two-way ANOVA, 'spectrometry': $F = 12.282$, d.f. = 1, 36, $P < 0.001$). Such a difference was observed between treated individuals but not between controls (two–way ANOVA, interaction between 'treatment' and 'spectrometry': $F = 5.509$, d.f. = 1, 36, $P = 0.025$). Beetles that spent several days boring into treated phloem (= 'Reem' in Table 1) incorporated the mark at significantly higher levels than the control beetles, though at significantly lower levels than beetles that developed on treated phloem (one-way ANOVA: $F = 452.693$, d.f. = 2, 25, $P < 0.001$).

Mean RbCl-concentrations differed significantly between treated and untreated *R. tutela* (Table 1) (three-way ANOVA, 'treatment': $F = 236.601$, d.f. = 1,52, $P < 0.001$). No difference between the results obtained by EAAS and FES was detected (three-way ANOVA, 'spectrometry': $F = 2.833$, d.f. = 1,52, $P = 0.098$) but higher levels of rubidium were detected in females (three-way ANOVA, 'sex': $F = 18.620$, d.f. = 1,52, $P < 0.001$) but only when untreated (three-way ANOVA, interaction between 'treatment' and 'sex': $F = 19.527$, d.f. = 1,52, $P < 0.001$). Parasitoid offspring produced on non-treated spruce phloem by labelled parents emerged from the RbCl-treated tree ('F1' in Table 1) contained significantly lower levels of RbCl than treated wasps, similar to unlabeled wasps (one-way ANOVA: $F = 12.934$, d.f. = 2, 18, $P < 0.001$), suggesting that the level of rubidium accumulated in each egg produced by a labeled female parasitoid is too low to be detected in the future parasitoid adult after development on an unlabeled host.

Coeloides bostrichorum adults were difficult to obtain as it appeared that they were diapausing. Only five males and one female were collected in both treatments (fed and unfed). For this reason, the sexes were combined for the analysis. There was a significant effect of treatment (Table 1, one-way ANOVA; 'treatment': $F = 79.812$, d.f. = 1, 10, $P < 0.001$). Pupae collected on RbCl-treated logs contained significantly more RbCl than untreated pupae (one-way ANOVA: $F = 683.135$, d.f. = 1, 14, $P < 0.001$).

Ropalophorus clavicornis attacked *I. typographus* adults. This endoparasitoid accumulated low levels of rubidium (Table 1), comparable to the background levels of *R. tutela* and *C. bostrichorum*. The egg remained inactive for several days. The parasitized host began boring into the phloem when the parasitoid began to develop and killed it. Levels of rubidium detected in *Ropalophorus* adults correspond to background levels plus some amounts accumulated by the host while boring on RbCl-treated phloem.

Using Stimmann's threshold (mean ± 3 SD of the control), 55% of the treated *R. tutela* females were not recognized as being marked while only 10% used the maximal value of the control (Table 2). Hopper & Woolson (1991)

Table 2 Threshold values in µg / g dry weight for scoring a positive response, i.e., the mean value of background levels ± 3 SD and the maximal value of background levels (max), and proportion of wasps (%) recognized as being marked according to these thresholds

	Mean + 3 SD		Max	
Species	value	% marked	value	% marked
Ips typographus	14.4	100	10.3	100
Rhopalicus tutela (males)	15.0	100	13.1	100
Rhopalicus tutela (females)	180.9	55	130.9	90
Coeloides bostrichorum	90.8	100	68.0	100

Table 3 Mean concentration of RbCl (µg/g dry weight ± SD) and proportion (%) of wasps marked according to the threshold based on the maximal value of background levels (see Table 1) 2–8 days after emergence from treated logs (also see Figure 1)

	Unfed males			Unfed females			Fed males			Fed females		
Age days	n	mean	%	n	mean	%	n	mean	%	n	mean	%
0	5	357.1 ± 74.6	100	5	778.3 ± 299.3	100	5	357.1 ± 74.6	100	5	778.3 ± 299.3	100
2	5	429.9 ± 141.2	100	5	374.1 ± 74.3	100	5	225.4 ± 125.5	100	5	391.6 ± 124.5	100
4	5	331.7 ± 234.5	100	5	516.6 ± 195.5	100	5	303.1 ± 253.5	100	4	209.4 ± 101.4	50
6	4	241.0 ± 161.4	100	6	382.5 ± 253.3	83	5	187.6 ± 86.5	100	5	401.7 ± 348.9	80
										10	141.8 ± 124.1	30

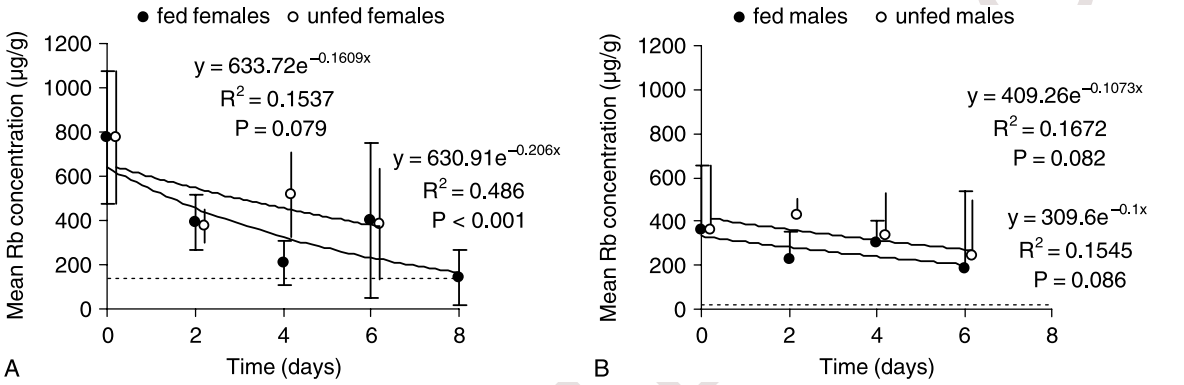

Figure 1 Mean RbCl concentration (µg/g dry weight ± SD) determined by EAAS in fed and unfed adult *Rhopalicus tutela* females (A) and males (B). Equation of trendlines, R^2 value and associated probability, threshold between labelled and unlabelled insects (maximal value of background levels – dotted line) have been added to the chart (see also Table 3).

also found that the maximal value of the negative control was more conservative than Stimmann's value because the concentration of RbCl in *Microplitis croceipes* (Cresson), a hymenopteran parasitoid of *Heliothis* spp., was not normally distributed. The distribution of our data was difficult to assess because of the small sample size. In *I. typographus* and *C. bostrichorum,* both thresholds were acceptable. We selected the maximal value of the control as the threshold to distinguish labeled from unlabeled *R. tutela* in the next experiment.

The effect of wasp age and feeding on mark retention in *R. tutela* was analysed by considering males and females separately because of the significant difference in mean concentration at emergence time (Table 3, day 0, males vs. females, t-test: t = – 3.053, d.f. = 8, P = 0.016). Regression analysis showed a significant decrease in marking with age only in fed females (Figure 1). However, the decreasing trend observed became insignificant if we only consider data collected from day 0 to day 6. Unfed insects rarely lived longer than 7 days. Proportions of wasps recognised as being marked (using the maximal value of the control as threshold) 2, 4, 6, and 8 days after emergence in both treatments are presented in Table 3. About 20% of the females lost the mark in both treatments after 6 days while the proportion of marked males did not vary with time or treatment. In this experiment, a significant difference in mean concentration at emergence time was detected between the sexes. In the previous experiment, a difference in this species was found only in background levels and not in treated individuals. Here, pupae of *R. tutela* were removed from the treated logs and kept in Petri dishes until emergence in order to control accurately the age of the adults. The RbCl levels found in these females kept in Petri dishes are much higher than levels found in females that were not removed from the logs and that emerged in the rearing cages (Table 1; EAAS). In this latter case, the wasps might have remained hidden in the cages and been collected only several days after their emergence, which would explain the lower RbCl level found in *R. tutela* treated females.

Discussion

The incorporation of rubidium using the glass-tubing technique is very efficient for marking insects living under the bark of trees as well as their parasitoids. The quantities of rubidium introduced into phloem tissues could be a key

factor in the success of this experiment because the RbCl level in the control tree was not insignificant: as RbCl uptake in plants is closely related to the soil RbCl content and is directly influenced by the soil chemical properties (Nyholm & Tyler, 2000), a great variability could be found between sites. Given that this experiment was preliminary and that the cost of marking (RbCl 99 + %: ≈ 100/50 g) and analyses (EAAS: ≈ 5€ per sample; FES: ≈ 4€ per sample) was relatively high, only one tree was treated in this study.

Various techniques were developed in elemental marking in tree systems using lower rates of rubidium: foliar sprays, stem well technique, pressurized syringes, low-pressure trunk injection, and flare root injection (see Fleischer et al., 1991). The technique developed here does not require any sophisticated equipment and gives efficient results. In Louisiana, Bridges et al. (1989) recorded elevated levels of RbCl in loblolly pine xylem and phloem, 1 week after injection (using a pressurized trunk injector) and the maximum mean RbCl concentration in phloem was recorded 5 weeks post-injection. The same period of 5 weeks between RbCl treatment and insect attacks gave significant results on spruces in southern Belgium.

EAAS (or GF-AAS: Graphite Furnace Atomic Absorption Spectrophotometry) is more sensitive than FES due to the methodology used for element analysis. Elimination of most interfering elements in the graphite furnace and analysis at the specific atomization temperature of the target elements results in a highly sensitive measurement. Despite its higher cost, EAAS is by far more common in elemental analysis due to its sensitivity and reproducibility. However, in elemental marking with rubidium, FES has been reported to be easy and sensitive (Bridges et al., 1989). In Bridges' study, the mean endogenous levels of RbCl in *D. frontalis* using FES was comparable to the results obtained similarly with EAAS by Thoeny et al. (1992): a mean of 4.6 ± 0.3 to 6.7 ± 0.3 µg RbCl/g dry weight (mean ± SEM) and 1.3 ± 0.2 to 2.2 ± 0.4 µg RbCl/g dry weight (mean ± SE), respectively. Our results with *I. typographus* adults using the two same techniques led to similar observations suggesting that FES is as sensitive as EAAS (Table 1).

The higher levels of rubidium in females may be due to selective incorporation of the marker into the ovaries and eggs. A sexual difference in the uptake of rubidium has also been observed in other species (Van Steenwyk et al., 1978; Fleischer et al., 1989; Knight et al., 1989) with higher levels of rubidium observed in females. The rubidium present in the eggs is probably excreted following egg resorption, when the females use the energy and materials obtained from the eggs to maintain themselves and to sustain oogenesis in the absence of hosts (Jervis & Kidd, 1996). This phenomenon is frequent in idiobiont hymenopteran parasitoids (Quicke, 1997) and could occur rapidly as the female is deprived of hosts. For example, egg resorption starts during the second day of host absence in *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (King, 1963). In our observations, feeding amplified the loss of rubidium (Figure 1).

Although the use of this internal marker could be of great help in mark-recapture experiments, some major aspects of the behaviour and biology of the spruce bark beetle parasitoids must still be studied to confirm the efficiency and stability of this marking technique and its limitations. Adult nutrition plays a major role in the biology and reproductive success of these wasps. These hymenopterans are synovigenic: the females emerge with a small number of large, nutrient rich eggs, and new eggs can be produced only if food is available. Furthermore, some species need a pre-oviposition period (Krüger & Mills, 1990) during which food (flower nectar, pollen, or honeydew) is vital for egg maturation. Attacked trees become attractive to females that have passed through this egg-maturation period. A previous study (E. Hougardy, unpubl.) has shown that the preoviposition period for *R. tutela* and *C. bostrichorum* lasts 3–5 days. If capture occurs just after pre-oviposition, when females start to become attracted by host odour (capture via infested logs, for example), their bodies will contain enough marker to be distinguished from unmarked insects. But if the time between release and capture increases, the probability of losing the label will also increase. The possibility of detecting the marker at this stage still requires further investigation. Increasing the amount of RbCl introduced into the host plant to a non-toxic level for the phytophagous insects and their natural enemies would probably balance the greater mark release observed in females. Thoeny et al. (1992) introduced 100 g RbCl per tree, which almost doubled the concentration of RbCl in *D. frontalis* as compared with individuals that developed on a tree treated with 50 g RbCl, without apparent toxic impact on the insects.

Another difficulty in mark-recapture experiments is that the percentage of recoveries must justify the cost of the marking and the analyses. Although substantial research efforts have been focused on this question (Pettersson et al., 2000, 2001a, b; Sullivan et al., 2000; Pettersson, 2001) no attractant as reliable as pheromones has been identified so far. Trapping might possibly be attempted using infested logs, coloured plates, glue traps, or Malaise traps, but this requires further testing considering the previous comment.

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