Eur. J. Entomol. **112**(3): 477–485, 2015
doi: 10.14411/eje.2015.054
ISSN 1210-5759 (print), 1802-8829 (online)

Evaluation of the diet of a carabid predator using fluorescent marking of prey

JAN OKROUHLIK[1,2,3] and PAVEL FOLTAN[1,2,3,*]

[1]Faculty of Science, University of South Bohemia, Branisovska 1760, 370 05 Ceske Budejovice, Czech Republic; e-mail: jan.okrouhlik@prf.jcu.cz

[2]i2LResearch Ltd., Lipova 1789/9, 370 05 Ceske Budejovice, Czech Republic; e-mail: pavel@i2LResearch.com

[3]Institute of Entomology, Biology Centre of the Czech Academy of Sciences, Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

Key words. Food web, prey marking, fluorescent dye, Coleoptera, Carabidae, *Pterostichus melanarius*, predator-prey interaction, gut content analysis

Abstract. We present a UV-fluorescent dye-based prey marking technique, using three different dyes, with later detection of the dyes in the guts of predators using a micro-plate fluorescent reader. By using this new method of detecting dyes based on their different excitation and emission characteristics it is possible to simultaneously screen 96 samples for multiple fluorescent-markers in less than 30 minutes. The marking is stable, cheap, non-toxic and had no effect on the choice of the carabid predator *Pterostichus melanarius* between marked and unmarked fly larvae (*Musca domestica*). Different fluorescent dyes provide variable detection intervals up to 100% detectability at 96 h post-ingestion by this predator. The simple marking, extraction and detection methods presented could be used in food web research to map individual trophic links and predator preference for different types of prey.

INTRODUCTION

Many different methods have been developed and used previously to study the diet of invertebrate predators and predator-prey interactions. Laboratory feeding trials have been shown to be inaccurate in predicting prey choice in the field (e.g. Rothschild, 1963; McKemey et al., 2003). Direct observation of predatory events in the field are impractical as both the predators and their prey are usually small, evasive and/or nocturnal (Hagler & Durand, 1994; Pfannenstiel & Yeargan, 2002). Visual gut content analyses were later replaced by immunological and more recently by PCR based approaches (reviewed in Symondson, 2002a, b; King et al., 2008) specifically identifying prey proteins or DNA in a sample of the gut contents of a predator. Even though these methods can provide accurate and valuable information on predator feeding habits their use is limited by cost and time/labour spent on sample extraction and analysis (PCR) or by a laborious and costly specific antibody development.

Different methods of marking prey for detection of predation have been developed. Labelling of prey with radioactive tracers (Baldwin et al., 1955; McDaniel et al., 1978; Grant & Shepard, 1985; Hamon et al., 1990) is rarely used, not only because of the expensive equipment and reagents required, but also because of the risk to health and the environment and the complicated process of obtaining permission for the use of radioactive elements in the field.

Several studies (i.e. Graham et al., 1978a, b, Johnson & Reeves, 1995, Prasifka et al., 2001; Kobelt et al., 2009) employed rubidium chloride marking of the prey and demonstrated reliable and long detectability (up to 8 days cited by Kobelt et al., 2009) of this metal in subsequently trapped predators, using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). Unfortunately, the advantages of this method are offset by the complicated machinery required and the very high cost of the ICP-MS analysis (Willbur, 2005).

Gibson et al. (1997) used neutral red marking for successfully recording predation by carabid beetles on an invasive New Zealand flatworm *Artioposthia triangulata*. However, unfortunately, there is no information on the stability or detectabilty of the dye and on possible effects of the dye on predator choice or predator/prey survival.

Marking prey using vertebrate immunoglobulin and their detection by ELISA (Hagler & Durand, 1994; Hagler, 2006, 2011; Buczkowski & Bennet, 2007; Mansfield et al., 2008; Lundgren et al., 2013; Williams et al., 2013) is currently the most frequently used method among the prey marking – predator gut analysis techniques. The main advantages of this method are that the antigens used for marking are readily available and the assays use relatively inexpensive and commercially available antibodies. The method can be modified for studies of more than one prey species, using more than one antigen, i.e. rabbit and chicken IgGs (Hagler, 2006). However, different antigens can be detected only by using a separate assay for each of the antigens, which increases the labour and cost per sample. As with other proteins, the IgGs are digested in the gut, which limits the use of this technique in food web tracking studies (Harwood et al., 2001; Calder et al., 2005).

A rocket red fluorescent dye (DAY-GLO colour division) was used by Hawkes (1972) for marking the eggs of the

* Corresponding author.

lepidopteran *Tyria jacobaeae* and detecting these in the gut of the European earwig, *Forficula auricularia*, by drying the guts in an oven and later identification using a portable UV light source in a dark room. However, the marking resulted in an anti-feeding effect (73% of non-dyed eggs were eaten compared to 44% of dyed eggs) and a last detection on day 3 post feeding, but only if the earwig did not feed again. Although, there is a large body of literature on the use of UV-fluorescent dyes in mark-release recapture studies (Medley, 1968; Cook & Hain, 1992; Hagler & Jackson, 2001; Foltan & Konvicka, 2008; Pechova & Foltan, 2008; Warner & Bierzychudek, 2009), as far as we are aware, there have been no further attempts to use UV-fluorescent dyes to mark prey for detecting predation.

In the present study, we introduce a UV-fluorescent dye based prey-marking technique, using three different dyes, with later detection of the dyes in the guts of predators. This detection technique is based on the different excitation and emission spectra of the dyes. The three dyes were selected from nine candidate dyes, based on their spectral characteristics measured in polyethylene glycol solution. The fluorescent characteristics of these dyes were confirmed using dye-fed and extracted fly larvae. To estimate the detection intervals of the dyes and possibility of detection when different dyes are used in multiple prey situations, a carabid predator was left to feed on dyed fly larvae. The dyes were then extracted and detected. To confirm that the dyes do not influence the beetle's choice of prey two-choice laboratory experiments with marked and unmarked prey were performed.

METHODS

Dyes used

Emission (375–675 nm, stepped by 5 nm) and excitation (350–600 nm, stepped by 5 nm) spectra of nine UV-fluorescent powder dyes (JST10 – Chartreuse, JST11 – Green, JST12 – Orange Yellow, JST15 – Red, JST16 – Cerise, JST17 – Pink, JST18 – Magenta, PS29 – Blue, JST43 – Orange, JST44 – Orange Red; Radiant colour N.V., Belgium) were measured using a RF-5301 fluorescent reader (Shimadzu, Japan). Ten percent w/w dye solution in polyethylene glycol (average molecular weight = 400; Sigma-Aldrich) was diluted in distilled water 1 : 1 (Foltan & Konvicka, 2008) and used for the analysis. Based on the different emission and excitation spectra and appropriate emission yields, JST10, JST18 and PS29 dyes were selected for further experimentation (see further details in the result section).

Animals tested

Pterostichus melanarius Illiger, 1798 (Coleoptera: Carabidae) beetles were collected from arable fields around Ceske Budejovice, Czech Republic. The beetles were kept individually in plastic containers (9.5 cm × 7 cm × 5 cm) half filled with moist peat and maintained in a controlled environment chamber under a 16L : 8D photoperiod and kept at 20 ± 1°C. The beetles were fed ad libitum on a diet of mixed pork and beef. Prior to the experiment, the beetles were starved for 7 days to ensure the same nutritional state in all individuals. All experiments were conducted under a 16L : 8D photoperiod at 20 ± 1°C.

A culture of the common house fly, *Musca domestica* L., 1758 was obtained from the Institute of Entomology in Ceske Budejovice. The larvae were maintained in 0.5 l plastic pots covered with fine netting and fed a diet consisting of 50 g of coarse grain feed mixed with 40 ml of skimmed milk.

Dye marking and extraction

Five grams of the dye was thoroughly mixed with 50 g of coarse grain before the addition of 40 ml of milk and fly larvae to the medium. The larvae were allowed to feed for 3 h on the diet. Subsequently, the larvae were separated from the diet, washed in a 2 : 1 (v/v) methanol:chloroform solution to remove external dye and remaining diet and left to dry for several seconds on a filter paper. They were then killed by placing them into liquid nitrogen and stored at −80°C until required. The same procedure was applied to control non-dyed larvae. Five larvae for each of the three dyes were dissected and the presence of the dye was checked using a portable UV-light source. Thawed larvae were weighed and used in the experiments.

In experiments requiring the extraction of dye, each sample was homogenised in a 1.5 ml microcentrifuge tube with 0.5 ml of methanol/chloroform solution (2 : 1, v/v). Samples were then centrifuged at 12,000 G for 5 min and 100 µl of the liquid supernatant were pipetted into individual wells of a 96- well black polypropylene micro-plate (NUNC), left to dry and loaded into a fluorescent multi-well reader as described below.

Preference testing

Two-choice laboratory experiments were conducted to determine whether the common generalist predator and scavenger *P. melanarius* preferred to eat either fly larvae dyed with one of the three UV-fluorescent dyes or non-dyed controls. The weight of each larva and each beetle used in the experiment was recorded before the experiment.

A single dyed larva was placed in a plastic Petri dish (7 cm diameter) lined with moist filter paper, together with a non-dyed control larva. The prey items were placed on opposite sides of the Petri dish, approximately 1.5 cm from the edge of the dish. Every subsequent Petri dish was turned 90° to prevent any effect of prey preference due to spatial orientation of the prey. Adult *P. melanarius* beetles (equal numbers of each sex) were placed individually in the middle of each dish and observed continuously for 30 min. The first attack by the beetle and feeding on the dyed or non-dyed larva were recorded as separate parameters. An attack was recorded when the beetle bit the prey. Feeding was recorded when biting occurred continuously for more than 10 s on any one prey. After 30 min, any non-feeding beetles were discarded and the experiment was repeated with a new beetle, until the final number of 40 replicates for each of the three dyes was reached (overall, 120 replicates).

To assess the effect of beetle weight, beetle sex, weight of dyed and non-dyed larvae and difference in weight between non-dyed larvae on beetle choice, binomial GLM regressions (link binomial) with preference as the dependent variable (and remaining variables as potential predictors) were conducted separately for response variables (attack and consumption) using the statistical software R (2008). The separate effects of all potential predictors on the beetle's choice were compared to the null model (that contained only the response variable); the potential decrease in deviance vs. model complexity was measured using the Akaike information criterion (AIC, Sakamoto et al., 1986).

Detectability of a single-dye-marked prey in the guts of predators

To test the detection interval of a single-dye marked prey in a predator's gut, single dyed larvae or non-dyed control larvae were offered to starved beetles (kept at 25°C, 12L : 12D light regime) and whether they fed or not over a 30-min period was recorded. Any beetles that did not feed were discarded. Separately for the

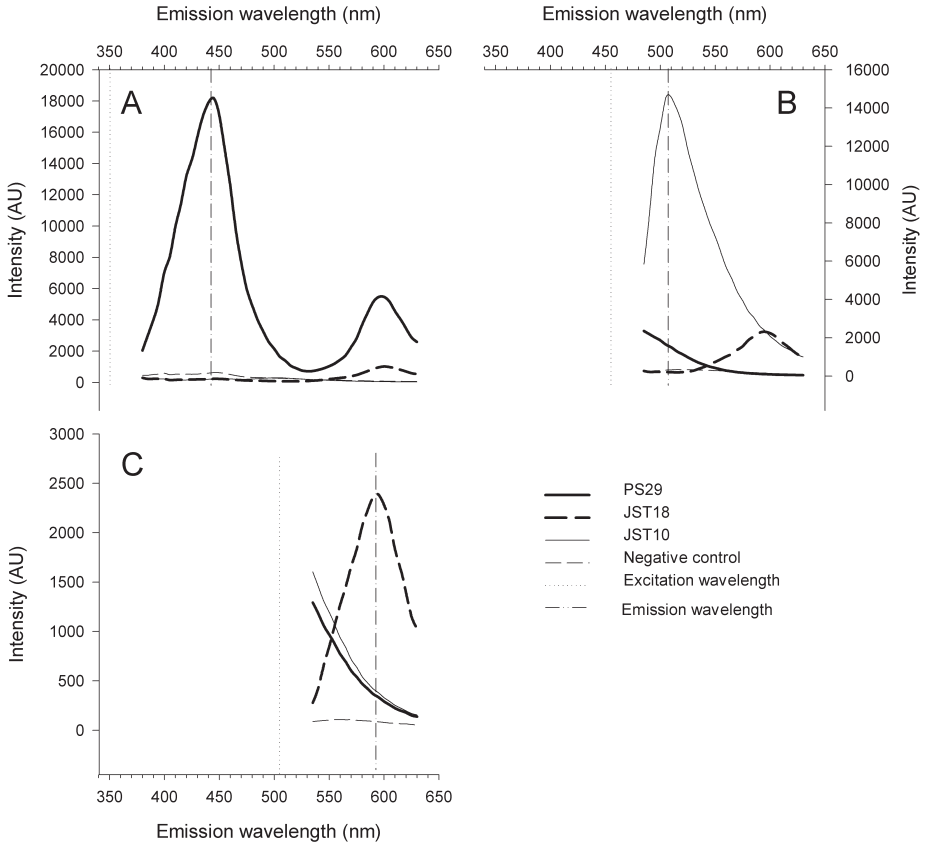

Fig. 1. Fluorescence spectra of dried methanol/chloroform (2 : 1) extracts of a single fly larva (N = 8) fed one of three fluorescent dyes (JST10, JST18 or PS29) after excitation with 350 nm (A), 455 nm (B) and 505 nm (C). Each of the dyes emitted maximal fluorescence at a specific wavelength (*specific fluorescence*) after excitation with a *specific excitation wavelength* at which the fluorescence emitted by the other dyes (*non-specific fluorescence*) has a very low intensity. Specific excitation/fluorescence wavelength of PS29 is 350 nm/442 nm (A); JST10 455 nm/505 nm (B) and JST18 505 nm/592 nm (C). The spectrum of a specific fluorescence with the lowest measured intensity is displayed together with the spectra of non-specific fluorescence and negative control (flies fed on a diet without dye) exhibiting the highest intensity measured. AU = arbitrary units.

three dyes tested and the negative control, 6 batches of 8 beetles (4 males and 4 females) were killed by freezing at 0, 12, 24, 48, 72 and 96 h post feeding and stored at −80°C. The weight of the individual larvae and beetles was recorded before feeding. Each beetle was thawed, decapitated and the remaining thorax and abdomen subjected to the dye extraction procedure.

The emission yield at the emission maximum (em. max. JST10: 455 nm, JST18: 505 nm, PS 29: 350 nm) was measured using a micro-plate fluorescent reader Infinite 200 (Tecan, Switzerland) at the excitation maximum (ex. max. JST10: 505 nm, JST18: 590 nm, PS 29: 445 nm).The emission yield of the controls was measured at the emission maxima corresponding to the three dyes, excited by the particular maximal excitation wavelength. The samples were considered dye-positive when the emission yield doubled the yield of the highest negative control sample.

To assess the effect of beetle weight, beetle sex and weight of dyed larvae on the maximal emission yield, a GLM regression (link gamma) with yield as the dependent variable (and remaining variables as potential predictors) was used, separately for each of the three dyes, using R (2008). An analogous analysis was conducted for the pooled data, where the dependent variable yield was replaced by the probability of detection (binomial link function). The potential decrease in deviance caused by particular predictors vs. model complexity was measured using AIC.

Preliminary multiple-dye distinction experiment

To test the potential of UV-fluorescent marking for identifying more than one prey item in a single gut sample, fly larvae were first marked with one of the three dyes, or were left unmarked for the negative control. Following this, extracts from larvae marked with each of the dyes were mixed in each of the possible pairwise combinations prior to detection. Finally, extracts from larvae marked with each of the dyes were mixed together so that all three dyes were present prior to detection. Each sample was scanned three times excited by optimal excitation wavelengths of the three dyes used and the whole emission spectra were measured starting with a wavelength 25 nm longer than particular excitation peaks. The above protocol was programmed into the micro-plate fluorescent reader and did not require any further assistance until all of the measurements were completed.

Each dye was identified based on their typical emission peaks. A blind trial was used to assess the credibility of the identification method using a researcher with no prior knowledge of the sample identity to sort the samples into appropriate categories.

Identification of differently marked prey in the guts of beetles

Starved beetles were fed two larvae simultaneously. Each larva was marked with a different dye (each pair combination of JST10, JST18 and PS29). The beetles were observed over a 30-min period and those that did not completely devour both larvae

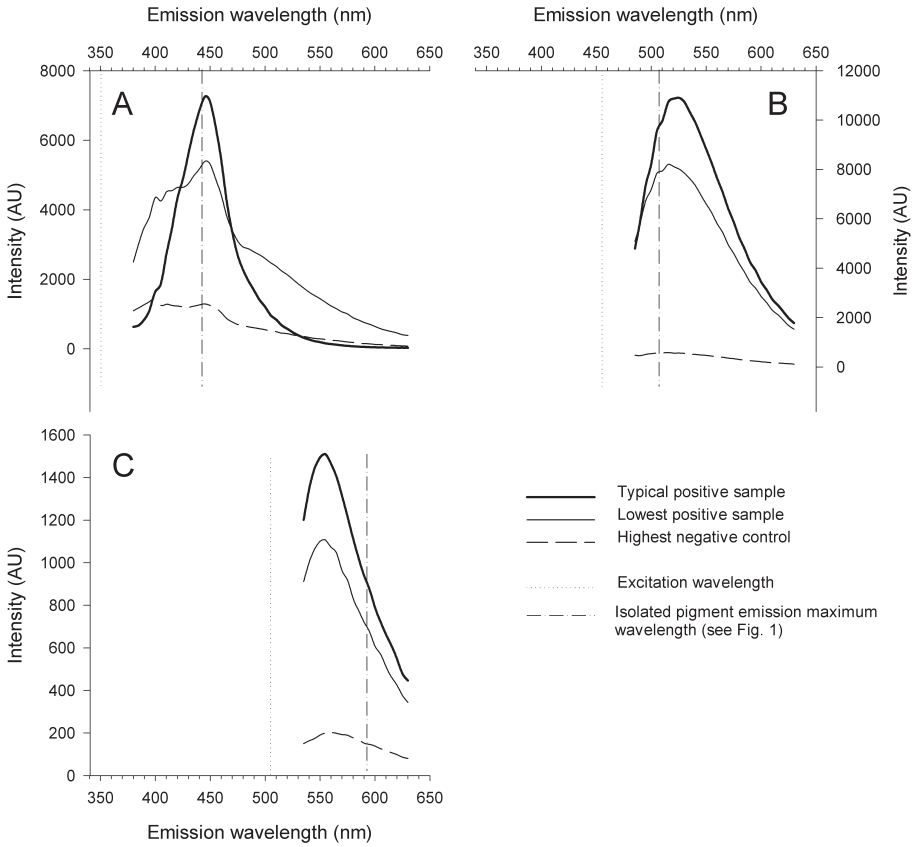

Fig. 2. Fluorescence spectra recorded for extracts of the gut of a carabid predator *Pterostichus melanarius*. The beetles were fed by a fly larvae marked with one of the three fluorescent pigments (A – JST10, B – JST18 or C – PS29) or by non-dyed control larvae. Batches of 8 beetles were killed at 0, 12, 24, 48 and 96 h post feeding. The extracts were excited using 350 nm (A), 455 nm (B) and 505 nm (C). Spectra of the samples with the lowest specific fluorescence considered positive (at least double the value of the highest negative control at the maximal emission wavelength) are displayed together with the negative control exhibiting the highest non-specific fluorescence and with a typical dye positive sample. AU = arbitrary units.

were discarded. Batches of 8 beetles (4 males and 4 females) fed each of the two-dye combinations were killed at 0, 48 and 96 h post feeding. Finally, batches of 8 beetles (4 males and 4 females) were killed at 0, 48 and 96 h after feeding on three larvae marked by the three different dyes (JST10, JST18 and PS29). The weight of the individual larvae and beetles was recorded before feeding. The extraction protocol and detection method were the same as used in the preliminary trial.

To confirm the presence of particular dyes in the sample and test for potentially false positive peaks when two or more dyes were present (the beetle gut can contain 0, 1, 2 or 3 dyes), the samples from the experiment in which single dyes were used, were checked for the presence of particular dye specific peaks by a researcher with no prior knowledge of the sample identity. PS29 created false positive peaks for JST10 in almost 50% of the samples. Similarly, JST18 created false positive peaks for PS29 in 12.5% of the samples. JST18 created false positive peaks for JST10 in less than 1.5% of the samples. After comparison of the particular JST10-false positives (actually marked by JST18), with proper JST10 samples, a difference in the shape of the peaks was identified. To reflect the different shape of the peaks, only those samples with 560 nm/535 nm emission yield ratio of > 1.3 were considered JST10 positive. Application of this rule resulted in no false positives recorded in the JST10 and JST18 samples. Only gut samples containing JST10 and JST18 marked larval remains were used for further analysis.

To assess the effect of beetle weight, beetle sex and weight of the dyed larvae on the probability of detection, binomial GLM regressions (link logit) with the probability of detection as the dependent variable (and remaining variables as potential predictors) were used, separately for each of the two dyes using R (2008). The potential decrease in deviance caused by particular predictors vs. model complexity was measured using AIC.

RESULTS

Dye selection and characteristics

The three dyes used (JST10, JST18 and PS29) were selected based on non-overlapping emission peaks and different excitation spectra characteristics. Each of the dyes emitted maximal fluorescence of a specific wavelength (emission maximum wavelength for JST10 = 505 nm, for JST18 = 592 nm and for PS29 = 442 nm) after excitation with a specific excitation wavelength (for JST10 = 455 nm, for JST18 = 505 nm and for PS29 = 350 nm) at which the fluorescence emitted by the two other dyes (non-specific fluorescence) had a very low intensity (Fig. 1). All other dyes tested exhibited overlapping emission peaks and usually more than one peak was present.

Table 1. Results of the GLM regression analysis used to separate the effects of potential predictors (weight of UV fluorescent marked larvae, weight of control larvae, difference in weights of the larvae, and sex and weight of the predatory carabid beetle, *Pterostichus melanarius*) on the preference of *P. melanarius* for fly larvae marked by one of three UV-fluorescent dyes (JST10, JST18, PS29) and negative controls (unmarked larvae).

Term	Df	Dev[1]	AIC[2]	F[3]	P[3]	Df	Dev[1]	AIC[2]	F[3]	P[3]
			JST10					*JST18*		
Null model		55.35	57.35				54.55	56.55		
Beetle sex	1	54.45	58.45	0.63	0.43	1	53.62	57.62	0.66	0.42
Beetle weight	1	53.63	57.63	1.22	0.28	1	54.51	58.51	0.03	0.87
Fluorescent larvae weight	1	53.32	57.32	1.45	0.24	1	53.14	57.14	1.00	0.32
Control larvae weight	1	54.19	58.19	0.81	0.37	1	49.12	53.12	4.20	<0.05
Difference in larvae weights	1	55.10	59.10	0.18	0.68	1	45.37	49.37	7.69	<0.01
			PS29							
Null model		53.84	55.84							
Beetle sex	1	53.84	57.84	0.00	1.00					
Beetle weight	1	53.72	57.72	0.09	0.77					
Fluorescent larvae weight	1	53.84	57.84	0.00	0.97					
Control larvae weight	1	53.74	57.74	0.07	0.80					
Difference in larvae weights	1	53.73	57.73	0.08	0.78					

[1]Residual deviance; [2]Akaike information criterion; [3]F-test of null vs. fitted model.

Preference testing

All of the fly larvae attacked by the beetles were consumed before they attacked the other larvae offered, thus attack and consumption were analyzed as identical variables. No significant feeding preferences were found between the control larvae and larvae marked by the fluorescent dyes for any of the three dyes [JST10 χ^2 (1) = 0, p = 1; JST18 χ^2 (1) = 0.529, p = 0.466 ; PS29 χ^2 (1) = 0.667, p = 0.414]. For JST10 and PS29 preference testing, no significant effects of potential predictors (beetle sex, beetle weight, weight of UV fluorescent marked larvae, weight of control larvae and difference in the weight of the larvae) on the beetle's choice of prey were recorded. In the case of JST18 testing, the weight of the control larvae and the difference in the weights of the larvae significantly influenced the beetle choice (heavier larvae were preferred). Due to the correlation between these variables, only the differences in larval weights were included in the final regression model, using a forward step selection and backward step elimination method (Table 1).

Detectability of a single-dye-marked prey in the guts of predators

Emission characteristics of the negative control samples with the highest emission yield of non-specific fluorescence and of the fluorescent dye marked sample with the lowest specific fluorescence considered positive for each of the three dyes tested are displayed in Fig. 2. For all three dyes, the specific emission yield significantly decreased with the time post-feeding at which the beetles were killed. None of the other predictors (beetle sex, beetle weight, larval weight) had a significant effect on the specific emission yield (Table 2).

As JST10 was always detected over the entire 96 h experimental period, the analysis of the effect of the potential predictors on its detectability was not necessary. For JST18 and PS29, the probability of detection significantly decreased with the time post-feeding and no other predictors (beetle sex, beetle weight, larvae weight) had a significant effect on the detectability of these dyes (Table 3). The fitted regressions for detectability of the particular dyes are presented in Fig. 3. A curve for the 100% detectable JST10 dye is not included.

Table 2. Results of GLM regression analysis used to separate the effects of potential predictors (time post-feeding, sex and weight of the predatory carabid beetle *Pterostichus melanarius*, weight of UV fluorescent marked larvae) on the specific emission yields of the three UV-fluorescent dyes tested (JST10, JST18, PS29). UV-fluorescent dye marked larvae were fed to *P. melanarius* beetles, batches of 8 beetles (4 males and 4 females) were killed 0, 12, 24, 48, 72, 96 h post-feeding and their gut samples analyzed.

Term	Df	Dev[1]	AIC[2]	F[3]	P[3]	Df	Dev[1]	AIC[2]	F[3]	P[3]
			JST10					*JST18*		
Null model		3.70	1233				180.37	1085.5		
Time	1	1.77	1199	50.15	$<10^{-8}$	1	95.47	1046.2	40.90	$<10^{-7}$
Beetle sex	1	3.70	1235	0.002	0.97	1	177.54	1086.1	0.73	0.40
Beetle weight	1	3.70	1235	0.0008	0.98	1	178.15	1086.4	0.57	0.45
Larvae weight	1	3.60	1233.7	1.19	0.28	1	178.81	1086.7	0.40	0.53
			PS29							
Null model		62.47	1109.5							
Time	1	33.61	1073.7	39.49	$<10^{-7}$					
Beetle sex	1	61.80	1110.7	0.50	0.48					
Beetle weight	1	60.36	1108.8	1.61	0.21					
Larvae weight	1	62.43	1111.5	0.0281	0.87					

[1]Residual deviance (for JST10 * 10^{12}); [2]Akaike information criterion; [3]F-test of null vs. fitted model.

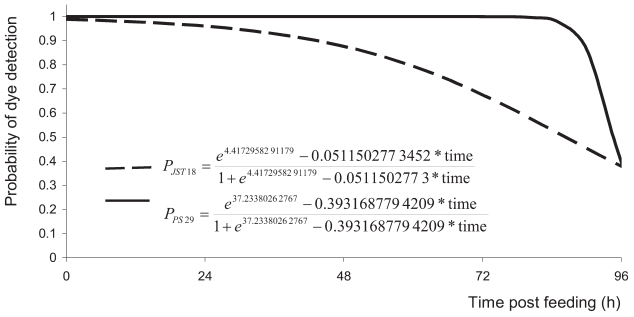

Fig. 3. Fitted regression lines for detectability of UV-fluorescent dyes (JST18, PS29) in the gut of a generalist carabid predator *Pterostichus melanarius* fed fly larvae marked with one of the three fluorescent pigments (JST10, JST18 or PS29). Batches of 8 beetles were killed at 0, 12, 24, 48, 72 and 96 h post feeding. As JST10 exhibited 100% detectability over the whole 96 h experimental period the results obtained using this dye were not analyzed.

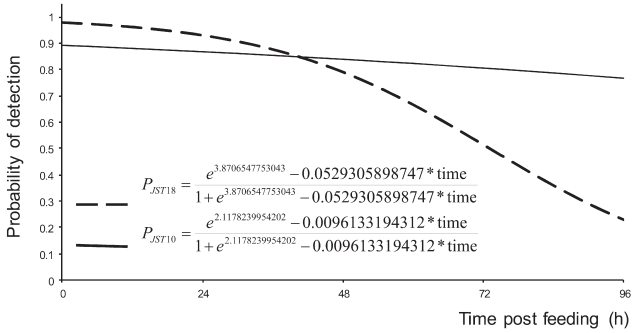

Fig. 4. Fitted regression lines for the detectability of UV-fluorescent dyes (JST10, JST18) in the gut of a generalist carabid predator *Pterostichus melanarius*. *P. melanarius* beetles were fed two larvae marked by two different dyes. Batches of 8 beetles were killed at 0, 48 and 96 h post feeding.

Identification of differently marked prey in the guts of the beetles

As all other combinations of dyes gave rise to false positives, only gut samples containing larvae marked with JST10 and JST18 were used in subsequent analyses. For JST18, the probability of detection significantly decreased with increase in the time post feeding. Fitted regression models for detectability of the two dyes are presented in Fig. 4. For both JST18 and JST10, the potential predictors beetle sex, beetle weight and larval weight did not have a significant effect on dye detectability (Table 4).

DISCUSSION

In the prey choice experiment, where fly larvae dyed with one of three UV-fluorescent dyes or non-dyed controls were offered to the carabid predator and scavenger *P. melanarius*, the beetles showed no preference for the control larvae or those marked with any of the three dyes. The beetle's choice of prey was controlled for by beetle weight, sex, the weight of the marked and unmarked larvae and their difference. The potential predictors did not have a significant effect except for a preference for heavier larvae in the case of JST18. In the study of Foltan & Konvicka (2008), identical dyes were used for injection marking of a slug (*Deroceras reticulatum*) to explore their potential in slug mark-recapture studies. To test whether a marked slug population was differently affected by predation compared to a non-dyed population, slugs were offered to *P. melanarius* in a cage arena choice experiment. No feeding preferences were recorded. Such results suggest that the marking of prey using the dyes tested does not influence the predator's choice of prey. This basic condition was not fulfilled in a previous study (Hawkes, 1972) or was often not considered (i.e. Gibson et al., 1997; Kobelt et al., 2009). Foltan & Konvicka (2008) further conclude that the injection of UV-fluorescent pigments dissolved in polyethylene glycol does not affect the survival over a period of 30 days post injection of any of the three species of slugs they tested. In our study, zero mortality of beetles that fed on fly larvae marked with each of the three types of dye, or combinations of these dyes, was recorded up to 96 h post-feeding. The UV-fluorescent pigments used are non-toxic, are naturally safe and are stable triazin-sulfonamid-formaldehyde polycondesation powders (Radglo®: safety sheet). These pigments have previously been used for tracking mammals (Mullican, 1988; Hruba, 1997) and frogs (Eggert, 2002) and apparently caused no harm to the animals coated in the UV-fluorescent powder. The powder that fell from the animals was used to track them with the aid of a portable UV lamp.

Marking prey using UV-fluorescent dyes resulted in a much longer detectability in the guts of predators compared to the recently most widely used technique of prey marking using vertebrate IgGs. The detectability of IgGs in the guts of predators usually ranges from 24 h (Mansfield

Table 3. Results of GLM regression analysis used to separate the effects of potential predictors (time post-feeding, sex and weight of the predatory carabid beetle *Pterostichus melanarius*, weight of UV fluorescent marked larvae,) on the probability of dye detection. UV-fluorescent dye marked larvae were fed to *P. melanarius* beetles and batches of 8 beetles (4 males and 4 females) were killed at 0, 12, 24, 48, 72, 96 h post-feeding and their gut samples analyzed. As JST10 exhibited 100% detectability over the whole 96 h experimental period the results obtained using this dye were not analyzed.

Term	Df	Dev[1]	AIC[2]	F[3]	P[3]	Df	Dev[1]	AIC[2]	F[3]	P[3]
			JST18					*PS29*		
Null model		39.88	41.88				32.08	34.08		
Time	1	24.7	28.07	30.23	$<10^{-6}$	1	10.59	14.59	93.40	$<10^{-12}$
Beetle sex	1	38.33	42.33	1.86	0.18	1	31.85	35.85	0.32	0.57
Beetle weight	1	38.89	42.89	1.17	0.28	1	31.40	35.40	0.10	0.32
Larvae weight	1	37.54	41.54	2.86	0.097	1	32.0	36.0	0.11	0.74

[1]Residual deviance; [2]Akaike information criterion; [3]F-test of null vs. fitted model.

Table 4. Results of GLM regression analysis used to separate the effects of potential predictors (sex and weight of the predatory carabid beetle *Pterostichus melanarius*, weight of UV fluorescent marked larvae) on the detectability of particular dyes (JST10, JST18). *P. melanarius* beetles were fed two larvae marked by two different dyes. Batches of 8 beetles (4 males and 4 females) were killed at 0, 48 and 96 h post feeding.

Term	Df	Dev[1]	AIC[2]	F[3]	P[3]	Df	Dev[1]	AIC[2]	F[3]	P[3]
			JST10					*JST18*		
Null model		21.62	23.62				30.55	32.55		
Time	1	21.17	25.171	0.47	0.50	1	18.42	22.42	14.49	$<10^{-3}$
Beetle sex	1	21.62	25.62	$<10^{-14}$	1.0	1	29.80	33.80	0.56	0.46
Beetle weight	1	20.86	24.86	0.81	0.37	1	30.35	34.35	0.15	0.71
JST10 larvae weight	1	21.35	25.35	0.29	0.60	1	27.69	31.69	2.27	0.14
JST18 larvae weight	1	21.17	25.17	0.48	0.49	1	30.25	34.25	0.22	0.64

[1]Residual deviance; [2]Akaike information criterion; [3]F-test of null vs. fitted model.

et al., 2008) to 72 h (Buczkowski & Bennett, 2007), but the detection probability decreases markedly with time. Beetle weight, beetle sex and weight of the larvae had no significant effect on detectability. The detection intervals of the different fluorescent dyes tested differed. Thus theoretically these dyes could be used for semi-quantitative estimates of prey consumption when prey are marked with two dyes that have different detection intervals (by eliminating false negatives and simultaneously providing information on the time of the predatory event). We assume that detection intervals for other fluorescent dyes may also vary. If that is the case, the method potentially enables the use of a single dye with a detection interval suitable for a particular purpose, i.e. confirming predatory links when the predators are not abundant or are difficult to sample (a dye with long detection interval), or for semi-quantitative estimates of prey consumption when the prey and predators are readily available at high densities (a dye with short detection interval). Concurrent marking with a fluorescent dye that is detectable over a long period of time with other marking methods (IgGs) might be used to calibrate marking methods in the field.

The principle of the detection method used for detecting single fluorescent-dyes practically excludes false positives, which is the major weakness of all ELISA based detection methods except those that use vertebrate IgGs, due to the potential of non-specific cross-reactivity (Symondson, 2002b; Mansfield et al., 2008). The method used for assessing the presence of a single dye in the gut of a predator is greatly simplified by measuring emission yield at a single maximal emission wavelength, whereas the sample is irradiated by a single maximal emission wavelength. This method enables evaluation of a sample within a few seconds. The detectability and accuracy of detection might be further increased by identifying the specific emission peaks of fluorescent dyes, as is apparent from Fig. 2, where the emission characteristics of the samples with the lowest emission yield considered positive are compared to negative control samples exhibiting the highest non-specific fluorescence at the emission maximum for particular fluorescent dyes.

The results of the preliminary multiple-dye distinction experiment suggest that the use of all three dyes in a single study to record consumption of three different types of prey is possible. Unfortunately, in the following experiment in which all combinations of dye marked larvae were fed to the predator and identified by a researcher unaware of the sample's identity, this hypothesis was compromised and only JST10 and JST18 were clearly differentiated (i.e. with no false positives) by employment of a method reflecting the specific shapes of the emission peaks.

The UV-fluorescent dye prey marking technique presented in this study enables fast, cheap and practical detection compared to other marking techniques currently used. In contrast to molecular or monoclonal antibody based gut content analyses, there is no need to design specific prey markers. Mixing the marker into the prey's diet, as in this study, has the effect that most of the marker is inside the prey and not exposed on surface structures of the prey, which reduces the possibility of false positives together with contamination of samples due to using different collection techniques (Greenstone et al., 2012; King et al., 2012). When researchers not equipped with a micro-plate fluorescent reader wish to use this method of marking, the gut samples can be desiccated, stored at ambient temperature on plates and sent for processing by laboratories possessing this equipment.

Although the advantages and potential of the marking method presented in this study are clear, there are further issues that must be addressed before employing it as a method for use in food web research in the field. Different types of predators (large and small, chewing and piercing, spiders) and prey, and marking techniques (for prey that do not consume the dye) should be examined. Detection intervals should be estimated at a finer scale and factors potentially influencing detection intervals (i.e. starvation of predator vs. presence of other food in the gut) should be tested. This marking method might be useful for experiments in the field or laboratory but not for screening predation of naturally occurring (unmarked) prey.

We propose that the method presented here might be of use in mapping trophic links, including predator-prey interactions, scavenging events or estimating the level of secondary predation in place of or in association with other methods of analysing gut contents. The possibility of using two different dyes in a study potentially enables direct measurement of prey selection in the field. We also propose that inclusion of UV-fluorescent dyes based on other different types of pigment may further increase the number of dyes that might be simultaneously used

in a single system. The methods for marking different invertebrates using fluorescent powders is well established for mark-release-recapture studies (Hagler & Jackson, 2001) and can be easily used for mass marking of prey that can be subsequently identified in the guts of predators, as presented in this study.

ACKNOWLEDGEMENTS. This study was supported by the Czech Science Foundation (grant no. 526/09/1249) and the Ministry of Education, Youth and Sports of the Czech Republic (grant no. 6007665801). The authors thank R. Neuzil for supplying the flies, S. Klementova and P. Kopacek for access to their equipment. Many thanks are due to the four anonymous referees for their comments, which greatly improved the quality of the manuscript. We would like to thank also Y. Moodley, P. McEwen, G. Small and A.F.G. Dixon for correcting our English.

REFERENCES

Baldwin W.F., James H.G. & Welch H.E. 1955: A study of predators of mosquito larvae and pupae with a radioactive tracer. — *Can. Entomol.* **87**: 350–356.

Buczkowski G. & Bennett G.W. 2007: Protein marking reveals predation on termites by the woodland ant, *Aphaenogaster rudis*. — *Insectes Soc.* **54**: 219–224.

Calder C.R., Harwood J.D. & Symondson W.O.C. 2005: Detection of scavenged material in the guts of predators using monoclonal antibodies: a significant source of error in measurement of predation? — *Bull. Entomol. Res.* **95**: 57–62.

Cook S.P. & Hain F.P. 1992: The influence of self-marking with fluorescent powders on adult bark beetles (Coleoptera: Scolytidae). — *J. Entomol. Sci.* **27**: 269–279.

Eggert C. 2002: Use of fluorescent pigments and implantable transmitters to track a fossorial toad (*Pelobates fuscus*). — *Herpetol. J.* **12**: 69–74.

Foltan P. & Konvicka M. 2008: A new method for marking slugs by UV-fluorescent dye. — *J. Mollusc. Stud.* **74**: 293–297.

Gibson P.H., Cosens D. & Buchanan K. 1997: A chance field observation and pilot laboratory studies of predation of the New Zealand flatworm by the larvae and adults of carabid and staphylinid beetles. — *Ann. Appl. Biol.* **130**: 581–585.

Graham H.M., Wolfenbarger D.A. & Nosky J.B. 1978a: Labeling plants and their insect fauna with rubidium. — *Environ. Entomol.* **7**: 379–383.

Graham H.M., Wolfenbarger D.A., Nosky J.B., Hernandez N., Llanes J. & Tamayo J. 1978b: Use of rubidium to label corn earworm and fall armyworm for dispersal studies. — *Environ. Entomol.* **7**: 435–438.

Grant J.F. & Shepard M. 1985: Techniques for evaluating predators for control of insect pests. — *J. Agric. Entomol.* **2**: 99–116.

Greenstone M.H., Weber D.C., Coudron T.A., Payton M.E. & Hu J.S. 2012: Removing external DNA contamination from arthropod predators destined for molecular gut-content analysis. — *Mol. Ecol. Resour.* **12**: 464–469.

Hagler J.R. 2006: Development of an immunological technique for identifying multiple predator-prey interactions in a complex arthropod assemblage. — *Ann. Appl. Biol.* **149**: 153–165.

Hagler J.R. 2011: An immunological approach to quantify consumption of protein-tagged *Lygus hesperus* by the entire cotton predator assemblage. — *Biol. Contr.* **58**: 337–345.

Hagler J.R. & Durand C.M. 1994: A new method for immunologically marking prey and its use in predation studies. — *Entomophaga* **39**: 257–265.

Hagler J.R. & Jackson C.G. 2001: Methods for marking insects: Current techniques and future prospects. — *Annu. Rev. Entomol.* **46**: 511–543.

Hamon N., Bardner R., Allen-Williams L. & Lee J.B. 1990: Carabid populations in field beans and their effect on the population dynamics of *Sitona lineatus* (L.). — *Ann. Appl. Biol.* **117**: 51–62.

Harwood J.D., Phillips S.W., Sunderland K.D. & Symondson W.O.C. 2001: Secondary predation: quantification of food chain errors in an aphid-spider-carabid system using monoclonal antibodies. — *Mol. Ecol.* **10**: 2049–2057.

Hawkes R.B. 1972: A fluorescent dye technique for marking insect eggs in predation studies. — *J. Econ. Entomol.* **65**: 1477–1478.

Hruba H. 1997: [*Spatial Activity of Dormice* (*Gliridae*) *within the Sandstone Rocks of the Labské Pískovce Protected Landscape Area*.] MSc. Thesis, University of South Bohemia, Ceske Budejovice, 19 pp. [in Czech].

Johnson P. & Reeves R. 1995: Incorporation of the biological marker rubidium in gypsy moth (Lepidoptera: Lymantriidae) and its transfer to the predator *Carabus nemoralis* (Coleoptera: Carabidae). — *Environ. Entomol.* **24**: 46–51.

King R.A., Read D.S., Traugott M. & Symondson W.O.C. 2008: Molecular analysis of predation: a review of best practice for DNA-based approaches. — *Mol. Ecol.* **17**: 947–963.

King R.A., Davey J.S., Bell J.R., Read D.S., Bohan D.A. & Symondson W.O.C. 2012: Suction sampling as a significant source of error in molecular analysis of predator diets. — *Bull. Entomol. Res.* **102**: 261–266.

Kobelt A.J., Yen A.L. & Kitching M. 2009: Laboratory validation of rubidium marking of herbivorous insects and their predators. — *Austral. J. Entomol.* **48**: 204–209.

Lundgren J.G., Saska P. & Honek A. 2013: Molecular approach to describing a seed-based food web: the post-dispersal granivore community of an invasive plant. — *Ecol. Evol.* **3**: 1642–1652.

Mansfield S., Hagler J.R. & Whitehouse M.E.A. 2008: A comparative study of the efficiency of a pest-specific and prey-marking ELISA for detection of predation. — *Entomol. Exp. Appl.* **127**: 199–206.

McDaniel S.G., Keeley L.L. & Sterling W.L. 1978: Radiolabeling *Heliothis virescens* eggs by 32P injection of adult females. — *Ann. Entomol. Soc. Am.* **71**: 432–434.

McKemey A.R., Symondson W.O.C. & Glen D.M. 2003: Predation and prey size choice by the carabid beetle *Pterostichus melanarius* (Coleoptera: Carabidae): the dangers of extrapolating from laboratory to field. — *Bull. Entomol. Res.* **93**: 227–234.

Medley J.G. & Ahrens E.H. 1968: Fluorescent dyes for marking and recovering fowl ticks in poultry houses treated with insecticides. — *J. Econ. Entomol.* **61**: 81–84.

Mullican T.R. 1988: Radio telemetry and fluorescent pigments – a comparison of techniques. — *J. Wildl. Manag.* **52**: 627–631.

Pechova H. & Foltan P. 2008: The parasitic nematode *Phasmarhabditis hermaphrodita* defends its slug host from being predated or scavenged by manipulating host spatial behaviour. — *Behav. Process.* **78**: 416–420.

Pfannenstiel A.S. & Yeargan K.V. 2002: Identification and diet activity patterns of predators attacking *Helicoverpa zea* (Lepidoptera: Noctuidae) eggs in soybean and sweetcorn. — *Environ. Entomol.* **31**: 232–241.

Prasifka J.R., Heinz K.M. & Sansone C.G. 2001: Field testing rubidium marking for quantifying intercrop movement of predatory arthropods. — *Environ. Entomol.* **30**: 711–719.

R Development Core Team 2008: *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. URL http://www.R-project.org

Rothschild G.H.L. 1963: The immature stages and biology of some mirid predators of Delphacidae, with notes on other predatory Heteroptera occurring in *Juncus* areas. — *Entomol. Mon. Mag.* **99**: 157–161.

Sakamoto Y., Ishiguro M. & Kitagawa G. 1986: *Akaike Information Criterion Statistics*. D. Reidel, Dordrecht, 290 pp.

Symondson W.O.C. 2002a: Molecular identification of prey in predator diets. — *Mol. Ecol.* **11**: 627–641.

Symondson W.O.C. 2002b: Diagnostic techniques for determining carabid diets. In Holland J.M. (ed.): *The Agroecology of Carabid Beetles*. Intercept, Andover, pp. 137–164.

Warner K.A. & Bierzychudek P. 2009: Does marking with fluorescent powders affect the survival or development of larval *Vanessa cardui*? — *Entomol. Exp. Appl.* **131**: 320–324.

Willbur S. 2005: *A Comparison of the Relative Cost and Productivity of Traditional Metal Analyses Techniques Versus ICP-MS in High Throughput Commercial Laboratories*. Agilent Technologies, Bellevue, 6 pp.

Williams L., Hagler J.R. & Tonkel K.C. 2013: Does dimethyl sulfoxide increase protein immunomarking efficiency for dispersal and predation studies? — *Entomol. Exp. Appl.* **148**: 275–286.

Received October 3, 2014; revised and accepted February 25, 2015
Prepublished online March 20, 2015