

## Role of surface chemical signals in egg cannibalism and intraguild predation in ladybirds (Coleoptera: Coccinellidae)

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**Summary.** The eggs of some ladybirds are known to be toxic to intraguild ladybird predators. However, this defence is of little value if the eggs are killed before their toxicity becomes apparent. The results presented in this paper indicate that chemicals on the surface of the eggs of two species of ladybirds signal the relative risk of cannibalism and intraguild predation. In *Adalia bipunctata* and *Coccinella septempunctata*, 87% of the chemicals are alkanes. Each species of ladybird is less reluctant to eat their own eggs than those of the other species. This asymmetry is to be expected because there is a greater risk to ladybirds from intraguild predation than cannibalism. Similar alkanes to those on the surface of the eggs of *A. bipunctata* are present in tracks left by larvae and on the elytra of the adults of this species. Those in the larval tracks deter females from ovipositing in patches of prey already being attacked by their larvae and those on the elytra are used in mate recognition. That different context dependent messages could be signalled by similar chemicals is an example of semiochemical parsimony.

**Key words.** Alkanes – intraguild predation – egg cannibalism – *Adalia bipunctata* – *Coccinella septempunctata*

### Introduction

Both in terms of intraguild predation and cannibalism the eating of eggs by ladybirds would appear to be advantageous in that it both supplies a source of food and removes potential competitors (Polis 1981). Although the disadvantages of consuming one's kin has been considered (Osawa 1992; Joseph *et al.* 1999) those associated with eating toxic eggs of other species, although well described (Agarwala & Dixon 1992), has been ignored. The larvae of *Adalia bipunctata* (L.) and *Coccinella septempunctata* L., which regularly co-occur in the field, are reluctant to eat conspecific eggs painted with water extracts of whole eggs of the other species,

and larvae of *C. septempunctata* are more likely to develop more slowly and/or die after eating a few eggs of *A. bipunctata* than *vice versa* (Agarwala & Dixon 1992; Hemptinne *et al.* 2000). However, the water extract of crushed whole eggs is likely to contain the species specific alkaloids that are known to be present in ladybird eggs (Pasteels *et al.* 1973). Some of these alkaloids are known to be toxic to vertebrates (Frazer & Rothschild 1960; Marples *et al.* 1989) and therefore it does not seem unreasonable to assume they are responsible for the toxicity of ladybird eggs. Starving adults of *Menochilus sexmaculatus* (F.) and *Coccinella transversalis* (F.) both consume their own eggs more readily than those of the other species, and *C. transversalis* is more reluctant to eat the eggs of *M. sexmaculatus* than *vice versa* (Agarwala *et al.* 1998).

Although the eggs of ladybirds are defended chemically such defence would be of little value if the eggs are killed before their toxicity becomes apparent. Therefore, it would be advantageous for both predator and egg if the toxicity of the latter could be flagged in some way. Close observation revealed that on encountering eggs larvae palpated their surface and then often quickly withdrew leaving the eggs intact. That is, whatever was responsible for a larva's change in behaviour appeared to be on the surface of the eggs, and therefore unlikely to be the species specific alkaloid.

Each species of ladybird prefers a particular habitat or range of habitats, and shows varying degrees of overlap in its preference(s) with other species (Honek 1985; Majerus 1994; Nedved 1999). Those species whose eggs and larvae regularly co-occur are the most likely to be well defended against one another. *C. septempunctata* shows a marked preference for herbaceous plants and although *A. bipunctata* is not so restricted its immature stages nevertheless frequently co-occur with those of *C. septempunctata* (Smee 1922; Banks 1955, 1956; Smith 1966; Radwan & Lövei 1982, 1983; Hemptinne & Naisse 1988; Hemptinne 1989; Barczak *et al.* 1996; Trouvé *et al.* 1996). As indicated above these two species are protected against predation by one another.

The following study was undertaken in order to test the hypothesis that substances on the surface of ladybird eggs afford them some protection from cannibal-

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ism and intraguild predation, and to identify these substance(s) in two species of ladybirds.

## Material and methods

### *Ladybird culture*

Two-spot ladybirds, *A. bipunctata*, were reared at  $15 \pm 1^\circ\text{C}$  and a photoperiod of 16 h light and 8 h darkness. Groups of approximately twenty males and twenty females were kept in 5 litre plastic boxes, which also contained a piece of corrugated filter paper to increase the surface area. Every other day the ladybirds were fed an excess of the pea aphid, *Acyrtosiphon pisum* (Harris). On that occasion, a piece of stem of broad bean, *Vicia faba* L., was put in each of the rearing boxes to provide a source of humidity and of food for the aphids. Once a week the ladybirds were transferred to clean containers to stimulate egg laying.

The same rearing procedure were followed for the seven-spot ladybird, *C. septempunctata*, except for the temperature, which was maintained at  $25^\circ\text{C}$  to encourage egg production.

The corrugated filter paper was changed every day and any egg batches were removed from the paper by cutting round them with fine scissors. Each piece of paper with a batch of eggs was incubated in 175 cm<sup>3</sup> boxes at  $15 \pm 1^\circ\text{C}$  and a photoperiod of 16 h light and 8 h darkness. After hatching the larvae were kept 15 to a box in order to reduce the risk of cannibalism. From the beginning of the third instar the larvae were isolated in 5-cm diameter Petri dishes lined with filter paper. Throughout their development the larvae were fed an excess of pea aphids.

### *Intraguild predation and cannibalism of eggs by fourth instar larvae*

Two days after moulting to the fourth instar, a larva of *A. bipunctata* was transferred to a 9-cm diameter Petri dish lined with filter paper and kept for 3 h without aphids. Then each larva was gently placed in the centre of a 9-cm diameter Petri dish lined with filter paper and containing a batch of a minimum 10 conspecific eggs located close to the rim of the dish. The larva was observed under a binocular microscope. On its first encounter with the eggs, it was noted whether it immediately backed away from the eggs or not. If it did not avoid the eggs, how many were crushed or eaten was recorded. There were 20 replicates, which each lasted for 15 min from the time of first encountering the batch of eggs. Twenty fourth instar larvae of *A. bipunctata* were then similarly offered a batch of seven-spot eggs. The same experiment was repeated but with larvae that were previously kept for 24 hours without food.

In the next experiment fourth instar larvae of *C. septempunctata*, which were starved for 3 or 24 hours, were offered, as above, conspecific or *A. bipunctata* eggs.

The numbers of larvae that avoided or ate the eggs were analysed using a standard  $\chi^2$  test. The numbers of encounters with the eggs in the following 15-min were analysed by means of a Mann-Whitney's test. Egg consumption in the different treatments was compared by a Kruskal-Wallis test (Zar 1996).

### *Are ladybird eggs protected against cannibalism and intraguild predation?*

Following the above procedure, a fourth instar larva of *A. bipunctata* was placed in a 9-cm diameter Petri dish containing either a batch of conspecific eggs, a batch of conspecific eggs washed in n-hexane or a batch of conspecific eggs previously washed in n-hexane and then painted with the aid of an Hamilton® micro-syringe with an hexane extract obtained from a corresponding number of eggs. There were 21 replicates of each treatment. Eggs to be washed were first removed from the filter paper on which they were laid by means of a fine paintbrush after wetting the filter paper with de-ionised water, then immersed in groups of 150 in 0.5 ml of n-hexane for 2 min. For the third treatment, the hexane

extract was carefully painted on to hexane washed eggs under a binocular microscope to avoid losses. In this way, the extract was delivered in quantities small enough that it did not spread onto the surrounding substrate. The washed and painted eggs were dried for 30 min in a fume cupboard on a piece of filter paper before being presented to the larvae. Hexane was chosen as a solvent because a preliminary study indicated that it did not penetrate into eggs during the 2 min extraction period, and only removes chemicals present on the surface of the eggs (Chan 1995), and it does not affect the hatching success (Guo & Dixon, unpublished). This experiment was then repeated using fourth instar larvae of *A. bipunctata* presented with similarly treated eggs of *C. septempunctata*. The proportions of larvae eating eggs were compared by means of a  $\chi^2$  test and the numbers of eggs eaten were analysed by means of a Kruskal-Wallis test.

Fourth instar larvae of *C. septempunctata* were also offered similarly treated eggs of *A. bipunctata*. The numbers of replicates varied from 20 to 16 depending on the treatment.

### *Chemical analysis of the n-hexane extract*

Eggs of *A. bipunctata* were collected daily from the ladybird culture, detached from the filter paper on which they were laid as described before and 50 of them placed in a small conical flask. They were washed for 2 min in 500  $\mu\text{l}$  of n-hexane. This extract was evaporated under a gentle stream of nitrogen and the dry residue dissolved in 100 ml of n-hexane. The extracts from 22 groups of 50 eggs were pooled prior to Gas Chromatography-Mass Spectrometry (GC-MS). The same procedure was followed to obtain an extract from 433 eggs of *C. septempunctata*.

The GC-MS was done on a Hewlett-Packard HP 5989 Mass Spectrometer coupled to a HP 5890 Serie II gas chromatograph equipped with a HP-5 (crosslinked 5% phenyl-methylpolysiloxane) column (30 m  $\times$  0.25 mm I.D.; film thickness 0.25  $\mu\text{m}$ ). The operating conditions were fixed as follows: split-splitless injector at  $275^\circ\text{C}$ ; carrier gas: helium at 0.9 ml min<sup>-1</sup>; temperature programme: from  $50^\circ\text{C}$  to  $140^\circ\text{C}$  at  $20^\circ\text{C}$  min<sup>-1</sup> then from  $140^\circ\text{C}$  to  $290^\circ\text{C}$  at  $5^\circ\text{C}$  min<sup>-1</sup> with a final hold of 30 min at  $290^\circ\text{C}$ . The mass spectra were recorded in the electron impact mode at 70 eV (Source Temperature:  $200^\circ\text{C}$ , scanned mass range: 35 to 600 amu).

The peaks were identified by their retention data and their characteristic fragmentation patterns. The identification of straight-chain hydrocarbons and free fatty acids were confirmed by co-injections of pure references. Their mass spectra were also compared with those of the NBS 75K.L and WILEY 138.L computer MS libraries. The structural assignments of internally methyl-substituted hydrocarbons were performed on the basis of the prominent mass ions  $\text{C}_n\text{H}_{2n+1}$  and  $\text{C}_n\text{H}_{2n}$  (McCarthy *et al.* 1968) formed by the  $\alpha$ -cleavage at the branch position(s). The 2-methylalkanes were fragmented to give a characteristic strong M-43 ion (loss of an isopropyl group) and a less intense M-15 peak (loss of a methyl group). For the 3-methylalkanes, a characteristic M-29 peak (loss of an ethyl group) together with a smaller M-57 fragment (loss of a secondary butyl group) were the diagnostic ions.

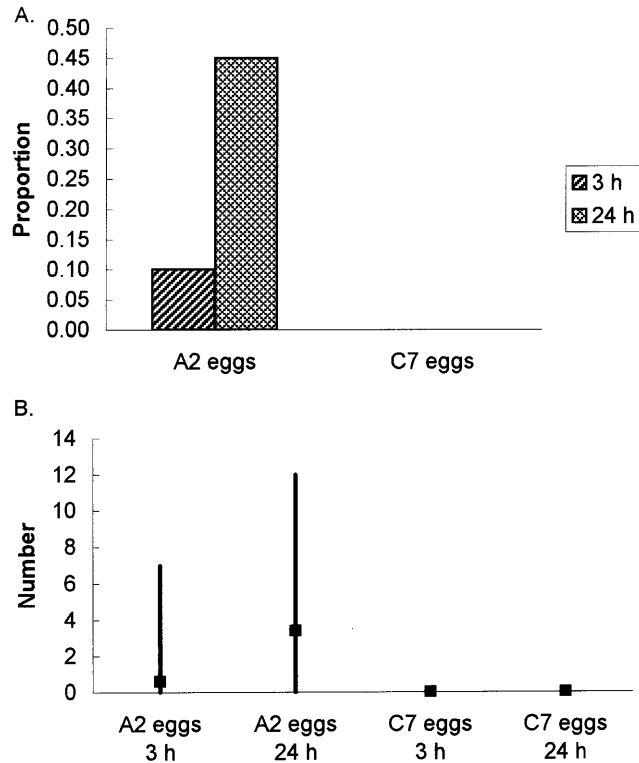
To measure the quantity of each of the alkanes present in the extracts, eggs of *A. bipunctata* were separated from filter paper as above. A group of 50 eggs was washed for 2 min in a solution of 400  $\mu\text{l}$  of n-hexane plus 100  $\mu\text{l}$  of a solution of 12.05 mg n-dodecane/20 ml of hexane, which was the internal standard. This extract was evaporated under a gentle stream of nitrogen, the dry residue was dissolved in 100 ml of n-hexane and then analysed by gas chromatography on a Carlo Erba 5060 Mega fitted with a cold on-column injector and a FID detector maintained at  $295^\circ\text{C}$ . A fused silica OPTIMA 1 column (30 m  $\times$  0.32 mm I.D.; film thickness 0.35  $\mu\text{m}$ ) from Macherey-Nagel was used for the analysis. The temperature programme was identical to that of the GC-MS analytical procedure. For diagnostic purposes, the relative retention times of the different peaks were calculated with n-tricosane as a reference and compared to the GC-MS chromatographic data and to the retention times of n-alkane homologs injected under the same conditions. This was repeated 9 times. Then three extracts of five batches of 34 to 47 eggs of *C. septempunctata* were treated similarly.

**Results**

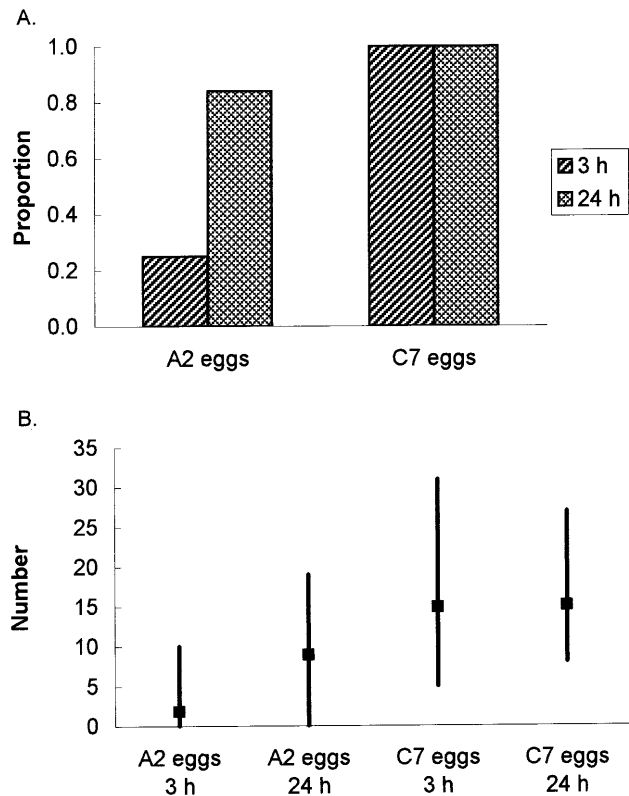
*Intraguild predation and cannibalism of eggs by fourth instar larvae*

After starving for 3 hours fourth instar larvae of *A. bipunctata* were reluctant to eat the eggs of their own species and did not eat those of *C. septempunctata* (Fig. 1). On first encountering two-spot eggs, 13 out of 20 larvae backed away; 11 out of 20 larvae behaved similarly on encountering seven-spot eggs. These two proportions do not differ significantly ( $\chi^2 = 0.417$ , 1 d.f.,  $P > 0.05$ ). During the course of the experiment, the two-spot larvae on average encountered batches of their own eggs twice as frequently as batches of seven-spot eggs (Mann-Whitney's  $U = 305.5$ ;  $P = 0.004$ ). Two-spot larvae appeared to be less active in the presence of seven-spot than two-spot eggs. After 24 hours starvation the proportion of two-spot larvae eating their own eggs increased ( $\chi^2 = 6.144$ , 1 d.f.,  $P = 0.013$ ) but they still did not eat *C. septempunctata* eggs (Fig. 1A). That is, the larvae of *A. bipunctata* ate more conspecific eggs after a long than a short period of starvation (Fig. 1B: Mann-Whitney's  $U = 271$ ;  $P = 0.015$ ).

The period of starvation, similarly, affected the consumption of eggs of *A. bipunctata* by larvae of *C. septempunctata*. The longer period of starvation resulted in a greater proportion of larvae eating ( $\chi^2 = 13.745$ , 1 d.f.,  $P = 0.000$ ) and a greater number of eggs



**Fig. 1** The proportion of fourth instar larvae of *Adalia bipunctata* starved for 3 or 24 hours that ate eggs (A) and the maximum, average and minimum numbers of eggs they ate (B) when offered either a batch of *Coccinella septempunctata* eggs or a batch of conspecific eggs



**Fig. 2** The proportion of fourth instar larvae of *Coccinella septempunctata* starved for 3 or 24 hours (A) and the maximum, average and minimum numbers of eggs they ate (B) when offered either a batch of *Adalia bipunctata* or a batch of conspecific eggs

eaten (Mann-Whitney's  $U = 61$ ;  $P = 0.000$ ). In contrast it had almost no effect on the consumption of conspecific eggs (Fig. 2).

That is, hungry two-spot larvae refused to eat seven-spot eggs and were reluctant to eat their own species eggs, whereas seven-spot larvae readily ate their own species eggs and avoided eating two-spot eggs unless very hungry.

*Are ladybird eggs protected against cannibalism and intraguild predation?*

The fourth instar larvae of *A. bipunctata* ate numerically and proportionally significantly more washed than unwashed, or painted conspecific eggs (Table 1). They were extremely reluctant to eat eggs of *C. septempunctata*. Although the differences between the three treatments were not significant, the proportion of larvae eating at least one seven-spot egg increased when the eggs were washed in hexane.

Similarly, fourth instar larvae of *C. septempunctata* were reluctant to eat two-spot eggs. Few of them consumed unwashed eggs or painted eggs. However, nearly all the larvae ate washed eggs. Significantly more washed eggs were eaten than unwashed or painted eggs (Table 2).

That is, *A. bipunctata* eggs are protected against both cannibalism and intraguild predation by *C. sep-*

	Eggs offered					
	Two-spot			Seven-spot		
	unwashed	washed	painted	unwashed	washed	painted
$n_t$	21	21	21	20	19	10
$n_e$	7	18	7	2	6	1
$\chi^2$ test		15.37 ( $P < 0.001$ )			3.61 ( $P > 0.05$ )	
Min	0	0	0	0	0	0
Max	10	10	10	1	4	2
Mean	2.7	7.9	2.5	0.1	0.6	0.2
KW test		18.07 ( $P = 0.00$ )			3.63 ( $P = 0.16$ )	

$n_t$  = total number of larvae;  $n_e$  = number of larvae eating eggs; KW test = Kruskal-Wallis test

*tempunctata* larvae by one or several n-hexane soluble substances on the surface of their eggs. Eggs of *C. septempunctata* are strongly protected against intraguild predation by *A. bipunctata* but not against cannibalism.

#### Chemical analysis of the n-hexane extract of the surface covering of eggs

87% of the molecules in the hexane extract of the surface of the eggs of *A. bipunctata* are either linear hydrocarbons [heneicosane (C21), docosane (C22) and tricosane (C23)] or branched hydrocarbons [7-methyl-tricosane (7Me-C23) and 9-methyl-tricosane (9Me-tricosane)]. 7Me-C23 and 9Me-C23 made up a fairly large percentage of the hydrocarbon profile (29% and 50% respectively); C22 made up only a small percentage (2%) (Table 3).

The hexane extract of the surface of the eggs of *C. septempunctata* is more complex and less concentrated. It contains hydrocarbons with an even number of carbon atoms [docosane (C22), tetracosane (C24), hexacosane (C26) and octacosane (C28)] and an odd number of carbon atoms [tricosane (C23), pentacosane (C25), heptacosane (C27) and nonacosane (C29)]. The latter are dominant and among them C27 makes up 40% of the total (Table 3). Although the chromatographic profile from the GC-MS analysis is similar to that obtained from gas chromatography in displaying ten major peaks, it only reveals the hydrocarbons with odd numbers of carbon atoms. The concentration of

**Table 2** The number of fourth instar larvae of *Coccinella septempunctata* that ate eggs, and the numbers of eggs eaten, when they were offered two-spot eggs that were unwashed, washed in hexane or washed in hexane and painted with extract

	Two-spot eggs		
	unwashed	washed	painted
	$n_t$	20	20
$n_e$	5	19	8
$\chi^2$ test		20.47 ( $P < 0.001$ )	
Min	0	0	0
Max	10	32	10
Mean	1.8	11.8	1.9
KW test		24.63 ( $P = 0.000$ )	

$n_t$  = total number of larvae;  $n_e$  = number of larvae eating eggs; KW test = Kruskal-Wallis test

**Table 1** The number of fourth instar larvae of *Adalia bipunctata* that ate eggs, and the numbers of eggs eaten, when offered either two-spot or seven-spot eggs that were unwashed, washed in hexane or washed in hexane and painted with extract

hydrocarbons with even numbers of carbons was probably insufficient to be detected. Peak number 10 corresponds to a mixture of C28 branched hydrocarbons. In addition to hydrocarbons, there are other compounds not yet identified. On average, there was less hydrocarbon material on the surface of *C. septempunctata* eggs (1.10  $\mu\text{g}/50$  eggs) than of *A. bipunctata* eggs (4.20  $\mu\text{g}/50$  eggs).

#### Discussion

There is an increasing body of evidence that all stages of ladybirds contain species specific alkaloids (Pasteels *et al.* 1973; Daloze *et al.* 1995; King & Meinwald 1996) and that these substances are toxic to other ladybirds (Agarwala & Dixon 1992; Hemptinne *et al.* 2000.). Accepting that they are toxic, then there are advantages in each developmental stage being able to flag its toxicity. The experiments reported here indicate that the hexane soluble molecules on the surface of the eggs flag this information. The hexane extract consists mainly of alkanes (87%).

The mechanism by which insect predators recognize their prey has been poorly studied. Although adult ladybirds have well developed eyes they nevertheless recognize conspecifics during mating by their surface

**Table 3** Concentration, in  $\mu\text{g}/50$  eggs, of the hydrocarbons in the n-hexane extract of the surface covering of the eggs of *Adalia bipunctata* and *Coccinella septempunctata*

Hydrocarbons	<i>A. bipunctata</i>		<i>C. septempunctata</i>	
	Mean	SD	Mean	SD
C21	0.5	0.1		
C22	0.1	0.0	0.04	0.01
C23	0.3	0.1	0.13	0.07
7Me-C23	1.2	0.2		
9Me-C23	2.1	0.5		
C24			0.11	0.10
C25			0.17	0.07
C26			0.05	0.02
C27			0.44	0.21
C28			0.04	0.00
C29			0.12	0.07
Total	4.2		1.10	

chemistry (Hemptinne *et al.* 1998). Ladybird larvae have poorly developed eyes and do not appear to use visual stimuli in prey recognition (Dixon 2000). Therefore, it is likely that larvae also use surface chemistry in prey recognition. Although the surface chemistry of different stages of an insect varies (Carlson *et al.* 1999; Nielsen *et al.* 1999; Wagner *et al.* 1998), nevertheless, it is likely to be more similar within than between species and thus could function as a species specific signal (Blows & Allan 1998; Lahav *et al.* 1999; Liu *et al.* 1999; Nielsen *et al.* 1999). If this is the case then the reluctance to eat conspecific eggs might indicate that well fed larvae generally avoid attacking individuals of the same species as there is a risk of the attacker becoming the victim in case of a size handicap. If starving, however, cannibalism becomes more advantageous.

Intraguild predation involves an additional risk, as not only might the attacker become the victim it may also be poisoned by eating its victim (Hemptinne *et al.* 2000). Thus generally, one would expect ladybirds to show greater reluctance to attack and eat other species eggs than their own eggs. This is supported by the results presented here. In addition one would expect this asymmetry to apply also to the interactions between larvae and, larvae and pupae: be more cautious if not conspecific!

The alkanes on the surface of the eggs of two-spot ladybirds are similar to those in the tracks left by their larvae, which are thought to deter females from ovipositing in patches of prey already attacked by their larvae (Doumbia *et al.* 1998; Hemptinne *et al.*, in prep.), and on the surface of adult elytra, which males appear to use in mate recognition (Hemptinne *et al.* 1998). That is, these alkanes probably signal a context specific message. In the context of predation, they signal relative risk; in mating, species recognition and in oviposition, the presence of conspecific larvae. This is probably an example of what Blum (1996) refers to as semiochemical parsimony.

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