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Artificial rearing of the olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) for use in the Sterile Insect Technique: improvements of the egg collection system

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Abstract

One major constraint in the development and implementation of a successful and cost-effective area-wide integrated pest management (AW-IPM) programme with a SIT component for *Bactrocera oleae* (Diptera: Tephritidae) is the ability to produce a large number of high quality mass-reared individuals. The aim of this study was to develop a more efficient and practical egg collection system in an attempt to improve the mass-rearing of this species. The following basic parameters were examined: egg production per female, egg hatch, pupal recovery, pupal weight, adult emergence and percentage of fliers. Three different strains (Israel wild-type, France wild-type, and Greece laboratory) were tested and each strain was evaluated for six generations. Female flies of the Israel strain produced significantly more eggs per female than the other two strains, but egg hatch was significantly lower. Egg hatch of the France wild type and the Greece laboratory strain was similar. For all other parameters, there was no significant difference between strains; however, there was a significant generational effect for all parameters observed. As a result of this study, a protocol was developed for the mass-rearing of this species that included the use of large adult holding cages that could house up to 96,000 flies per cage. The newly developed method of egg collection using a flat wax panel as one of the sides of an adult holding cage proved to be cost-effective, efficient, making colony growth easier for industrial mass-rearing.

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Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi) is a serious pest of economic importance for the production of olive fruits in the Mediterranean basin and the Middle East. It is highly invasive and has become established in North America after its first

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detection in 1998 in California (California, Arizona and northern Mexico) (Rice *et al.*, 2003). *B. oleae* larvae infest olive fruits and causes premature fruit drop (Collier and Van Steenwyk, 2003), mediates bacterial and fungal contamination of remaining fruit (Zalom *et al.*, 2009), causes undesirable staining of olive oil (Neuenschwander and Michelakis, 1978) and renders table olives unmarketable (Kapatos, 1989).

Traditionally, *B. oleae* infestations have been controlled using indiscriminative applications of synthetic insecticides. The insecticides have however serious limitations due to their toxic effects, residues in the olive fruit and olive oil, causing outbreaks of secondary insect pests and other adverse effects on the environment (Ferreira and Taínha, 1983). Therefore, there is a great demand to develop control methods which are environmentally friendly, efficient and cost effective. The sterile insect technique (SIT) is an eco-friendly control tactic for the management of selected insect pests. It relies on the mass-rearing of the target insect, sterilizing the male sex using ionizing radiation and releasing the sterile males in the target area where they will mate with virgin wild females and transfer their sterile sperm which results in unviable eggs (Knipling, 1955). Successive, regular and sustained releases of sterile insects will gradually reduce the density of the target population to a very low, economically acceptable level and in some cases eradication might be achievable (Knipling ,1955). The SIT has been proven to be very effective for the suppression, containment, prevention or eradication of several fruit flies species such as the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) in Mexico (Hendrichs *et al.*, 1983), the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) in Thailand (Orankanok *et al.*, 2007), the Mexican fruit fly, *Anastrepha ludens* (Loew) in north-western Mexico (Reyes Flores *et al.*, 2000), the melon fly, *Bactrocera cucurbitae* Coquillett in the Okinawa Islands, Japan (Koyama *et al.*, 2004) and the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) in western Australia (Fisher, 1996;Jessup *et al.*, 2007).

Despite its many successes and increased applications world-wide, there is still a need to improve several aspects of the SIT package for certain pest insect species. The SIT requires not only the ability to produce large quantities of insects at a reasonable cost but the mass-produced must display a behavioural repertoire that allows the released sterile males to compete with wild males for wild females, transfer their sterile sperm and induce sterility in the target population.

The development and implementation of the SIT as part of area-wide integrated pest management (AW-IPM) strategies (Vreysen *et al.*, 2007) against *B. oleae* has great potential in view of the extensive economic losses it causes. In addition, due to the monophagous nature of this pest that limits greatly its distribution, the probability of successful application of the SIT against this pest is considered high. However, the main limitation for its successful and efficient application remains the lack of efficient mass-rearing methods for this species.

The main bottleneck for the use of the SIT against B. oleae is the lack of consistency in producing large numbers of flies. The challenges does not only include the adaptation of wild flies to an artificial laboratory environment (Ahmad *et al.*, 2014), but also the development of efficient egg collection systems using artificial devices, proficient larval cultivation in artificial diets and adequate pupal recovery. With respect to oviposition substrates, the common practice has been to use paraffin domes that were exchanged daily or sometimes at longer intervals (Moore, 1959; Cantarino and Rey, 1970; Hagen *et al.*, 1963), parafilm (Silva, 1973), smooth surface or paraffin coated nylon gauze (Tzanakakis, 1971) and nylon gauze (Cavalloro and Girolami, 1968). All of these methods however are labour intensive and the number of egg obtained and their percentage hatch was not satisfactory. The production of large numbers of olive fruit flies required for the application of the SIT therefore necessitated the development of a better adult holding and egg collection system. The objective of this study was to assess improvements in egg collection system for olive fruit fly.

Materials and Methods

Origin of flies

The *B. oleae* populations were maintained at the FAO/IAEA Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria under the conditions described below. Insects originated from: a) a colony maintained at the Demokritos laboratory of the National Centre of Scientific Research, Athens, Greece that was initiated from wild material collected in 2003 and reared in the laboratory for > 500 generations before being shipped to the IPCL, where a colony was established and reared for a further > 90 generations. This colony is referred to as 'lab strain' b) a colony that was established from wild pupae collected in Israel and cultured at the

IPCL since 2006. F1 males were cross-mated with females from the Greece "lab strain", and this colony is referred to as 'Israel strain', and c) a colony that was established from wild pupae collected in Neziganl'ereque, France and cultured at the IPCL since 2010. F1 males were cross-mated with females from the Greece "lab strain", and this colony is referred to as 'French strain'. Insects of all life stages were held under constant environmental conditions at 25 ± 1 ºC, 60 ± 5% RH, and a photoperiod of 14 h:10 h light: dark. Light was supplied by four ceiling-mounted fluorescent tubes (153 cm long) that provided 2500-3000 lux.

Traditional cages with oviposition cones

Wax cones were placed inside laboratory cages that had a size of 40 × 30 × 30 cm for the flies to oviposit. Three sides of the cages were covered with netting to allow air flow, whereas the top and bottom parts were made out of plexi glass. The top part had three holes (diameter of 8 cm) to put the wax cones in the cages. Three funnel-shaped wax cones (8 cm diameter × 30 cm height) were placed inside the cages for oviposition. Each cone was covered with a plexi glass lid, on which a piece of sponge was glued to maintain humidity.

Modified cages with flat wax-coated panel for oviposition

Flat aluminium cages (201 cm long × 100 cm high × 20.5 cm wide) that have been traditionally used for the mass-rearing of the Mediterranean fruit fly e.g. in the Metapa facility in Mexico (Vargas, 1984) were modified by covering one of the larger sides (201 cm long × 100 cm high) with a muslin cloth net and the other side with a wax coated net as an oviposition panel. The oviposition panel was made of wax-coated fine Terylene cloth mesh (In design, Lahnau, Germany). The frame of the cages (100 cm high × 20.5 cm wide) was made of aluminium sheeting.

Each cage had two water sources, made of clear 0.5 cm-thick plastic tubing (outside diameter of 5 cm and a length of 210 cm) that extruded 5 cm at each end of the cage through 5 cm holes that were cut into the end walls of the cage. After adding the water, the tubes were sealed at one end with a rubber plug or a short plastic elbow joint with the upright opening to allow re-filling with water. The other end was sealed with a solid, leak-proof plug.

The modified cages with the flat wax-coated panel also contained two trays constructed of 2 mm-thick aluminium sheeting to hold the adult food. Each tray measured on the outside 200 × 9.5 × 2 cm (length x width x depth), was open on the top and was pop-riveted to the inside ends of the cage. All experimental adult flies were supplied with the same adult diet containing sugar, hydrolyzed yeast, and egg yolk in a ratio of 8:2:0.6 (Economopoulos and Tzanakakis, 1967; Tsitsipis and Kontos, 1983). The adult holding cages were suspended from ceiling-mounted tracks, side-by-side, 200 cm from the wall and 100 cm apart (each cage was suspended half-way between lights).

Eleven days after initiation of the pupal stage, 1.2 litres of pupae (~ 96,000 pupae) were placed in the adult holding cages for adult emergence. After loading the cages with water, adult food and pupae, the oviposition panel was fixed using elastic loops at each corner of the cage to stretch the panel flat across the cage. The oviposition panel was then covered with a frame (201 × 100 cm (length x height)) holding a sheet of thick plastic (0.1 mm) foil to protect the oviposition panel and the oviposited eggs from desiccation.

Egg collection from traditional cages with oviposition cones

The cones were rinsed interiorly with water and eggs were collected in a petri dish at the bottom part of the oviposition cones.

Egg collection from modified cages with flat panel for oviposition

In the modified flat panel mass-rearing cages, eggs were collected in a water-filled galvanized iron trough measuring 220 × 35 × 15 cm (length × width × depth) placed directly under the oviposition panel. The trough was fixed with two metal holders, one at either end of the trough, such that one end of the trough was slightly higher than the other. The lower end had a 4 cm drain hole cut through the base of the trough and sealed with a rubber plug. The drain hole was situated directly over a 10 L plastic container (25 × 25 × 10 cm). Eggs were collected from day 3 to days 10-14 after adult emergence. The oviposition panel cover was opened and all eggs were washed into the egg collection trough. Both the oviposition panel and cover were washed using a garden sprayer (Size 25 L). After collecting the eggs, the water trough plug was removed and all water and eggs were allowed to flow into a plastic tray placed under the plug hole. The water trough was washed a second time to collect any remaining eggs. After a 10 min-settling time, excess water was drained

from the plastic container and eggs and remaining water were decanted into a measuring cylinder. After an additional 10 min of settling time, egg volumes per cage were measured and recorded. This procedure occurred on each day of egg collection, Monday through Friday.

Eggs were stored at 24-25 °C in measuring cylinders filled with tap water so that there was at least 4 cm of water above the egg layer. Eggs were placed onto larval rearing diet within 2 to 3 h of collection.

Egg hatch

After each egg collection a sample of 300 eggs was taken to evaluate egg hatch. The eggs were arranged on a white filter paper soaked in 0.3% propionic acid (Manoukas and Mazomenos, 1977) and this filter paper was placed on a wet piece of sponge placed in a petri dish (9 cm × 2 cm) and kept at 25 °C and 65 % humidity. The petri dishes with eggs were covered with top lid to keep the humidity. Five d after egg collection, total number of eggs that had not hatched was counted to measure egg hatch (%).

Larval rearing

Plastic trays of the following dimensions; 38.3 × 8.4 × 4.7 cm (length x width x height) were used for larval rearing. Five of these larval rearing trays were kept side by side with a gap of about 3 cm between them, and placed on top of Petri dish lids to raise the trays 5 mm from the bottom of a Hawaiian tray, which is the standard larval rearing tray currently used in many fruit flies mass-rearing programs and they measured 77 × 40 × 3.8 cm (length x width x depth at the sides, and 7 cm depth at the ends). The differential in depth from the sides to the ends allowed trays to be stacked one on top of each other leaving a gap of 3.2 cm between the top of the diet layer in one tray and the bottom of the next tray, providing essential ventilation for the developing larvae. A fine layer of teak sawdust was placed around the larvae trays for use as a pupation medium (Vargas *et al.*, 1986). To achieve additional ventilation each Hawaiian tray holding the five larval rearing trays was covered with another Hawaiian tray placed upside down, thus creating a 10 to 12 cm gap between the trays. These pairs of Hawaiian trays (i.e. one with the five larval rearing trays and the other as a lid) were stacked up to six pairs. Each pair of Hawaiian trays was covered tightly with thin plastic film to optimize incubation. This cover was left on the stack for three days after eggs were added to the larval diet. After 3 d, the plastic film was removed and replaced with a finely woven Terylene cloth. This cover allowed ventilation for second and third instar larvae and, most importantly, precluded contamination of the diet by *Drosophila* species.

The larval diet used was based on a diet described by (Tsitsipis, 1975). For 1 kg of larval diet the following ingredients were used: tap water (550 mL), extra virgin olive oil (20 mL), Tween® 80 emulsifier (7.5 mL), potassium sorbate (0.5 g), Nipagin® (2 g), sugar (20 g), brewer's yeast (75 g), soy hydrolysate (30 g), hydrochloric acid 2N (30 mL) and cellulose powder (275 g) as the bulking agent.

Pupal weight

Using a Sartorius balance or scale that had an accuracy of 1 mg, a sample of 100 pupae two days before adult emergence was weighed, after taking out all visible trash (e.g., vermiculite, sawdust) from the sample along with any pupae that had trash sticking to their puparia.

Adult emergence and adult flight ability

To assess adult emergence from pupae, 100 pupae were placed within the ring of paper (1cm wide and 4 cm in diameter), which was centred in the bottom of the Petri dish two d before emergence and these pupae were placed on the bottom of a 3-mm thick walled PVC tube (8.9 cm diameter × 10 cm height). Before use, the inside of the tube was lightly coated with unscented talcum powder to avoid flies crawling out of the cylinder. To provide a resting place for newly emerged flies, talcum powder up to 1 cm of height was removed from the bottom of the tube. After all flies had emerged, the number of emerged flies, non-emerged pupae, and flies that had flown out of the tube were counted. The flight ability test was carried out in a separate room that was maintained at 25 ± 1°C ,65 ± 15% RH, and a photoperiod of 14 h : 10 h light : dark. Light intensity was 1,500 lux at the top of the tubes (FAO/IAEA/USDA, 2014).

Data collection and analysis

All three *B. oleae* strains were evaluated for egg production, egg hatch, pupae production, pupae recovery and pupae weight, adult emergence and flight ability for each of the six generations and data for these parameters was collected during eight consecutive days. The duration of the data collection period is normally 10 d for Mediterranean fruit fly (Cáceres *et al.*, 2000). However, mortality of olive flies after 8 d was significant and therefore, egg collection data were only collected

for 8 d. Eggs produced per female in the modified cages with flat wax panel were counted by dividing the number of eggs by the number of females; egg hatch was calculated by subtracting the number of unhatched eggs by the total number of sampled eggs, pupae production was assessed by measuring the volume of pupae, pupae recovery was calculated by dividing the number of pupae by the total number of seeded eggs, mean pupal weight was estimated by measuring the weight all pupae divided by the number of pupae, adult emergence percentage was calculated by the number of emerged adults divided by the number of pupae and flight ability was calculated by the number of flies remained inside the PVC tube subtracted the total number of sampled pupae. All the parameters were evaluated following the procedures described in the quality control manual of FAO/IAEA/USDA (2014). Data from each generation was considered as one replicate. Differences in the means among three strains for these parameters were analysed by analysis of variance (ANOVA).

Results

Egg production in traditional cages with oviposition cones

The number of eggs produced per female in laboratory cages with oviposition cones was assessed only for the lab strain and egg production per female was not significantly different across generations ($F = 3.53$, $df = 1$, $p = 0.09$).

Egg production and hatch in the modified cage with flat panel

There were no significant differences in egg production ($F = 1.59$, $df = 2$, $p = 0.24$) and egg hatch ($F = 0.76$, $df = 2$ $p = 0.48$) among strains (Table 1).

Pupal recovery and pupal weight in flat panel cages

There were no significant differences in pupal recovery ($F=0.11$, $df=2$, $p=0.89$) and pupal weight ($F=0.06$, $df=2$, $p = 0.93$) among strains. All three stains shared a similar trend for pupal weight by generations; i.e.an increase in pupal weight from generation 1 to 3, then a decrease in subsequent generations.

Adult emergence and percentage of flyers from the flat panel cages

There was no significant difference in adult emergence ($F = 2.18$, $df = 2$, $p = 0.15$) and percentage flyers ($F = 0.67$, $df = 2$, $p =0.52$) between strains (Table 1). The overall trend was again an increase in adult emergence and percentage flyers between generations 1 to 3 and then a decrease from generations 4 to 6.

Discussion

Efficient mass-rearing of fruit flies requires standardized protocols, appropriate adult holding and oviposition cages and egg collection systems. Since 1950s many efforts have been made to develop systems for the mass-production of olive fruit fly. These efforts were mainly to develop adult holding cages, larval diet and an appropriate rearing environment. Previous research has resulted in the development of various types of olive fruit fly holding and oviposition cages and egg collection methods. With respect to egg collection methods, (Moore, 1959) introduced paraffin domes that consisted of paraffin wax or mixtures of paraffin with bee wax, vaseline or other similar substances (Hagen, Santas, and Tsecouras ,1963). However, the preparation and use of

Table 1. Mean egg production, egg hatch, pupal weigh, pupal recovery, adult survival and percentage flyers for 3 strains of B. oleae that were maintained for 6 generations in modified flat wax panel holding cages. Means for each strain followed by the same lower case letter within columns are not significant (Tukey's test: $p = 0.05$)

Strain	Egg production (number of eggs per female)	Egg hatch (%)	Pupal recovery (%)	Pupal weight (mg)	Pupal emergence (%)	Flyers (%)
Israel	35.7±6.0a	76.6±2.4a	14.9±1 a	2.64±0.3a	79.1±3a	70.0±5a
France	24.6±4.3a	77.1±1.7a	14.2±1a	2.83±0.4a	62.1±6a	62.4±5a
Olive lab	25.1±4.6a	79.6±1.9a	13.9±2a	2.72±0.4a	72.1±6a	60.6±6a

paraffin domes as an egg collection system was time-consuming and therefore, costly.

A different approach was taken by (Cavalloro and Girolami, 1968), who maintained the olive fruit flies in all-screen cages without oviposition devices. The pressure of the developing eggs in their ovaries forced the females to oviposit on the bottom of the cage and most of the eggs passed through the netting. Although this set-up was very simple, it suffered from low egg production. It seems that to ensure the fertilization of the eggs, a situation is required where the fly is offered the opportunity to puncture some substrate (like a membrane) and where it can adopt the natural position during oviposition, i.e. a curved abdomen with the ovipositor projected forwards that puts the necessary pressure on the spermathecae to facilitate the release of the sperm.

Tzanakakis (1971) reviewed several designs of adult holding cages and egg collection systems for olive fly rearing. Historically, small colonies of olive fruit fly have been housed in screen cages with a wooden frame (screen on 3 sides, front side out of glass, a Formica floor and a wooden back with door) but these cages (30 × 30 × 30 cm) could only hold 300 flies and were therefore only used for maintaining small cultures of olive fruit flies mainly for research and development purposes (Hagen, Santas, and Tsecouras ,1963).

Silva (Silva, 1973) introduced a holding cage that had on one or two sides a stretched Para film membrane. The olive fruit flies oviposited by puncturing the membrane with their ovipositor, and the eggs were removed from the membrane with a flushing device that delivered water every 15-20 minutes. Using Para film on domes, the olive fruit flies produced less than half the number of eggs as compared with paraffin cones. (Cavalloro and Girolami ,1968) reported similar results when comparing paraffin domes with Para film.

For mass-rearing purposes, a larger cage (100 × 40 × 30 cm) was developed at the National Centre of Scientific Research, Athens, Greece (Tsitsipis, 1977b). This cage could hold 1800-2400 flies and the diet were provided in an elongated container that also provided surface for resting of flies and four funnels for egg collection. These funnels were cone shaped and made of ceresin coated nylon gauze, and inserted vertically through openings from the ceiling and placed on the bottom of the cage. Females oviposited their eggs by inserting their ovipositor through the cone ceresin and eggs were rinsed with water from the interior of the cone and collected at the bottom of the cage (Tsitsipis ,1977b). These cages allowed rearing of olive fly but only at a small scale and these cages were less efficient in terms of flies produced per unit area and less numbers of eggs produced per female. Females were ovipositing large numbers of eggs on the bottom of the cage showing that ceresin cones were probably inappropriate for oviposition.

This study presents a new modified adult holding cage that has a flat wax panel as an oviposition substrate and egg collection system, and in which it is possible to maintain nearly 100,000 olive fruit flies. These modified cages provided a larger surface area for oviposition by females and the cages were easy to set-up and easy to handle as compared with the earlier cages that had wax cones for egg collection.

Large numbers of eggs can only be produced when the adult holding cages have an appropriate size that can hold a large number of ovipositing females that produce large numbers of eggs which is a main goal for mass-rearing of fruit flies. Good illumination in the rearing room can ensure a homogenous distribution of flies in the cage and a random selection of oviposition sites on the available oviposition substrate. As such, small cages are obviously not ideal for mass-rearing (Tsitsipis, 1977a). The present study used cages that had dimensions of 201 × 100 × 20.5 cm that could successfully hold 96,000 adult flies in a single cage, i.e. or 238.8 flies per cm^3, whereas the traditional cages with the wax cones could only house 33.3 flies per cm^3 . This is obvious as the cage was smaller and could only hold 13,400 flies. In addition, the modified cages with flat wax panels requires less space which is one of the important cost factors in a large mass-rearing facility. As an example, to maintain a100 million olive fruit flies using traditional cages, a surface area of ca.19, 696 m^2 would be needed, whereas maintaining the same number of olive fruit flies in our modified flat wax panel cages would require ca.9, 395 m^2, i.e., a reduction of ca.10, 300 m^2.

In addition, experiments conducted at the IPCL have shown that replacing the oviposition cones with flat panelled, wax netting almost doubled egg production per female in both type of cages, i.e. 25.1 ± 4.6 and 13.3 ± 3.9 mL of eggs, respectively (unpublished data). In the current study, egg production increased significantly from the first to the sixth generation i.e. 19.8±4.9 mLand 44.0±3.0 mL, respectively, revealing the flies' ability to adapt gradually to the new cage and egging system under laboratory conditions.

Table 2. Egg production, egg hatch, pupal recovery, pupal weight, emergence and flyers from F-1 to F-6 generation of three different strain of olive fly in modified flat panel cages

Total production data for analysis							
Strain	Generation	Fecundity (Eggs/ female)	Egg hatch(%)	Pupal recovery(%)	Pupal Weight(mg)	Emergence(%)	Flyers(%)
Israel	1	26.13	66.83	16.23	3.05	85.80	77.80
Israel	2	52.54	73.70	10.67	2.69	68.80	49.00
Israel	3	26.05	79.01	16.61	3.21	66.80	64.80
Israel	4	18.19	76.24	13.90	3.14	80.80	64.60
Israel	5	41.27	81.13	14.44	1.93	85.00	87.33
Israel	6	50.05	82.55	17.65	1.83	87.67	76.67
Olive lab	1	10.26	83.17	21.91	2.84	81.00	76.40
Olive lab	2	31.6	78.13	9.18	2.74	53.40	49.80
Olive lab	3	14.99	84.03	17.16	4.46	64.50	53.50
Olive lab	4	24.63	74.41	11.46	2.86	58.60	44.00
Olive lab	5	27.81	78.56	8.54	1.74	88.67	87.00
Olive lab	6	41.47	79.59	15.33	1.69	86.33	52.67
France	1	23.13	80.68	13.13	2.86	79.60	74.40
France	2	24.98	69.27	9.94	2.85	41.20	38.40
France	3	16.79	80.58	19.49	4.47	52.75	55.88
France	4	10.77	77.08	15.79	3.01	82.40	66.80
France	5	31.56	76.23	13.67	1.94	67.67	77.33
France	6	40.61	78.83	12.93	1.82	49.33	

*Number of eggs per female was collected in eight d.

The present study demonstrated that the use of the new flat panel cages increased production of eggs, pupae and adults of all strains in the first three generations with respect (Table 2) to all studied parameters.

Adequate egg production for mass-rearing of olive fruit flies can significantly be enhanced by using the flat wax panel egg collection system, which is an important improvement in the overall rearing of olive fruit fly. There are however, other problems that need to be overcome such as stabilizing larvae production, and the use of cheaper components for the larval diet so that the SIT could be become a viable economic option for managing wild olive fruit fly populations.

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Disclosure

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