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Effect of different narcosis procedures on initiating oviposition of prediapausing *Bombus terrestris* queens

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Abstract

Four experiments aimed at the stimulation of starting oviposition were carried out with bumblebee queens (*Bombus terrestris* L.) from colonies belonging to the ecotype of Central Western France and reared in a glasshouse. After mating, queens were narcotized with carbon dioxide, confined singly in small boxes (11 × 5 × 4.5 cm) and kept in a dark room at 28–29 °C and 60 %–65 % r.h. They were fed on a sugar solution and a pollen-syrup mixture. No effects were discernible if the narcosis was applied 20 to 30 days after mating instead of 5 days, nor if the queens were submitted to a 4 to 5 day period at 34 °C following narcosis. Survival rates ranged from 65 % to 68 %. If the queens were reared under fluorescent tubes (L8:D16) after narcosis the mean delays to egg-laying were significantly reduced compared to a dark treatment (21 days instead of 39), as was their variability (s.e. = 1.6 day instead of 3.1 days). The survival rates were respectively 73 % and 67 %.Under the same photoperiod (L8:D16) the CO_2 narcosis repeated at a 24h interval had the same efficacy whether its duration was 10 min or 5 min. The delays to egg-laying were respectively 20 days (s.e = 1.5) and 25 days (s.e. = 4.8) with survival rates close to 73 %. Egg-laying could also be induced in non-narcotized queens with a survival rate of 54 % and delays to oviposition close to those of queens narcotized 2 × 10 min.

Introduction

Since mass-rearing of *Bombus terrestris* L. began in 1987 to supply glasshouse tomato growers in Europe, bumblebee producers have used several methods to rear colonies year-round, despite the long ovarian diapause of this species (Alford, 1975; Pouvreau, 1976). Röseler & Röseler (1984) demonstrated that narcotizing prediapausing queens with carbon dioxide (a 30 min narcosis repeated twice) inhibited the formation of fat reserves, increased the size of the *corpora allata*, which produced high rates of juvenile hormone *in vitro*, and induced oogenesis. In 1985, Röseler, giving the first description of a year round rearing system, stated that after mating, *B. terrestris* queens would start laying eggs within a week following the double narcosis.

Part of the great commercial development of bumblebee rearing is due to Röseler's narcosis method which was also recommended by van den Eijnde *et al.* (1991) who described how to produce colonies of *B. terrestris* artificially. Carbon dioxide narcosis of 10 min repeated at a 24 h interval was already used by Mackensen (1947) to stimulate egg-laying in newly inseminated honey bee queens. Enhancement of ovarian maturation after Co_2-narcosis was also reported by Nicolas (1979) in *Locusta migratoria*. From the information released by commercial producers of bumblebees it is obvious that the results of carbon dioxide narcosis procedures on *B. terrestris* queens are variable and that the treatment does not always reach an optimum efficiency, sometimes causing detrimental side effects.

Several parameters are expected to be involved in the physiological action of carbon dioxide. In this article four of them were studied in order to assess their effect on egg-laying delays:

- the duration of narcosis,
- the age of the mated queens submitted to narcosis,
- the temperature following narcosis,
- the photoperiodic regime to which narcotized queens were submitted.

Materials and methods

Origin of queens. All colonies that produced experimental queens were created by queens captured from the wild in February 1991. Bumblebees were reared either in glasshouse compartments (3 × 3 × 2 m) or in a dark climate room. Glasshouse colonies were raised in cubic wooden boxes (25 × 25 × 25 cm) and fed on a solution containing 73 % sugar, pollen collected by honeybees and flowers of *Salix* and *Brassica napus oleifera.* In these colonies the production of queens occurred from the 20^{th} of June until the 4^{th} of November. In the climate room the bumblebees were kept in wooden boxes (25 × 25 × 25 cm) each fitted with two feeders. They were supplied with sugar solution and a mixture of honeybee collected pollen and syrup. The temperature was 28 °–29 °C and the relative humidity 60–65%. In both rearing conditions experimental queens emerging from the colonies were mated in the glasshouse with males from other colonies flying in screened cages of 0.7 m^3. After mating, queens were maintained in groups in other cages of the same size in glasshouse conditions. They were fed with sugar solution and pollen collected by honeybees.

Current narcosis technique. At least five days after mating queens were cooled to 3 °–4 °C for one to two hours, then narcotized for 10 min at the same temperature in plastic boxes. The carbon dioxide used in all the trials was 99.9% pure. After flowing one min into a narcosis box, its concentration in weight varied from 75% to 85% at the begining of anaesthesia, to 51% to 77% after 10 min. CO_2 narcosis was repeated after 24 h. In the interval queens remained in the dark at 23 °–24 °C.

Standard rearing conditions after narcosis. After the second narcosis queens were put into single wooden boxes (11.3 × 4.5 × 4.3 cm) with a transparent cover. They were equiped with two feeders, one was filled with a syrup containing 73% sugar, the other received small daily deposits of a pollen-syrup mixture. To prevent *Nosema* multiplication, Fumidil powder at 2 g/l was added to the sugar solution during periods of 14 days alternating with two week intervals without the antibiotic. A three cm round plastic lid with a beeswax bottom was also at the disposal of the queen for egg-laying. The rearing boxes were kept in a dark climate room at 28 °–29 °C and 60 %–65% R.H. A worker *B. terrestris* was introduced in each box to stimulate the queen. Daily observations were performed under red light to note the date of the first egg-cell building or the queen's death. We defined the survival rate as the percentage of experimental queens which laid eggs, whatever might be the duration of their life after the first oviposition. Oviposition delays are given as: $\overline{x} \pm se$.

Variations of the procedure.

a) *Trial 1.* Some unpublished results suggested that queens would lay eggs if they were submitted to a temperature higher than the nest temperature for a short period. Thus we applied 34 °C for four to six days in a dark room to 44 queens just after narcosis and before introduction into the dark climate room at 28 °–29 °C. A control sample of 47 queens was reared in the dark climate room just after narcosis.

b) *Trial 2.* The standard CO_2-narcosis was applied to 62 queens five days after mating and to 29 queens 20–30 days after mating. Following the CO_2 treatment all these bumble bees were reared in the climate room.

c) *Trial 3.* The standard CO_2-narcosis was applied to 136 queens, then 91 of them were kept in the dark climate room at 28 °–29 °C, and 45 were reared at the same temperature under fluorescent tubes supplying 800 lux with the photoperiodic regime: L8:D16. The 50 HZ electric equipment fed three tubes on three phases, thus suppressing light flickering. Eight hours is the lowest value of the day length in January, the month when early *B. terrestris* queens emerge from their hibernation lodge.

d) *Trial 4.* Three groups of queens were reared in the climate room under the photoperiod L8:D16 after three different treatments applied to 13, 9 and 36 queens respectively: no narcosis, five min narcosis repeated at a 24h interval, 10 min narcosis repeated at 24h interval.

Results

Influence of high temperature treatment after CO_2-narcosis and of the age of mated queens on oviposition delay. The four treatments applied:

Table 1. Influence of a high temperature period following narcosis on the mean days to egg-laying

Treatment	Egg-laying delay (days) n	$\bar{x}$	s.e.	Value of *t*
CO_2 narcosis	31	39.5[a]	4.7	0.22
CO_2 narcosis + 4–6 days at 34 °C	30	38.2[a]	4.0	

Values bearing the same letter are not different according to Student *t* test ($P<0.05$).

Table 2. Influence of the delay between mating and narcosis on the mean days to egg-laying

Delay between mating and narcosis (days)	Egg-laying delay (days) n	$\bar{x}$	s.e.	Value of *t*
5	42	40.0[a]	3.9	0.56
20–30	19	36.3[a]	5.0	

Values bearing the same letter are not different according to Student *t* test ($P<0.05$).

- standard narcosis,
- period of 4 to 5 days at 34 °C after narcosis,
- narcosis 5 days after mating,
- narcosis 20–30 days after mating

resulted in identical survival rates: 66% - 68% - 68% and 65% respectively. The delays to egg-laying were also identical according to the Student 't' test: 39 - 38 - 40 - 36 days with standard errors close to each other ranging from 3.9 to 5.0 (Table 1 and 2). As shown in Fig. 1 and 2, the dispersion of data presents common characteristics: a large range from 0–10 days to 90–100 days, and modes between either 10 and 20 days or 20 and 30 days.

Influence of a photoperiod applied after narcosis on oviposition delays. The survival rate of control queens (dark treatment) was 67% compared to 73% of queens laying eggs under artificial light. The photoperiod L8:D16 reduced the mean egg-laying delays compared to the dark, respectively: 21.1±1.6 days (33) and 38.9±3.1 days (61). Fig. 3 shows that the distribution of data in the light-dark treatment was quite different from that of the data concerning queens reared in the dark. The range of the delays in the dark was twice as large as that in the light. The modes were identical, between 10 and 20 days. Within 40 days, 97% of the queens in the light-dark regime laid their first eggs, compard to 59% of the queens in the dark. These proportions are significantly different (Table 3: $\chi^2_1 = 13.56$; $P = 0.0003$). Similarly, queens in L:D regime showed a higher frequency of oviposition within 20 days than queens in the dark ; respectively: 60.6 % and 29.5 % (Table 3: $\chi^2_1 = 7.36$; $P = 0.006$).

Table 3. Influence of photoperiod on oviposition delays of narcotized queens

	Treatment	Oviposition delays (days) 0–20	21–40	>40
Number of queens	dark	18	18	25
	photoperiod L8:D16	20	12	1

Influence of narcosis duration. If no CO_2 treatment was applied only 7 out of the 13 queens survived whereas 33 of the 45 narcotized queens did, all reared in the L8:D16 regime. The egg laying delays were identical in the non-narcotized queens and those submitted to the narcosis (2 × 10 min) respectively 17.3±2.1 days (7) and 19.8±1.5 days (25) (Student 't' = 0.8; df = 30; $P>0.05$). No significant difference was found between oviposition delays of non narcotized queens and those narcotized 2 × 5 min respectively 17.3±2.1 days (7) and 25±4.8 days (8) (Student 't' = 1.4; df = 13; $P>0.05$). Chi-square analyses did not show significant differences between the proportions of queens laying eggs within 20 or 30 days (Fig. 4), respectively $\chi^2_2 = 2.1$; $P = 0.35$ and $\chi^2_2 = 4.6$; $P = 0.09$.

Discussion

Duration of narcosis. In bumblebees a 5 min exposure to CO_2 at 4 °C, repeated once, may be the minimum narcosis duration for a reliable stimulation of egg-laying. It is noticeable that some queens could start a brood in artificial conditions without being narcotized. In a larger sample of pre-diapausing non-narcotized

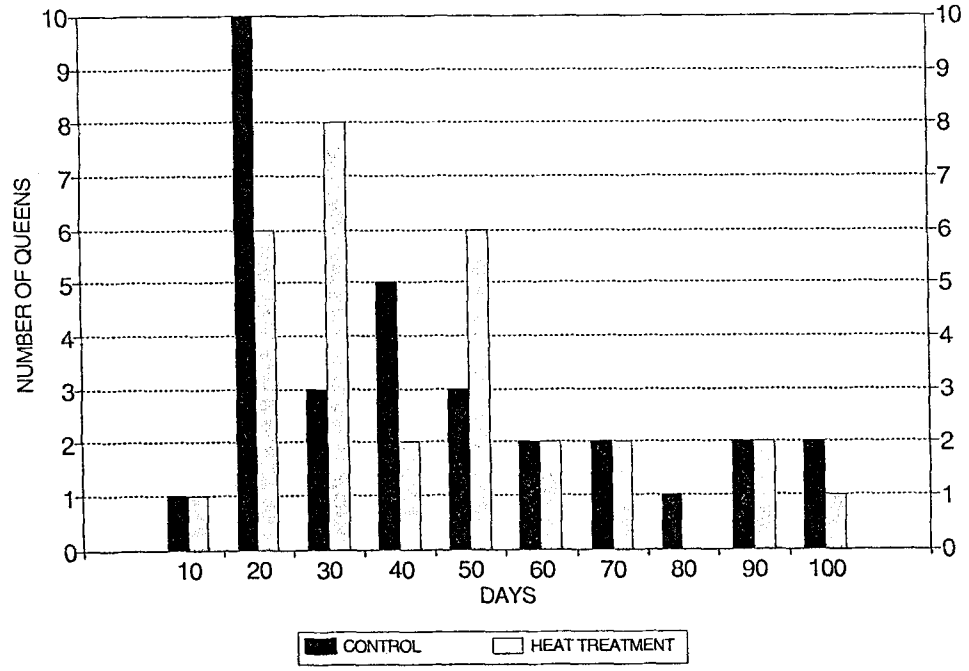

Fig. 1. Distribution of delays to initial oviposition of narcotized queens submitted to a post-narcosis temperature treatment (34 °C for 4 to 6 days) compared to a control.

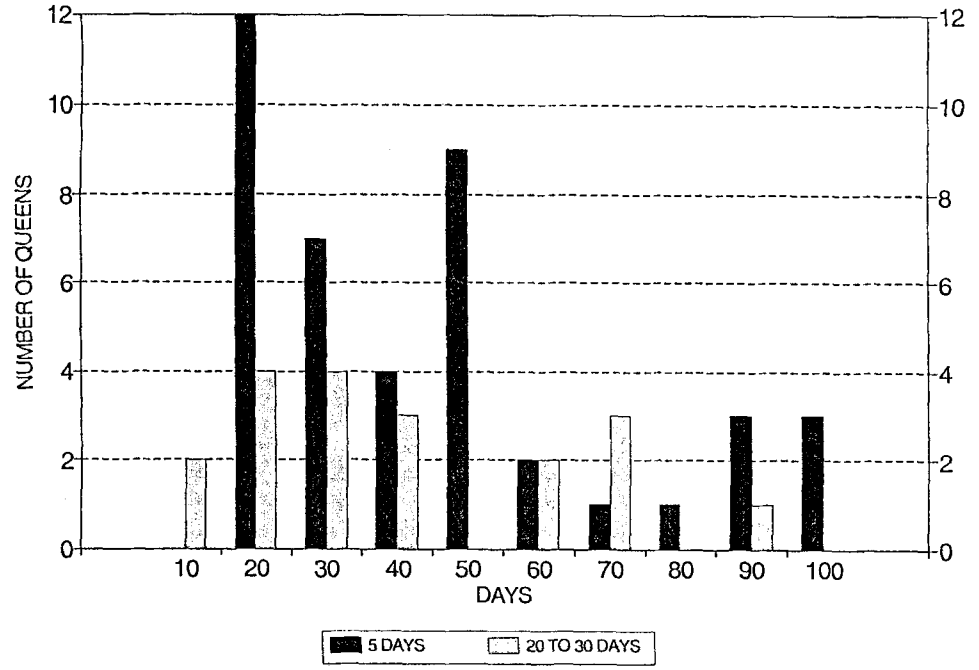

Fig. 2. Distribution of delays to initial oviposition of queens narcotized either 5 days or 20 to 30 days after mating.

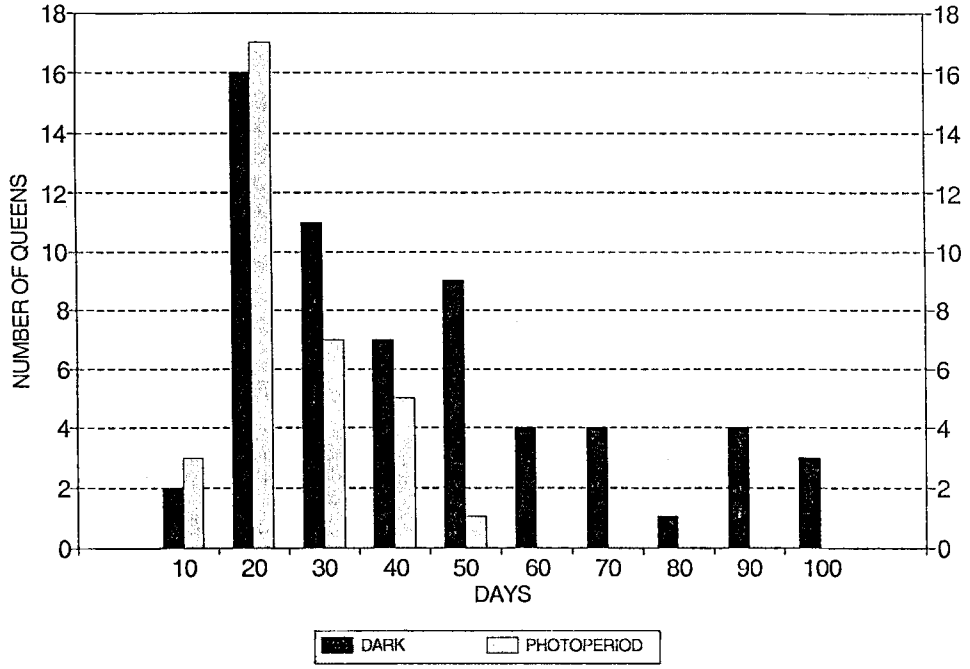

Fig. 3. Distribution of delays to initial oviposition of narcotized queens reared in a photoperiodic regime L8 : D16, compared to control queens reared in the dark.

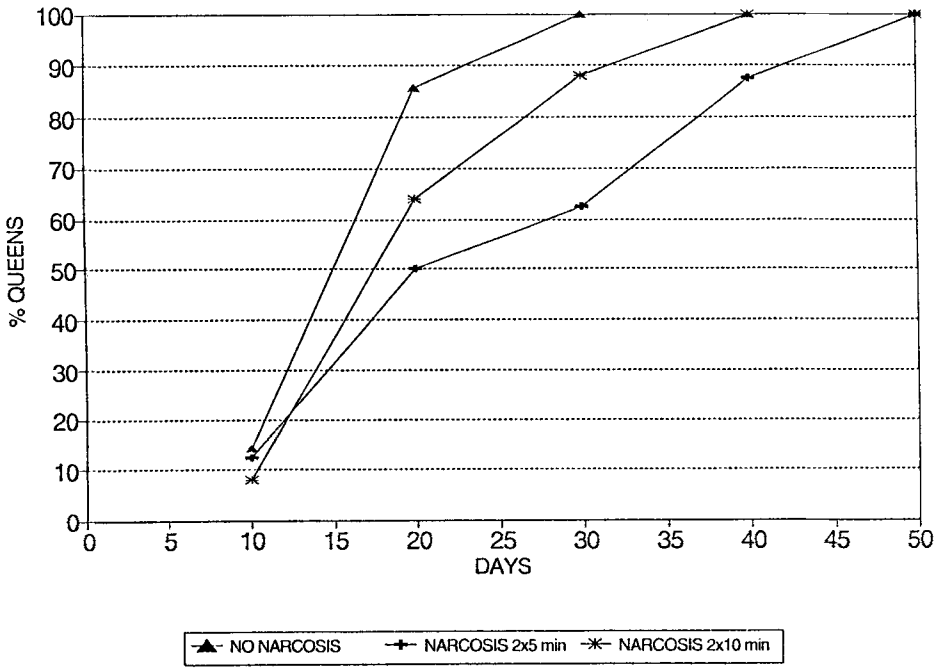

Fig. 4. Cumulative proportions of queens starting oviposition as a function of time since start of rearing at 28–29 °C.

queens (unpublished results) we observed that 30% of queens founded a nest. Presumably their physiological state was different from that of other queens which died in identical rearing conditions. This latter category of queens would probably have needed exposure to CO_2 to activate oognesis. Röseler's (1985) narcosis of bumblebees lasted three times as long as ours and resulted in oviposition delays of about one week instead of 19 days as shown in our study. In *Locusta migratoria*, Nicolas (1979) showed that short exposures to CO_2 (one min every day) were able to stimulate oviposition of females inhibited by a long photophase. The concentration of CO_2 should also be considered as a parameter of the narcosis efficiency. According to Nicolas (1989), variations in the concentration of carbon dioxide may result in opposite physiological effects.

Age of queens. The timing of CO_2 narcosis after mating did not affect the delays of egg-laying within the range of 5–30 days. According to Röseler (1985) narcosis can be also applied to queens during hibernation to terminate their diapause. Larrere *et al.* (1993) reported similar experiments demonstrating that overwintering queens submitted to a 10 min CO_2 narcosis repeated once, emerged from the soil earlier than non-treated diapausing insects.

Social stimulation. In our experiments each queen was supplied with one worker bumblebee. The contact between the queen and the worker proved efficient in reducing egg laying delays as it has been demonstrated in other studies. Engels *et al.* (1976) demonstrated that honeybee queens submitted to a double CO_2 exposure required social contacts with workers to initiate the vitellogenin metabolism. In bumblebees various stimulating treatments have been used: brood cells containing old larvae or pupae with some accompanying workers (Röseler, 1985) or 4 to 5 honeybee workers as recommended by Ptacek (1983, 1985) and van den Eijnde *et al.* (1991). Oviposition may be influenced by the kind of social stimulation to which narcotized queens are submitted. Brood cell supply might have accounted for the shorter egg-laying delays reported by Röseler (1985).

Temperature. The short exposure of queens to 34 °C after narcosis did not result in any significant modification of egg-laying delays. Further experiments should assess the influence of temperature on CO_2 efficiency through exposures during narcosis, within the range of 4 °C - 20 °C, so as to avoid lethal high temperatures. In *Drosophila*, Nicolas (1989) reported a reduced anaesthetic potency of CO_2 at high concentration if the temperature was increased. On the contrary, if the temperature varied between 27 °C and 35 °C, CO_2 at low concentrations (1.5%) caused an increased production of the juvenile hormone titre in honeybee workers (Bühler *et al.*, 1983).

Photoperiod. Exposure to artificial light (L8:D16) had a remarkable influence on narcotized bumblebee queens. In other species a number of experiments demonstrated the importance of photoperiod on oviposition: in *Adalia bipunctata* egg-laying onset was possible with L13:D11 and inhibited by L12:D12 (Iperti & Prudent, 1986). *Aelia acuminata* laid eggs earlier in L18:D6 (Hodek, 1974). *Myrmica rubra* workers laid eggs in L16:D8 and L:L more readily than in L8:D16 or in the dark (Hand, 1983). In *Plodia interpunctella* L12:D12 or the dark was more efficient than constant light (Mbata, 1985). More recent studies (unpublished results) confirm the beneficial action of light-dark regimes on overwintered *B. terrestris* queens: L8:D16 stimulated oviposition more effectively than L16:D8, L:L or D:D. In the natural cycle of *B. terrestris*, queens fly and forage for two to three weeks before founding their nest and remaining in the dark. If we consider that the prefounding period in our region lies from the beginning of February to mid-March the day length varies from about 9 to 12h. Presumably the photoperiod L8:D16 is relatively close to the natural regime. Difficulties remain owing to the different spectrum of day-light and fluorescent lamps and to the lack of knowledge on the wave length to which queen's brain is susceptible. It is expected that further studies will provide a better appraisal of light requirements of bumblebees in climate room.

Physiological and side-effects of CO_2 narcosis. Insect metabolism is influenced in various ways by carbon dioxide exposure and by other environmental factors. In *Locusta migratoria* short narcosis induces the same effects as electric stimulation of the pars intercerebralis (Nicolas, 1979). Carbon dioxide concentrates in the central nervous system and affects the amount of neurotransmitters such as dopamine and octopamine (Fuzeau-Braesch & Nicolas, 1981). In *B. terrestris*, Larrere (1993) observed that neurosecretory cells of diapausing queens resumed their activity 72 h after CO_2 narcosis. In the honeybee, histological studies brought evidence of CO_2 effects on neurosecretory cells of brain, *corpora allata* and juvenile hor-

mone titres (Nicolas, 1989). In bumblebees Röseler and Röseler (1984) found that *corpora allata* of narcotized queens became enlarged and produced more juvenile hormone. Their fat reserves for hibernation were not built up and oogenesis had started. The same authors noted opposite results in queenright workers whose oogenesis was inhibited by carbon dioxide. Lum and Flaherty (1972) demonstrated that CO_2 narcosis reduced oviposition and hatchability in *Plodia interpunctella* Ebadi *et al.* (1980) noted adverse effects of CO_2 on honeybee longevity, and Pain *et al.* (1967) found a reduction of the secretion rate of the royal mandibular glands in narcotized honeybee queens. Pomeroy & Plowright (1979) found that CO_2 induced ejection of larvae by bumblebee workers in narcotized colonies. Röseler (1985) reported the emergence of some males among the first workers batch. On the contrary, Mackensen (1947) did not note any change in anaesthetized honeybee queens performances.

Apart from its effects on oogenesis, carbon dioxide used on bumblebee queens may affect some other characteristics. They sometimes produce males instead of workers and their nests may be of smaller size than those of overwintered queens. These detrimental side-effects are worth investigating and a better knowledge of physiological modifications due to CO_2 narcosis would allow setting up new procedures, that will take into account important parameters, such as CO_2 concentration, narcosis timing, temperature, and light.

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