

Diapause and winter survival of two *Orius* species from southern Africa

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Abstract The cold hardiness and overwintering potential of the southern African pirate bugs, *Orius thripoborus* (Hesse) and *Orius naivashae* (Poppius) (Hemiptera: Anthocoridae), were assessed in the laboratory. Diapause traits were studied by observing nymphal development and reproductive performance of adults at 18 °C and three photoperiods (10:14, 12:12 and 14:10 (L:D) h). A 12 h light regime was also tested at 23 °C. A 12 h photoperiod and 18 °C induced reproductive diapause in 84 and 42 % of *O. naivashae* and *O. thripoborus* females, respectively. Cold tolerance of adults was measured by determining the supercooling point (SCP, the temperature at which the insect's body fluids freeze) and lethal time (LT₅₀, the time required to kill 50 % of the population) at 0 and 5 °C. All observed SCPs ranged from −21 to −17 °C. Significantly lower SCP values were observed for

acclimated (seven days at 10 °C) *O. naivashae* females. LT₅₀-values averaged 6.4 and 4.4 days at 0 °C and 11.6 and 7.8 days at 5 °C, for adults of *O. thripoborus* and *O. naivashae*, respectively. The findings indicate that *O. naivashae* is less cold tolerant and has a higher diapause incidence compared with *O. thripoborus*. Therefore, the latter species may have better potential for use in biological control programmes in the cooler regions of southern Africa or elsewhere.

Keywords Cold tolerance · Supercooling point · Diapause · *Orius* · Hemiptera: Anthocoridae

Introduction

Predatory bugs of the genus *Orius* (Hemiptera: Anthocoridae) are used worldwide for the control of different thrips pests (van den Meiracker and

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Ramakers 1991; Riudavets 1995). In southern Africa, both *Orius thripoborus* (Hesse) and *Orius naivashae* (Poppius) are commonly found in open cropping systems providing a refuge for various thrips species, including the sugarcane thrips *Fulmekiola serrata* Kobus, the citrus thrips *Scirtothrips aurantii* Faure, the two avocado thrips pests *Heliothrips haemorrhoidalis* (Bouché) and *Selenothrips rubrocinctus* (Giard), and the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Hesse 1940; Dennil 1992; Hernández and Stonedahl 1999; Way et al. 2006; EPPO 2014). Because of their cryptic lifestyle and fast development of resistance to pesticides, thrips are difficult to control. Moreover, many invasive thrips species may remain active outdoors during mild winters as they often lack obligate diapause (Morse and Hoddle 2006). For instance, *F. occidentalis* has been observed to survive the mild to cold winters in the southern regions of USA and Europe, and in Australia (Kirk and Terry 2003). Therefore, information on the winter ecology of their natural enemies is indispensable in order to achieve an effective biological control. Bonte et al. (2012a) showed that *O. thripoborus* is adapted to a slightly cooler temperature range as compared with *O. naivashae*. Based on a linear degree-day model, lower threshold temperatures for total development were estimated to be 10.2 °C for *O. thripoborus* and 11.6 °C for *O. naivashae*, with thermal requirements of 258.4 and 236.3 degree-days, respectively (Bonte et al. 2012a). Further, very little is known concerning the behavior of *O. thripoborus* and *O. naivashae* during southern African winters. Southern Africa has a wide variety of climatic conditions ranging from Mediterranean in the south-western corner, to temperate on the interior plateau (Highveld), subtropical in the northeast, and desert in the northwest. At higher elevation in the interior part of South Africa, average winter temperatures can be low (e.g., 7 °C in Lesotho Highlands) and occasionally drop to below freezing during winter nights (Brand South Africa 2015; South African Weather Service 2015).

Diapause is an essential life-cycle element underlying the overwintering success of many temperate and colder climate arthropod species (Tauber et al. 1986; Bale and Hayward 2010). In most *Orius* species studied so far, only adult females overwinter in a state of reproductive diapause and day length appears to play a key role in diapause induction in these insects

(van den Meiracker 1994; Ruberson et al. 2000; Musolin and Ito 2008; Kobayashi and Osakabe 2009; Saulich and Musolin 2009). However, for (sub)tropical anthocorids, diapause responses are poorly studied (Nakashima and Hirose 1997; Shimizu and Kawasaki 2001; Saulich and Musolin 2009). In general, insects show a weakened diapause response towards the tropics and subtropics (Tauber et al. 1986; Shimizu and Kawasaki 2001; Saunders 2002; Musolin and Ito 2008; Pazyuk et al. 2014).

In the present study, the cold hardiness and diapause responses of *O. thripoborus* and *O. naivashae* were studied in order to assess their overwintering strategies in southern Africa. Cold hardiness was evaluated by determining the supercooling point (SCP) and lower lethal times (LTs) in the laboratory (e.g., Hart et al. 2002a, b; Hatherly et al. 2005, 2008; Berkvens et al. 2010; Maes et al. 2015; van Damme et al. 2015). SCP measurements evaluate an insect's resistance to a brief cold exposure, whereas LT measurements assess its cold hardiness when faced with a long-term cold exposure (Chown and Terblanche 2006). Further, our study assessed the effect of photoperiod and temperature on the development and reproduction of *O. thripoborus* and *O. naivashae* in order to improve insights in the diapause potential of these predators.

Materials and methods

Stock culture

Cultures of *O. thripoborus* and *O. naivashae* were established in 2008 and 2009, respectively, with nymphs and adults collected in and around sugarcane (*Saccharum officinarum* L.) fields in South Africa, from Umzimkulu (KwaZulu-Natal) to Malelane (Mpumalanga). Stock colonies of both anthocorids were established at Ghent University (Ghent, Belgium) and maintained in climatic cabinets at 25 ± 1 °C, 65 ± 5 % RH, and a 16:8 (L:D) h photoperiod. *Orius* species were cultured in transparent Plexiglas containers (9 cm diameter, 4 cm high) containing a sharp pepper plant (*Capsicum annuum* L. 'Cayenne Long Slim' variety) as a water source and oviposition substrate. The food of nymphs and adults consisted of frozen *Ephestia kuehniella* Zeller eggs (Koppert B.V., Berkel en Rodenrijs, The Netherlands). Adults were also given dry honey bee pollen

(N.V. Weyn's Honingbedrijf, Ghent, Belgium). To reduce cannibalism, a wrinkled piece of wax paper was placed in each container (Bonte and De Clercq 2011).

Diapause induction

To study the diapause responses of *O. thripoborus* and *O. naivashae*, the anthocorids were exposed to three photoperiods at 18 °C during their entire nymphal and adult life. The tested photoperiods are close to the average day lengths that occur during the four seasons in South Africa, i.e. 14:10 (L:D) h in summer, 12:12 (L:D) h in spring and autumn, and 10:14 (L:D) h in winter. The selected temperature (18 °C) represents the mean autumn/spring temperature in the south-western part of South Africa. Relative humidity in the incubators was maintained at 65 %. In all treatments, both nymphs and adults were offered frozen eggs of the flour moth *E. kuehniella* and a flat green bean pod (*Phaseolus vulgaris* L.) was provided as a water source and oviposition substrate. Flour moth eggs and bean pods were refreshed every three days. Eggs (<24 h old) were collected from the stock colony and transferred to an incubator set at 18 °C and one of the three photoperiods. For each treatment, 70–120 nymphs (<24 h old) were then caged in individual plastic containers (4.5 cm diameter, 3 cm high) sealed with a lid having a ventilation hole covered with a fine mesh-gauze. Development and survival of nymphs were monitored daily and newly emerged adults were sexed and weighed using a Sartorius Genius ME 215P balance (Sartorius, Goettingen, Germany). Adults (<24 h old) were paired and transferred to similar plastic containers and placed in the same incubator as during their nymphal life. Bean pods were checked daily for eggs in order to determine the preoviposition period. When the first egg was laid, bean pods were replaced every three days and eggs were counted. On day 20 after adult emergence, supercooling points of females and males were assessed (see below). Afterwards, females were dissected to quantify oocyte development (Callebaut et al. 2004; Bonte et al. 2012b). Ovipositing females and females carrying mature eggs in the ovaries upon dissection were treated as non-diapausing females (Ruberson et al. 1991; Kohno 1997). Additionally, to better understand the process of diapause induction, the experiment was repeated with both species being exposed to a 12:12

(L:D) h photoperiod at 23 °C during their entire nymphal and adult life.

Cold hardiness

The SCP is reached when body fluids of freeze-intolerant individuals freeze in response to exposure to below zero temperatures. However, chill injury caused by temperatures above the insect's SCP can lead to death as well (Bennett and Lee 1989; Denlinger 1991; Bale 1993). Therefore, LTs have been used as an additional index of cold hardiness (Bale et al. 1988; Watanabe 2002) and were quantified at different temperatures, i.e., 0 and 5 °C.

Supercooling point

The supercooling capacity was determined separately for males and females of *O. thripoborus* and *O. naivashae*, subjected to six different experimental conditions. Three treatment groups consisted of 20-day-old adults obtained from the diapause induction experiments at 18 °C and the three different photoperiods. Their responses were compared with those of 20-day-old adults collected from the stock colony (16:8 (L:D) h and 25 °C). Two further groups were set up to test the influence of acclimation. These consisted of seven-day-old acclimated insects (exposed to 10 °C before testing, see section below) and seven-day-old adults directly taken from the stock colony. The SCP was measured using a Picotech TC-08 thermocouple data logger (Pico Technology, UK) and a low temperature programmable Haake Phoenix II CP30 alcohol bath (Thermo Electron Corporation, USA). Insects were placed individually in a 1.0 ml pipette tip with a thermocouple attached to the dorsal side of the body using Vaseline (Unilever, UK). After the pipette tip was sealed with Parafilm M, it was placed in an individual glass test tube and subsequently immersed in the alcohol bath (Berkvens et al. 2010). For each treatment 18–61 adults of each sex were tested. The starting temperature was set at 18 °C (as in the diapause induction experiment), 25 °C (the rearing temperature) or 10 °C (the acclimation temperature) and then lowered to –25 °C at a rate of 0.5 °C min⁻¹. The SCP of each individual was detected by the release of exothermal heat when the insect's body fluids froze.

Lower lethal time

Lower lethal time estimates how many days are needed to kill 10 (LT₁₀), 50 (LT₅₀) or 90 % (LT₉₀) of the tested adults at a certain temperature. Lethal times were determined at 0 and 5 °C for acclimated male and female adults of *O. thripoborus* and *O. naivashae* (see below). Adults were placed in closed polystyrene insect breeding dishes (10 cm diameter, 4 cm high; SPL Life Sciences, Republic of Korea), with a mesh hole (4 cm diameter) in the lid. Throughout the predators' exposure to 0 or 5 °C, eggs of *E. kuehniella* were provided as food, but none of the predators were observed to feed on them. Water was supplied by means of a moist piece of cotton wadding. For exposure to 0 or 5 °C, 20 (*O. naivashae*) or 30 (*O. thripoborus*) breeding dishes each containing eight *Orius* adults (four males and four females) were transferred to climatic cabinets (Type ET 2028, Weiss Technik, Belgium) set at the respective temperatures. No light was provided and RH was not controlled during the cold exposure, although humidity was likely in a range of 55–75 %. After 12 h for *O. naivashae* at 0 °C and after 48 h for all other treatments, two containers of either species were removed from the cabinets every 24 h. To avoid temperature shock, the containers were first held for 1 h at 10 °C in complete darkness. The insects were finally transferred to an incubator set at 25 °C and 16:8 (L:D) h and maintained for 24 h with water but without food, after which survival was recorded. The anthocorids were deemed to have died if they were incapable of moving upon prodding with a fine brush. All adults were allowed to acclimate before being subjected to 0 or 5 °C. For this purpose, newly molted adults (<24 h) from the stock colony (25 °C) were transferred to an incubator set at 10 °C and a photoperiod of 16:8 (L:D) h for seven days. During this period, adults were kept in an insect breeding dish and provided with *E. kuehniella* eggs and a piece of bean pod. Free water was also provided by way of a moist cotton plug fitted into a 1.5 cm (diameter) plastic dish. As for both species no differences in LTs between male and female adults were detected at either temperature, data of males and females were pooled resulting in four remaining data sets.

Statistical analysis

Data analysis was carried out using IBM SPSS Statistics 21 (IBM Corp. 2012). If the data were

continuous and a Kolmogorov–Smirnov test indicated that the data were normally distributed, the parameter was analyzed using analysis of variance (ANOVA). When continuous data were not normally distributed, a non-parametric Kruskal–Wallis test was used. In case of non-continuous data, a generalized linear model was used with a link function and error distribution depending on the nature of the data. Each analysis started with a saturated model and interactions and non-significant main factors were dropped at a significance level of 0.05. Countable data were analyzed using a generalized linear model, with a Poisson distribution if applicable or a negative binomial distribution in case of overdispersion, as determined by the deviance and Pearson goodness-of-fit statistics (Hilbe 2011). If none of the generalized linear models were applicable, a non-parametric model was applied. Parameters expressed as percentages (binary) were compared by means of a logistic regression. This regression is a generalized linear model using a probit link and a binomial error function (McCullagh and Nelder 1989). For all studied parameters, a two-factor analysis was applied using the appropriate model (two-way ANOVA or generalized linear model). In case a factor was found to be significant, a post-hoc analysis was performed to separate means. When a significant interaction between the factors was found, means were compared pairwise. Sex ratios were tested versus an equal female:male distribution (1:1 ratio) by means of χ^2 tests. Lethal times were analyzed using Probit analysis in order to estimate the time required to kill 10, 50 and 90 % of the population at a temperature of 0 and 5 °C. Significant differences were identified by non-overlapping fiducial limits (Hart et al. 2002b).

Results

Diapause induction

A two-factor analysis at 18 °C with *Orius* species and photoperiod as factors indicated no interaction between these factors for the parameters male and female adult weight, preoviposition period and proportion of ovipositing females (Table 1). For the remaining parameters at 18 °C, and for those observed at a 12:12 (L:D) h photoperiod, means were compared pairwise given significant interactions (Tables 2, 3).

At 18 °C, survival rates of nymphs ranged from 80.8 to 92.2 % for *O. thripoborus* and from 44.1 to 74.6 % for *O. naivashae* (Table 2; $\chi^2 = 67.118$, $df = 5$, $P < 0.001$). Hence, at this temperature, nymphal survival of *O. thripoborus* was higher than that of *O. naivashae*, except at a 14 h photoperiod ($\chi^2 = 1.594$, $df = 1$, $P = 0.207$). For *O. thripoborus* nymphs reared at 18 °C, the highest survival occurred under short day conditions (10 h) and the lowest under a 14 h photoperiod. For *O. naivashae* at 18 °C, the lowest nymphal survival (44.1 %) was observed at a 12:12 (L:D) h photoperiod. The highest mortality was recorded during the final (fifth) instar. However, when at the same photoperiod temperature was set to 23 °C, nymphal survival was more than doubled for *O. naivashae* and increased by 10 % as well for *O. thripoborus*, resulting in a similar survival rate of both anthocorids under the latter conditions ($\chi^2 = 0.611$, $df = 1$, $P = 0.434$).

At 18 °C, male and female nymphal developmental times were shorter for *O. thripoborus* than for *O. naivashae* (females: $U = 96.00$, $P < 0.001$; $U = 0.50$, $P < 0.001$ and $U = 10.00$, $P < 0.001$ at 14, 12 and 10 h light, respectively; males:

$U = 157.00$, $P < 0.001$; $U = 2.50$, $P < 0.001$ and $U = 18.00$, $P < 0.001$ at 14, 12 and 10 h light, respectively), and fluctuated between 27.1 and 30.6 days for *O. thripoborus* and between 31.3 and 40.6 days for *O. naivashae*. For *O. thripoborus* reared at 18 °C, the fastest development was observed at a 10-h light regime, whereas development was slowest at 12 h light. For *O. naivashae*, no differences between developmental times were observed when reared at 10 or 14 h light and 18 °C ($U = 425.00$, $P = 0.890$ and $U = 215.00$, $P = 0.135$ for females and males, respectively), but primarily due to a prolongation of the fifth nymphal stadium, nymphal development of *O. naivashae* was extended by 25 % when the predator was maintained at a 12 h day length. At the latter photoperiod and 23 °C, nymphal developmental times of both anthocorids were much shorter than at 18 °C (all $U = 0.00$ and $P < 0.001$), and development of *O. naivashae* was as long as (males: $U = 354.00$, $P = 0.216$) or shorter than (females: $U = 419.00$, $P = 0.001$) that of *O. thripoborus* (Table 2).

No effect of photoperiod on adult weight was observed at 18 °C (Table 1). For all tested photoperiods at the latter

Table 1 Results of a logistic regression or a two-way ANOVA indicating the effect of species (*O. thripoborus* and *O. naivashae*) and photoperiod (14:10, 12:12 and 10:14 (L:D) h) on developmental and reproductive parameters of *Orius* spp. reared at 18 °C

Parameter	Species			Photoperiod			Species × photoperiod		
	F/ χ^2	df	P	F/ χ^2	df	P	F/ χ^2	df	P
Nymphal survival rate ^a	31.131	1	<0.001	12.229	2	0.002	9.164	2	0.010
Female developmental time ^b	356.545	1, 180	<0.001	103.209	2, 180	<0.001	28.210	2, 180	<0.001
Male developmental time ^b	313.682	1, 168	<0.001	118.503	2, 168	<0.001	52.239	2, 168	<0.001
Female adult weight ^b	26.090	1, 178	<0.001	0.029	2, 178	0.972	0.722	2, 178	0.487
Male adult weight ^b	16.782	1, 168	<0.001	1.724	2, 168	0.181	1.809	2, 168	0.167
Preoviposition period ^c	25.156	1	<0.001	6.345	2	0.042 ^e	0.021	1	0.884
Number of oviposited eggs during the first 20 days ^d	16.21	1	<0.001	68.228	2	<0.001	15.775	2	<0.001
Number of oocytes in females dissected on day 20 ^b	0.377	1, 83	0.541	4.510	2, 83	0.014	5.347	2, 83	0.007
Proportion of non-diapausing females ^a	12.986	1	<0.001	12.685	2	0.002 ^f	1.580	2	0.454

^a Probit (Wald χ^2)

^b Two-way ANOVA

^c Poisson (Wald χ^2)

^d Negative binomial (Wald χ^2)

^e Poisson post-hoc test: 14:10 versus 12:12 h: $\chi^2 = 6.344$, $df = 1$, $P = 0.012$; 14:10 versus 10:14 h: $\chi^2 = 1.584$, $df = 1$, $P = 0.208$; 12:12 versus 10:14 h: $\chi^2 = 2.722$, $df = 1$, $P = 0.099$

^f Probit post-hoc test: 14:10 versus 12:12 h: $\chi^2 = 7.689$, $df = 1$, $P = 0.006$; 14:10 versus 10:14 h: $\chi^2 = 0.452$, $df = 1$, $P = 0.501$; 12:12 versus 10:14 h: $\chi^2 = 11.313$, $df = 1$, $P = 0.001$

Table 2 Developmental parameters (mean \pm SE) of *O. thripoborus* and *O. naivashae* at two temperatures and three photoperiods

Temperature (°C)	Photoperiod (L:D) (h)	Species	n ^a	Nymphal survival (%)	Female		Male		Sex ratio (♂:♀) ^c
					Developmental time (days)	Adult weight (mg) ^b	Developmental time (days)	Adult weight (mg) ^b	
18	10:14	<i>O. thripoborus</i>	77	92.2 \pm 3.1a	27.1 \pm 0.19a	41.1 \pm 0.77	27.3 \pm 0.23a	34.5 \pm 0.73	1:1.03
		<i>O. naivashae</i>	71	74.6 \pm 5.2bc	32.8 \pm 0.51d	37.2 \pm 0.88	32.7 \pm 0.67d	30.7 \pm 0.64	1:1.41
	12:12	<i>O. thripoborus</i>	84	85.7 \pm 3.8abB	30.6 \pm 0.35cC	40.9 \pm 0.65A	30.5 \pm 0.25cB	32.8 \pm 0.53A	1:0.64
		<i>O. naivashae</i>	118	44.1 \pm 4.6dC	40.6 \pm 0.57eD	37.7 \pm 0.84B	40.3 \pm 0.66eC	30.8 \pm 0.92AB	1:1.55
23	14:10	<i>O. thripoborus</i>	73	80.8 \pm 4.6bc	29.0 \pm 0.29b	40.5 \pm 0.61	29.5 \pm 0.32b	32.3 \pm 0.61	1:1.18
		<i>O. naivashae</i>	75	72.0 \pm 5.2c	32.7 \pm 0.53d	38.3 \pm 0.62	31.3 \pm 0.32d	31.0 \pm 0.65	1:2.12*
	12:12	<i>O. thripoborus</i>	80	96.8 \pm 2.2A	15.6 \pm 0.19B	40.4 \pm 0.60A	15.5 \pm 0.12A	29.7 \pm 0.54B	1:0.79
		<i>O. naivashae</i>	63	98.8 \pm 1.2A	14.9 \pm 0.13A	41.7 \pm 0.57A	15.3 \pm 0.21A	31.1 \pm 0.50AB	1:2.04*

Means within a column at 18 °C followed by the same lower-case letter are not significantly different ($P > 0.05$); Mann–Whitney U test (developmental times); or binary probit test (nymphal survival)

Means within a column at 12:12 (L:D) h followed by the same upper-case letter are not significantly different ($P > 0.05$); Tukey test (adult weights); Mann–Whitney U test (developmental times); or binary probit test (nymphal survival)

^a Initial number of first instars tested

^b Statistical differences (at 18 °C), as indicated by a two-factor analysis and post-hoc analysis, see Table 1

^c Values with an asterisk differ significantly from a 1:1 ratio; χ^2 and P values were 0.906, 0.014; 0.216, 1.528; 0.515, 0.424; 0.001, 10.383; 0.059, 3.556; 0.123, 2.373; 0.370, 0.803; and 0.002, 9.228, respectively (χ^2 test, df = 1)

Table 3 Reproductive parameters (mean \pm SE) of *O. thripoborus* and *O. naivashae* at two temperatures and three photoperiods

Temperature (°C)	Photoperiod (L:D) (h)	Species	n ^a	Preoviposition period (d) ^b	No. of oviposited eggs during the first 20 days ^c	No. of oocytes dissected on day 20 ^c	Proportion of non-diapausing females (%) ^b
18	10:14	<i>O. thripoborus</i>	31	10.00 \pm 0.50	32.6 \pm 4.3a	6.53 \pm 0.53a	83.9 \pm 6.7
		<i>O. naivashae</i>	21	15.50 \pm 1.23	2.1 \pm 0.9c	4.23 \pm 0.82bc	57.1 \pm 11.1
	12:12	<i>O. thripoborus</i>	26	11.73 \pm 0.86B	30.7 \pm 4.4aC	6.00 \pm 0.57aA	57.7 \pm 9.9B
		<i>O. naivashae</i>	19	— ^d	0.0 \pm 0.0dD	9.67 \pm 3.38acA	15.8 \pm 8.6C
23	14:10	<i>O. thripoborus</i>	27	9.11 \pm 0.63	32.2 \pm 5.7a	6.18 \pm 0.73ab	74.1 \pm 8.6
		<i>O. naivashae</i>	25	13.78 \pm 1.00	7.0 \pm 2.0b	3.43 \pm 0.54c	56.0 \pm 10.1
	12:12	<i>O. thripoborus</i>	26	5.32 \pm 0.60A	89.1 \pm 9.6A	6.7 \pm 0.79A	88.5 \pm 6.4A
		<i>O. naivashae</i>	25	6.68 \pm 0.32A	54.9 \pm 5.4B	5.7 \pm 0.66A	88.0 \pm 6.6A

Means within a column and at 18 °C followed by the same lower-case letter are not significantly different ($P > 0.05$); generalized linear model with negative binomial distribution (oviposited eggs); or Mann–Whitney *U* test (oocytes in dissected females)

Means within a column and at 12:12 (L:D) h followed by the same upper-case letter are not significantly different ($P > 0.05$); generalized linear model with Poisson distribution (preoviposition period); Mann–Whitney *U* test (oviposited eggs; oocytes in dissected females); or binary probit test (non-diapausing females)

^a Initial number of first instars tested

^b Statistical differences at 18 °C are indicated by a two-factor analysis and post-hoc analysis, as presented in Table 1

^c Only females were included which produced at least a single egg and/or had oocytes on day 20 after emergence

^d No eggs were deposited in this treatment

temperature, *O. naivashae* males and females were heavier than those of *O. thripoborus*. However, when reared at a 12 h photoperiod, *O. naivashae* females were heavier at 23 than at 18 °C ($F = 6.958$, $df = 3$, 135 , $P < 0.001$), whereas for *O. thripoborus* males the opposite was observed ($F = 5.825$, $df = 3$, 120 , $P < 0.001$) (Table 2).

Sex ratios of *O. naivashae* were female biased at a 14 h day length at 18 °C and at 12 h light and 23 °C. For all other treatments, no significant deviations from a 1:1 sex ratio were observed (Table 2).

Preoviposition period at 18 °C was influenced by species and photoperiod (Table 1). At this temperature, first eggs were always laid earlier by *O. thripoborus* than by *O. naivashae* females. *O. naivashae* females did not oviposit during the 20-day observation period at a 12 h day length and 18 °C, although 16 % of them showed a preoviposition period exceeding 20 days. This regime led to the longest preoviposition period for *O. thripoborus* and *O. naivashae*, although only for the latter species a significant difference between its preoviposition periods at 12 and 14 h light was found. At 23 °C and 12 h light, preoviposition periods of both *Orius* species were similar ($\chi^2 = 3.394$, $df = 1$, $P = 0.065$) and about twice shorter than those observed for *O. thripoborus* at 18 °C and 12 h light ($\chi^2 = 25.393$, $df = 1$, $P < 0.001$ and $\chi^2 = 44.006$, $df = 1$, $P < 0.001$ for *O. naivashae* and *O. thripoborus*, respectively) (Table 3).

In *O. thripoborus* females at 18 °C, the number of eggs produced within the first 20 days of adult life did not differ between the three photoperiods. This number was also higher than egg numbers produced by *O. naivashae* females at both temperatures ($\chi^2 = 96.399$; $df = 5$; $P < 0.001$) (Table 3). Likewise, the number of oocytes in females dissected on day 20 at 18 °C was higher for *O. thripoborus* than for *O. naivashae*, except at a 12 h light period. At the latter regime, oocyte counts in dissected *O. naivashae* females were as high as those in *O. thripoborus* females ($U = 14.00$, $P = 0.302$), despite the fact that the former species did not oviposit during the first 20 days after adult emergence. For *O. naivashae*, differences in the number of oviposited eggs occurred between photoperiods, but the total potential egg production by day 20 (i.e., numbers of oviposited eggs plus oocyte counts) was not affected ($F = 0.062$, $df = 2$, 86 , $P = 0.939$). At 23 °C, a 12 h photoperiod led to similar oocyte numbers as those counted at

18 °C for both species ($\chi^2 = 2.128$, $df = 3$, $P = 0.546$), but more eggs were oviposited by day 20 at the higher temperature ($U = 55.00$, $P < 0.001$ and $U = 0.00$, $P < 0.001$ for *O. thripoborus* and *O. naivashae*, respectively) (Table 3).

Species and photoperiod influenced the proportion of non-diapausing females at 18 °C (Table 1). At this temperature, the relative number of egg producing couples was higher in *O. thripoborus* than in *O. naivashae*, ranging from 57.7 to 83.9 % in *O. thripoborus* and from 15.8 to 57.1 % in *O. naivashae*. Whereas for both anthocorids no difference in the proportion of non-diapausing females between the photoperiods 10 and 14 h was recorded, a day length of 12 h at 18 °C was associated with the lowest proportion of ovipositing females. However, at 12 h light and 23 °C, the proportion of egg producing females was equally high in both *Orius* species ($\chi^2 = 0.003$, $df = 1$, $P = 0.959$).

Cold hardiness

Supercooling point

Supercooling points of *Orius* males and females were affected by experimental conditions ($F = 8.017$, $df = 5$, 310 , $P < 0.001$ and $F = 16.717$, $df = 5$, 359 , $P < 0.001$ for males and females, respectively), but not by species ($F = 1.709$, $df = 1$, 310 , $P = 0.192$ and $F = 3.295$, $df = 1$, 359 , $P = 0.070$, respectively). The interaction between both factors was significant ($F = 4.086$, $df = 5$, 310 , $P = 0.001$ and $F = 4.346$, $df = 5$, 359 , $P = 0.001$ for males and females, respectively). At all tested conditions, no differences were found between average SCP values of *O. thripoborus* and *O. naivashae* (Table 4). When reared at 25 °C and a 16 h light regime, SCP values of (non-acclimated) seven- and 20-day-old adults did not differ for both species and sexes. At 18 °C, no differences in SCP values were detected between the three tested regimes, for both species and sexes. Lowest SCP values were found in acclimated adults of both species, but test significances among SCPs at non-acclimating conditions were inconsistent. Whereas SCP values of acclimated seven-day-old females of *O. naivashae* were significantly lower compared to those of non-acclimated seven-day-old females, no differences were found between acclimated and non-acclimated *O. naivashae* males.

Table 4 Supercooling points (SCP) (mean \pm SE) of females and males of *O. thripoborus* and *O. naivashae* reared under six different conditions

Conditions			Species	Female		Male	
Photoperiod (L:D) (h)	Temperature ($^{\circ}$ C)	Age (d)		n ^a	SCP ($^{\circ}$ C)	n ^a	SCP ($^{\circ}$ C)
10:14	18	20	<i>O. thripoborus</i>	32	-18.79 ± 0.29 bc	30	-19.80 ± 0.30 ac
			<i>O. naivashae</i>	27	-18.03 ± 0.24 cd	21	-19.07 ± 0.36 bc
12:12	18	20	<i>O. thripoborus</i>	26	-19.21 ± 0.57 acd	27	-19.14 ± 0.28 c
			<i>O. naivashae</i>	18	-17.09 ± 0.33 d	18	-19.53 ± 0.48 ac
14:10	18	20	<i>O. thripoborus</i>	29	-18.91 ± 0.33 ac	24	-19.76 ± 0.47 ac
			<i>O. naivashae</i>	21	-18.32 ± 0.42 bcd	23	-18.41 ± 0.34 c
16:8	25	20	<i>O. thripoborus</i>	28	-17.16 ± 0.34 de	30	-18.71 ± 0.24 c
			<i>O. naivashae</i>	26	-17.85 ± 0.33 cd	25	-18.28 ± 0.61 bc
16:8	25	7	<i>O. thripoborus</i>	61	-18.32 ± 0.18 cd	30	-19.07 ± 0.37 bc
			<i>O. naivashae</i>	39	-18.70 ± 0.27 bce	27	-20.51 ± 0.24 ab
16:8/Darkness ^b	25/10 ^b	7	<i>O. thripoborus</i>	30	-20.16 ± 0.33 ab	33	-21.01 ± 0.24 a
			<i>O. naivashae</i>	34	-20.47 ± 0.31 a	34	-20.03 ± 0.37 ac

Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tamhane test)

^a Number of adults tested

^b Acclimated treatment group, consisting of newly molted adults (<24 h) from the stock colony (25 $^{\circ}$ C and 16:8 (L:D) h), transferred to an incubator set at 10 $^{\circ}$ C and a photoperiod of 16:8 (L:D) h for seven days before determination of SCP

Acclimated seven-day-old *O. thripoborus* adults had lower SCP values than those of non-acclimated seven-day-old males and females and of 20-day-old adults of both sexes reared at 25 $^{\circ}$ C and a 16 h photoperiod. Also, the SCP of seven-day-old *O. thripoborus* males was higher when they were transferred from 18 $^{\circ}$ C and 12 h light without acclimation as compared with those which were allowed to acclimate at 10 $^{\circ}$ C ($F = 5.566$, $df = 11, 310$, $P < 0.001$).

Lower lethal time

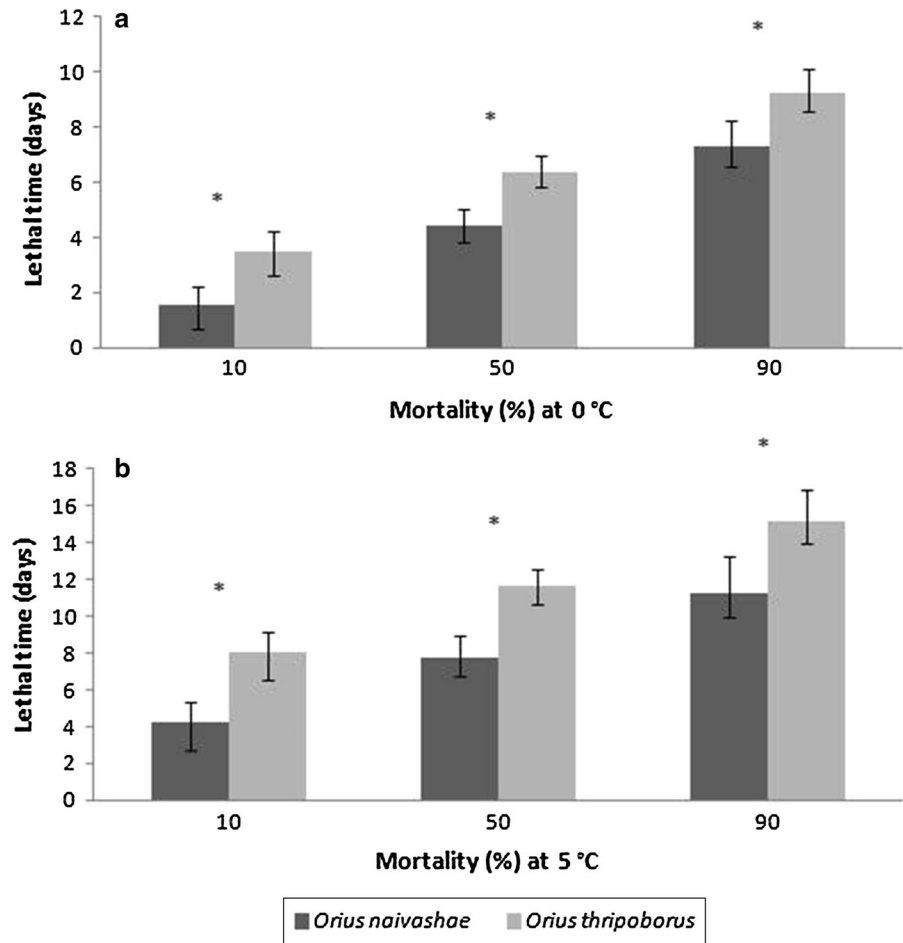
Adults of both *Orius* spp. survived longer at 5 $^{\circ}$ C (Fig. 1b) than at 0 $^{\circ}$ C (Fig. 1a), but LTs were overall lower in *O. naivashae* than in *O. thripoborus*. At 5 $^{\circ}$ C, the time required to kill 50 % of the population of *O. naivashae* and *O. thripoborus* was 7.8 and 11.6 days, respectively, whereas at 0 $^{\circ}$ C it took 4.4 and 6.4 days, respectively.

Discussion

In the subtropics, where the mean winter temperature falls close to the developmental threshold of a

particular species very occasionally, the benefit of diapause varies from year to year, and therefore the species may not achieve and maintain accurate seasonal synchronization and pronounced adaptation to local seasonal conditions (Masaki 1990). In many *Orius* species the photoperiodic response becomes weaker towards the (sub)tropics and some populations do not enter diapause at all (Tommasini and Nicoli 1995, 1996; Ito and Nakata 2000; Shimizu and Kawasaki 2001; Musolin and Ito 2008; Saulich and Musolin 2009). In the present study, however, the low percentage of egg producing females of *O. naivashae* reared at 18 $^{\circ}$ C and a 12 h day length indicated a relatively high incidence of reproductive diapause in this species, at least under some environmental conditions. Although not a single *O. naivashae* female produced eggs within 20 days at the latter conditions, 16 % of females had vitellogenic oocytes in their ovarioles upon dissection. These females were not considered to be in reproductive diapause (Musolin and Ito 2008), but demonstrate a delayed oviposition. Conditions of 18 $^{\circ}$ C and 12 h light, representing the southern African autumn, induced diapause in twice as many females of *O. naivashae* as in those of *O. thripoborus* (84 % as compared with 42 % of females,

Fig. 1 Lethal times (LT₁₀, LT₅₀ and LT₉₀) (means \pm 95 % fiducial limits) at 0 °C (a) and 5 °C (b) for acclimated adults of *O. thripoborus* and *O. naivashae*. Asterisks indicate significant differences in lethal time values among species



respectively). At 18 °C, shorter and longer days induced reproductive diapause in 43 and 44 % of the *O. naivashae* population, respectively, whereas for *O. thripoborus* diapause incidence remained below 26 %. Most *Orius* species enter diapause under short-day conditions, but critical day length varies from species to species as well as within the same species for populations occurring at different latitudes (Tauber et al. 1986; Danks 1987; Leather et al. 1995; Shimizu and Fujisaki 2006; Musolin and Ito 2008). Based on our results, critical day length for diapause induction in the studied populations of *O. naivashae* and *O. thripoborus* appears to be around 12 h. As shorter days did not increase the proportion of diapausing females, we assume that, when the autumn photoperiod prevails in South Africa, temperature further enhances diapause induction and then prevents premature diapause termination in these species.

Photoperiodic control of developmental time is a well known phenomenon in many insects (Saunders 2002; Beck 1980). Short day lengths have been observed to cause decelerating or accelerating effects to some extent in a number of heteropteran species (e.g., Ruberson et al. 1991; Musolin and Saulich 1997; Lopatina et al. 2007; Saulich and Musolin 2009), but can also lead to a pronounced prolongation of development in other true bugs (Kiritani 1985; Musolin and Saulich 1997; Tanaka and Zhu 2003). In this study, a 12 h light regime at 18 °C prolonged development of *O. naivashae*, accompanied by a low nymphal survival, mainly in the last instar. However, at 23 °C development of *O. naivashae* at a 12 h photoperiod was more successful and data were comparable with those reported for this species in previous studies performed under long day conditions (16:8 (L:D) h) and 23 °C (Bonte et al. 2012a, b). At

18 °C, short day conditions accelerated growth of *O. thripoborus* nymphs compared to long days, whereas for *O. naivashae* no differences in developmental times were observed. Short-day acceleration of nymphal growth at low temperatures (18–20 °C) has been reported earlier in some *Orius* species (Ruberson et al. 1991; van den Meiracker 1994; Musolin et al. 2004; Musolin and Ito 2008), but overall trends were inconsistent and likely geographically driven (Tauber et al. 1986; Tommasini and Nicoli 1995, 1996). These seasonal adaptations are always local and differ among species and populations (Saulich and Musolin 2009).

Lethal times (50 % mortality) were 6.4 and 4.4 days at 0 °C and 11.6 and 7.8 days at 5 °C, for acclimated adults (males and females combined) of *O. thripoborus* and *O. naivashae*, respectively. To our knowledge, lower LTs have not been documented for any anthocorid species although responses to near-zero temperatures have been studied only in a few *Orius* species (Ito and Nakata 1998a; Bueno et al. 2014; Zhang et al. 2008). Despite similar SCP values for both species, recorded LT values suggest that *O. thripoborus* is more cold tolerant than *O. naivashae*. Several factors related to the experienced rearing conditions or origin of the population under study may influence an insect's cold tolerance. Maes et al. (2012) noted that acclimation period, infection status with endosymbionts and diet may influence the supercooling ability of the predator *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae). Also physiological changes associated with diapause, reproductive maturation or ageing can affect an insect's cold tolerance (Bowler and Terblanche 2008; Saulich and Musolin, 2009). In the present study, no influence of adult age on SCP was found, and values recorded for males and females were similar. Females in treatment groups showing higher diapause incidence did not have lower supercooling points. The only factor influencing the SCP was the seven-day-acclimation of adults at 10 °C. A similar trend towards lower SCP values for acclimated individuals was found in *M. pygmaeus* (Maes et al. 2012). However, for another predatory mirid bug, *Nesidiocoris tenuis* Reuter, no significant decrease in SCP was detected after acclimation for seven days at 10 °C (Hughes et al. 2009). Also bacteria can affect an insect's freezing tolerance as they may act as heterogeneous ice nucleators inside the body of their host (Lee et al. 1991; Worland and

Block 1999). As a result, intracellular freezing may occur at higher temperatures when hosts are infected with bacterial endosymbionts. Maes et al. (2012) reported that *M. pygmaeus* infected with *Wolbachia pipientis* and *Rickettsia* spp. had higher SCP values than uninfected conspecifics. *Wolbachia* sp. was also detected in the studied *O. naivashae* population (J. Bonte, unpublished data). It is hypothesized that these bacterial endosymbionts are involved in the female biased sex ratios of *O. naivashae* observed in this and previous studies (Bonte et al. 2012a, 2015). It remains to be investigated whether SCPs of uninfected adults are lower than those measured in the present study. It again deserves emphasis that SCP data alone are not sufficiently reliable and comprehensive indicators of cold tolerance since the vast majority of species are freeze-avoiding and SCP temperatures are rarely experienced by individuals in their natural habitats (Bale 1996). Lethal time data are therefore believed to best indicate naturally occurring cold stress as they do not only test temperature but also exposure time (Allen 2010).

In many *Orius* species studied so far, only females (and usually fertilized ones) can properly accumulate fat body and enter diapause to ensure successful overwintering, whereas males usually mate before winter and do not survive until spring (Ito and Nakata 1998a, b; Shimizu and Kawasaki 2001; Kobayashi and Osakabe 2009; Saulich and Musolin 2009). However, in this study, LTs did not differ between *Orius* males and females and it is thus expected that both sexes survive southern African mild winters with reasonably similar success. During field observations at the end of winter (July) in the provinces Mpumalanga and KwaZulu-Natal male and female adults as well as nymphs of both *Orius* species were collected (J. Bonte, unpublished data). This implies that, in these areas, at least some nymphs and adults of both sexes remain active in winter and do not enter reproductive diapause, or that diapause in these individuals is very weak and/or short. Along with the results of the present laboratory study, these field observations further suggest that winter diapause is not likely to be a critical trait in *O. thripoborus* and *O. naivashae*, at least in populations occurring in regions with less pronounced temperature extremes. Diapause may, however, be more prevalent in populations of these anthocorids occurring in cooler regions. Further, it is worth noting that research on overwintering strategies

based solely on laboratory populations may not be representative of field situations. Prolonged laboratory rearing of *O. thripoborus* and *O. naivashae* at a 16 h photoperiod and 25 °C could have weakened their diapause response and tolerance to cold conditions.

Previous studies have shown that *O. thripoborus* is adapted to a slightly cooler temperature range as compared with *O. naivashae* (Bonte et al. 2012a) and that the overall performance of *O. thripoborus*, in terms of its developmental rates, reproduction and predation capacity, is generally superior to that of *O. naivashae* at moderate temperatures (Bonte et al. 2012a, b, 2015). Our present findings indicate that *O. naivashae* is less cold tolerant and has a stronger tendency to enter reproductive diapause as compared with *O. thripoborus*. In regions of southern Africa where average autumn/winter temperatures are around or below 18 °C, *O. thripoborus* thus appears to have stronger potential for use in biological control than *O. naivashae*. However, for successful biocontrol programs using these anthocorids not only species-specific (e.g., type of diapause), but also population-specific diapause-related traits (e.g., critical day length) should be taken into consideration (Musolin et al. 2004). Our laboratory study indicates that a combination of a 12 h day length and low temperatures (e.g., 18 °C) induces (weak) diapause in *O. thripoborus* and *O. naivashae*, but year-round field observations are warranted to elucidate whether the studied predators remain active and are able to contribute to the suppression of crop pests in different parts of southern Africa during different parts of the season.

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