Comparison of low temperature adaptation ability in three native and two hybrid strains of the rotifer *Brachionus plicatilis* species complex

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ABSTRACT: Low temperature adaptation ability of five selected strains of *Brachionus plicatilis* species complex: Japanese (NH1L), Australian, German, and two hybrid strains (♀NH1L and ♂Australian; *N*× *A*, and ♀NH1L and ♂German; *N*× *G*) was investigated with life history, reproductive characteristics and mobility under different thermal conditions at 12 and 25℃. The life history parameters at 12℃ showed longer lifespans, reproduction periods and generation times with reduced lifetime egg and offspring productions compared to those at 25℃. At 12℃, the intrinsic rate of natural increase was higher in NH1L and *N*× *A* strains. Reproductive characteristics determined at 12℃ with batch culture showed active population growth for NH1L and *N*× *G* strains, while no resting egg production was observed in all the tested strains. The ratio of swimming rotifers at 12℃ was monitored every hour for 6 hours (short-term) and every day for 10 days (long-term). NH1L strain maintained over 81.3% of swimming, while other strains exhibited low rates (< 60 %) in the short-term observations. In the long term observations, NH1L and two hybrid strains showed over 75% of swimming rate from the initial day. The obtained results recommended that outcrossing of rotifer strains is useful to obtain live food resources for cold water fish larviculture.

***KEYWORDS*: *Brachionus plicatilis* sp. complex; Low temperature adaptation; Life history; Swimming activity**

INTRODUCTION

The rotifer *Brachionus plicatilis* sp. complex including *B. plicatlis* sensu stricto and *B. manjavacas* is widely used in aquaculture facilities as live food for the initial stage of larviculture [1]. For effective larviculture, live food species should remain at a certain level in the water column of larval rearing tanks, while rotifers lose their availability in cold water temperatures that are needed for the proper larval growth of cold water species. Temperature is known to be a significant factor that controls rotifer physiological conditions related to life history [2], survival [3], reproduction [4] and mobility [5]. The rotifer *Brachionus* has a wide range of temperature tolerance, but active population growth and locomotion can occur temperatures ranging from 25 to 30℃ [2, 3, 5]. Under low temperature conditions (typically below 20℃), the rotifers showed inactivity in the aforementioned parameters [2, 5, 6], but larvae of cold water species such as Japanese flounder *Paralichthys olivaceus* [7], Pacific cod *Gadus macrocephalus* [8] and Atlantic cod *Gadus morhua* [9] must be cultured at 11-16℃ during the initial feeding within 2 days after yolk exhaustion to obtain optimal growth. To prevent cold shock by rapid thermal decrease, the rotifers should be cultured at the same temperature as the larviculture [10]. The cold shock of rotifers results in a low survival rate and a decreased mobility of rotifers (*i.e.*, sinking to the bottom of larval rearing tanks or sluggish movement) [10, 11]. These symptoms should make the rotifers unavailable for cold water fish larvae in aquaculture facilities [3, 5, 12]. However, rotifers are difficult to mass-culture at low temperature and show stagnation (or decreasing trends) of population growth [11, 13]. Given the above factors, rotifer strains with strong tolerance to low temperature are required for an efficient larval rearing of cold water fishes.

The present study was established in response to the requirement for appropriate live food for cold water fish larvae. To date, the low temperature effects on physiological conditions of rotifers were considered for preservation and explaining negative effects on population growth [3, 11, 14]. The low temperature tolerance appears to differ among various species and strains of the rotifer *Brachionus* [2]. In addition, the hybrid strains obtained by cross-mating of two strains of the rotifer *Brachionus* sp. showed distinct biological characteristics from their parental strains [4, 12]. Therefore, we employed three native strains (NH1L, Australian and German) and two hybrid strains (♀NH1L and ♂Australian; *N*× *A*, and ♀NH1L and ♂German; *N*× *G*) for this study (Table 1). Rotifers can use alternative strategies to maintain their normal metabolism under excessive stress conditions. Their metabolic activities under stressful conditions are mainly assessed by life history parameters, reproductive characteristics and mobility [15-18]. This study compared the low temperature adaptation ability of the employed rotifer strains in the *B. plicatilis* s. s., *B. manjavacas*, and their hybrids in terms of their (1) life history parameters, (2) reproductive characteristics (*i.e.*, sexual and asexual reproduction) and (3) mobility (swimming activity) that were investigated under the different thermal

conditions at 12 and 25℃.

MATERIALS AND METHODS

Strain selection

To determine potential strains from the rotifer collection in our laboratory, four strains of *B. plicatilis* s. s., three strains of *B. manjavacas*, and two strains of their hybrid (Table 1) were pre-cultured at 20℃ and a salinity of 17 ppt (parts per thousand) under ad libitum food condition for one month. These strains were collected from different geographic regions and were empirically known to have a strong tolerance to low temperature compared to other stock strains. We tested the effect of low temperature on the population growth rate of these strains. The population growth at 10℃ was estimated through 14-day batch culture with or without thermal acclimation at 15℃ under the optimal food condition for population growth of these strains, at 7×10^6 *Nannochloropsis oculata* cells/ml [18]. The culture medium was prepared by diluting natural seawater with Milli-Q water (Millipore 0.22μm) followed by GF/C filtration and autoclave sterilization at 121℃ for 20 min. The microalgae *N. oculata* cultured in modified Erd-Schreiber medium [19] was centrifuged with cell grown medium at 5000 rpm for 10 min, and only precipitated algal cells were re-suspended daily into the rotifer culture medium. To compare asexual reproductive activity at low temperature, the population growth rate (r) on the last day of culture was calculated using the following formula: $r=\ln(N_t/N_0)/t$, where N_0=initial density of rotifers, N_t=the number of individuals on day t, t= culture days. Based on these results (Fig. 1 in the results of *Rotifer strain selection*), the low temperature adaptation ability evaluated by the life history, reproductive characteristics and mobility at 12℃ (described below) were compared among the following five selected strains: three native strains *i.e.*, Japanese (NH1L), Australian, German, and two hybrid strains *i.e.*, *N*× *A* (♀NH1L and ♂Australian), *N*× *G* (♀NH1L and ♂German).

Individual culture

The individual cultures of the five selected rotifer strains were carried out to determine the effects of low temperature on the following life history parameters: (1) lifespan, (2) reproduction period, (3) generation time, (4) lifetime egg production and (5) lifetime offspring production. With these data, (6) the intrinsic rate of natural increase (r_m) was calculated by the following formula: $\sum l_x \cdot m_x \cdot e^{-r_m x} = 1$, where x=age interval, e=natural logarithm, lx=the

probability of surviving to age x, m_x=the number of female offspring per female of age x born during the interval x to x+1.

The rotifers were cultured at 25℃ and 17 ppt with daily feeding of *N. oculata* at 7×10^6 cells/ml. From these cultures, a certain number of rotifers carrying amictic eggs were transferred into a screw capped bottle containing 15 ml of fresh culture medium, and then agitated to shake off their eggs. About 200 of the separated eggs were each incubated in a well of a 12-well microplate under the same conditions as the stock cultures. Hatchlings (F_1) carrying an amictic egg were individually inoculated into a 48-well microplate, and F_2 (< 4 h) hatched from these eggs were acclimatized to the experimental temperature. To initiate experimental culture, F_2 individuals were individually transferred into a well of 24-well microplate containing 1 ml of fresh medium suspending *N. oculata* at 7×10^6 cells/ml. The experimental set-up was established with the same methods as described in the *Strain selection* section. The inoculated rotifers (n=3) of each strain were cultured at 25 or 12℃ under complete darkness and were transferred daily into a new well containing fresh medium. The observation of the life history parameters was consecutively made with the rotifer transference.

Batch culture

Batch cultures were initiated with five F_2 individuals carrying amictic eggs in 5 ml of food suspension which were prepared by the same methods as described in the part of *Individual culture*. These cultures were maintained in the microalgae *N. oculata* suspension at the constant concentration of 7×10^6 cells/ml without changing the medium for 14 days. A culture trial was maintained in a well of a 6-well microplate and was incubated at 25 or 12℃ under complete darkness (n=6). The number of female rotifers in each culture was counted daily with the following categories based on the types of eggs carried [14]: female carrying no eggs, amictic female carrying female eggs (FF); mictic female carrying male eggs (MF); mictic female carrying resting eggs (RF). The r was calculated by the same formula as mentioned in the *Strain selection* ($r=\ln(N_t/N_0)/t$), and mixis and fertilization rates were calculated using the following formula:

Mixis (%) = {(MF+RF)/(FF+MF+RF)}×100

Fertilization (%) = {(RF)/(MF+RF)}×100.

Swimming activity

The cultures of the five selected strains were maintained under the same culture conditions as the previous tests. From the culture of each strain, 50 individuals were randomly selected and transferred into a well of 12-well microplate containing 3 ml of fresh medium at 17 ppt and 12℃. The microplates containing the rotifers were wrapped around with foamed styrol to prevent thermal fluctuation of medium. These were adapted to complete darkness for 5 min and then the swimming rotifers were immediately counted. After the counting, the plates were maintained at 12℃ until the next observation. As a short term observation for 6 hours, the number of swimming rotifers was monitored every hour. For a long term observation for 10 days, the whole rotifer cultures (at 25℃) were transferred to 12℃ and were monitored every day with the same methods as the short term observation. Swimming rate (%), which is the proportion of swimming individuals among the total individuals, was estimated by the average of 5 independent replicates (n=5).

Statistical analysis

For the selection of rotifer strain, the population growth rate of the nine rotifer strains at low temperature associated with a treatment *i.e.*, with or without the acclimation were compared by Tukey-Kramer *post hoc* test, when a one-way ANOVA detected a significant difference. The indices of life history parameters, reproductive characteristics, and swimming rate of the 5 selected strains between the set-up temperatures (at 12 and 25℃) was compared using Tukey-Kramer *post hoc* test after one-way ANOVA. All statistical analyses were performed by StatView version 5.0 software (SAS Institute, Inc., USA).

RESULTS

Rotifer strain selection

The population growth rate (r) with or without the thermal acclimation is shown in Figure 1. For the 14-days cultures at 10℃ without the acclimation, the Shizuoka and the Australian strains showed negative growth rates compared to the 7 other strains (P<0.05, Fig. 1a). In the culture trials with the acclimation at 15℃, the Shizuoka strain was extinct during the acclimation. The other strains cultured at 10℃ showed no significant differences in population growth rate (Fig. 1b). The NH1L, Amami, and Russian strains maintained their population growth rate at low temperature, but the five other strains: Makishima, Australian, German, $N\times A$, and $N\times G$, needed the acclimation (P<0.05) to maintain their

population growth rate. Through the comparison of population growth rate, the rotifer strains which showed active propagation at 10℃ with acclimation (Australian, German, *N*× *A*, and *N*× *G*) and without acclimation (*i.e.*, NH1L) were selected to evaluate low temperature tolerance.

Individual culture

Table 2 shows life history parameters of the rotifer strains at different culture temperatures (12 and 25℃). There were no significant differences in the lifespan and the generation time among the all strains cultured at 25℃. On the other hand, other parameters showed differences at 25℃. The reproduction period was the longest in the German strain (P<0.05). The lifetime egg production (P<0.05) and lifetime offspring production (P<0.05) were the highest in the Australian strain. The intrinsic rate of natural increase (r_m) at 25℃ was the highest in the Australian strain (P<0.0001). When rotifers were cultured at 12℃, there were no significant differences in the reproduction period, lifetime egg production, and lifetime offspring production among the tested strains. For the other parameters at 12℃, the longest lifespan and generation time were observed in the German strain (P<0.0001), and the highest r_m was in the NH1L and the *N*× *A* strains (P<0.0001).

Batch culture

The characteristics of sexual and asexual reproductions determined by batch cultures under different water temperature (12 and 25℃) are shown in Table 3. For asexual reproduction, the *N*× *A* strain most actively propagated for 14 days at 25℃ (P<0.0001), while the NH1L and *N*× *G* strains exhibited higher population growth rate at 12℃ (P<0.05). Activity of sexual reproduction was defined by the following 4 parameters: mixis rate, male density, fertilization rate, and the total number of produced resting eggs. These parameters were higher in the Australian strain at 25℃ (P<0.001). For the two hybrid strains at 25℃, the *N*× *A* showed the abnormality of un-hatched mictic eggs (male eggs) and no resting egg production in the *N*× *G* strain even males being presented. At 12℃, the 5 tested strains showed no resting egg production. Higher mixis rate and male density at 12℃ were observed in the NH1L and *N*× *A* (P<0.001), and in the Australian culture (P<0.05), respectively.

Swimming activity

The swimming activity of the tested rotifer strains at 12℃ is shown in Figure 2. In the short-term observations (for 6

h), a higher proportion of swimming individuals (81.3±6.7 to 91.3±5.3%) was observed in the NH1L strain (P<0.05). The other strains showed < 60% swimming rates during the observation period (P<0.0001). In the long-term observations (for 10 d), NH1L, *N*× *A*, and *N*× *G* showed higher swimming rates (79.2±4.6 to 93.2±5.2%) than the other strains (P<0.0001) after one day from the beginning of observation at 12℃. The five tested strains showed over 75% of swimming rate on the last day of long-term observation.

DISCUSSION

This study compared the low temperature adaptation ability of various rotifer strains of *B. plicatilis* s. s., *B. manjavacas* and their hybrids by testing life history parameters, reproductive characteristics and mobility at 12℃, which can provide information to help supplying a suitable live food for effective larviculture of cold-water fishes. The outcrossing between two different species: *B. plicatilis* s. s. (NH1L) and *B. manjavacas* (Australian and German), was possible by a close phylogenetic distance [20]. Such hybrid strains show different morphological and physiological characteristics from their parents [4, 12]. In this study, the strains even in the same species showed different low temperature adaptation ability. For strain selection, the population growth of nine rotifer strains (Table 1) was determined at 10℃ with or without thermal acclimation at 15℃. Acclimation is important for survival at low temperature [6], and thus most strains showed higher growth rate after acclimation (Fig. 1). However, the Shizuoka strain did not survive during the acclimation, and experienced mortality at 10℃ without acclimation. It may imply that the critical temperature for survival of the Shizuoka strain is higher than for the other strains, which may be around 20℃. No differences associated with the acclimation were observed with the NH1L, Amami and Russian strains. These differences might reflect the geographic features of their habitats, because organisms are adapted to the ambient conditions where they live [21, 22]. Miracle and Serra [2] reported that temperature ranged as rotifer habitat had negligible influences on their physiological conditions, while low temperature induces species– and strain–related properties because of the restricted temperature tolerances of rotifers. The results of this study showed that there are strain–related properties (Table 2) and these may be affected by the temperature ranges of their original habitats.

Life history parameters, a tool for diagnosing the physiological condition of rotifers [23, 24], were evaluated by the individual culture of each strain. The tested rotifers at 12℃ (Table 2) had similar propensities when they were exposed to extreme stresses [17, 25]. In the rotifer *Brachionus* sp., low temperature prevented normal metabolism as shown by the expression of the *hsp*-20 at 10℃, which is for cellular defense under environmental stress conditions [26]. The embryonic development period of rotifers is elongated by decreasing temperature [27], and thus it probably retards

242 reproduction. The calculated r_m evaluates the reproductivity of animals [28]. In this study, the r_m value at 25℃ was
243 similar to that reported by King and Miracle [29] under favorable conditions (0.32-0.38), while that at 12℃ was below
244 0.11. This implies that the physiological status of the rotifers in our study was kept at good condition at 25℃, which
245 resulted in the active reproduction. The highest value of r_m at 12℃ was shown by the NH1L and $N \times A$ strains (Table 2)
246 and shows the possibility of mass-culture of rotifers at low temperatures [30].
247 Batch cultures of the selected rotifer strains were tested to evaluate the possibility of mass culture at low temperature.
248 The intrinsic feature (e.g., thermal tolerance) derived from the temperature of maternal culture, determines the sexual
249 reproduction characteristics of rotifer descendants [31]. Therefore, this study employed F_2 rotifers from mothers
250 cultured under the same environmental conditions to reduce the hereditary effects. Thermal fluctuation to low
251 temperature was reported to negatively affect sexual [31] and asexual reproduction [3] of rotifers. This study showed
252 that sexual reproduction of rotifers was negatively affected by low temperature that caused an inactive mixis induction
253 and no resting egg production, compared to asexual reproduction (Table 3). The asexual reproduction of the five
254 tested rotifer strains maintained a certain level of population growth. The required level of food can be varied with
255 culture temperature to maintain stable population growth of rotifers [13]. It is expected that populations of the selected
256 rotifer strains could maintain steady growth at the low temperature because of being sufficiently fed. Active
257 propagation was observed in the batch culture of NH1L associated with the determined r_m at the low temperature (Table
258 2). The hybrid strains, $N \times A$, and $N \times G$ strains also showed high population growth rates, perhaps due to the inherited
259 genetic background from the NH1L strain [12].
260 The frequency and duration of immobility after thermal fluctuation determines the efficiency as live foods for fish
261 larvae [5, 32]. The short term observation for 6 h was set up to compare with the study by Fielder et al. [32]. They
262 reported that *B. plicatilis* should be cultured at lower temperature or acclimated for at least 6 h to larval rearing
263 conditions before transfer to larval rearing tanks. In this study, the NH1L strain showed the highest swimming activity
264 during the short term observation (Fig. 2a), suggesting its potential to be used as live food for cold water species.
265 Moreover, in the long term observation, the two hybrid strains originated from the NH1L mothers also showed over 60%
266 of mobility on the first day (Fig. 2b). This phenomenon may also indicate that two hybrid strains are better adapted to
267 low temperatures by inherited characters from their mother compared to the other strains [12].
268 Recently, the application of rotifer strains acclimated to low temperatures has been used to acquire economic benefits
269 in the hatchery production of the cold water fish, Pacific cod *Gadus macrocephalus* [8]. The present study
270 demonstrated that the low temperature adaptation ability of the rotifer *B. plicatilis* sp. complex is indeed strain–related.
271 Among the five tested strains in this study, the NH1L strain has the highest ability to adapt at low temperature and is
272 expected to maintain their availability in the larval rearing tanks for the optimal growth of cold water aquaculture

species [5, 10, 32]. In addition, the outcrossing of rotifer strains is useful to obtain live food resources for cold water fish larval rearing.

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Tables

Table 1 Origin of the nine strains of *Brachionus plicatilis* sp. complex from the rotifer collection of Nagasaki University that were used in this study

Strain (Species)		Origin
Japanese (*Bp*)	NH1L	Japanese eel culture pond in 1970s (Hagiwara et al., 1994)
	Amami	National Center for Stock Enhancement, Fisheries Research Agency in Kagoshima, Japan
	Makishima	Fisheries Research Agency in Makishima, Japan
	Shizuoka	National Center for Stock Enhancement, Fisheries Research Agency in Shizuoka, Japan
Russian (*Bm*)		Russia (Snell et al., 1995)
Australian (*Bm*)		Marchland, Albany in Australia
German (*Bm*)		Schlei-Fjord in Germany (Kotani et al., 2006)
Hybrid	*N*× *A*	Hybrid between the NH1L (♀) and the Australian (♂)
	N× *G*	Hybrid between the NH1L (♀) and the German (♂)

Bp: *Brachionus plicatilis* sensu stricto
Bm: *Brachionus manjavacas*

Table 2 Life history parameters of the five selected strains of rotifer *Brachionus plicatilis* sp. complex at 25 and 12℃

	℃	NH1L	Australian	German	*N*× *A*	*N*× *G*
Lifespan (d)	25	14.6±5.5	14.3±4.4	12.4±2.5	12.2±5.4	14.5±4.8
	12	28.6±11.1^{b}	35.4±11.8^{b}	46.8±5.1^{a}	32.0±6.1^{b}	39.0±11.7^{ab}
Reproduction period (d)	25	7.0±2.0^{ab}	7.6±0.6^{ab}	8.0±1.8^{a}	5.9±1.0^{b}	7.0±1.0^{ab}
	12	21.3±10.1	23.7±10.6	28.7±5.2	23.3±5.2	30.8±10.9
Generation time (d)	25	1.8±0.5	1.5±0.2	1.7±0.3	1.7±0.1	1.6±0.2
	12	4.9±0.4^{c}	7.3±0.8^{b}	11.0±1.6^{a}	5.6±1.1^{c}	7.0±0.1^{b}
Lifetime egg production	25	20.1±4.4^{c}	26.6±1.7^{a}	22.4±2.1^{abc}	21.4±4.4^{bc}	23.4±1.7^{ab}
(eggs)	12	14.9±6.1	15.3±5.8	15.7±2.9	16.5±2.6	18.8±4.9
Lifetime offspring	25	19.6±4.9^{c}	26.3±1.5^{a}	22.1±2.4^{abc}	21.2±4.2^{bc}	23.1±1.7^{ab}
production (ind.)	12	14.4±5.9	13.5±5.5	15.2±3.0	15.0±3.5	18.0±4.6
Intrinsic rate of natural	25	0.30±0.01^{c}	0.36±0.01^{a}	0.34±0.00^{b}	0.34±0.01^{b}	0.32±0.01^{bc}
increase (r_m)	12	0.11±0.00^{a}	0.08±0.01^{b}	0.06±0.00^{c}	0.10±0.00^{a}	0.08±0.00^{b}

Values are means ± SD (n=3). Superscripts denote significant differences (a>b>c) in each life history parameter at a certain temperature (P<0.05).

Table 3 Reproduction parameters of the five selected strains of rotifer *Brachionus plicatilis* sp. complex at 25 and 12℃ for 14 days

	℃	NH1L	Australian	German	*N*× *A*	*N*× *G*
Population growth (*r*)	25	0.36 ± 0.01^{c}	0.37 ± 0.01^{bc}	0.36 ± 0.00^{bc}	0.38 ± 0.00^{a}	0.37 ± 0.00^{b}
	12	0.27 ± 0.01^{a}	0.26 ± 0.02^{ab}	0.24 ± 0.01^{b}	0.25 ± 0.01^{ab}	0.26 ± 0.01^{a}
Mixis (%)	25	4.4 ± 0.9^{bc}	14.6 ± 3.4^{a}	11.7 ± 3.7^{a}	7.3 ± 1.2^{b}	0.6 ± 0.8^{c}
	12	16.0 ± 3.0^{a}	3.8 ± 2.3^{b}	7.2 ± 2.3^{b}	15.2 ± 4.4^{a}	6.9 ± 1.2^{b}
Male (ind./ml)	25	1.1 ± 0.2^{b}	3.9 ± 1.1^{a}	0.4 ± 0.3^{bc}	0^{c}	0.01 ± 0.01^{c}
	12	0.2 ± 0.1^{ab}	0.3 ± 0.3^{a}	0.1 ± 0.2^{ab}	0^{b}	0.02 ± 0.02^{ab}
Fertilization (%)	25	17.6 ± 10.3^{b}	34.9 ± 9.1^{a}	12.1 ± 12.7^{bc}	0^{c}	0^{c}
	12	0	0	0	0	0
Resting egg (total No.)	25	1.7 ± 2.0^{b}	52.3 ± 17.3^{a}	5.3 ± 7.8^{b}	0^{b}	0^{b}
	12	0	0	0	0	0

Values are means ± SD (*n*=6). Superscripts denote significant differences (a>b) in each reproduction parameter at a certain temperature ($P<0.05$).

Figure legends

Fig. 1 Population growth rate of the nine rotifer strains of *Brachionus plicatilis* sp. complex cultured at 10℃ for 14 days without (a) or with acclimation at 15℃ (b) for 14 days. Columns and error bars describe means and standard deviations of replication (n=5-6), respectively. Small alphabetic letters (a>b>c in the panel A) indicate significant differences among the rotifer strains (P<0.05) and asterisks (in the panel B) are differences between two treatments without and with acclimation (P<0.05).

Fig. 2 Swimming rate (% of 50 individuals) of the five selected rotifer strains of *Brachionus plicatilis* sp. complex at 12℃ with 6 hours of short (a) and 10 days of long (b) term observation. Plots and error bars describe means and standard deviations of replications (n=5), respectively. Small alphabetic letters (a>b>c>d) indicate significant differences among the rotifer strains (P<0.05).

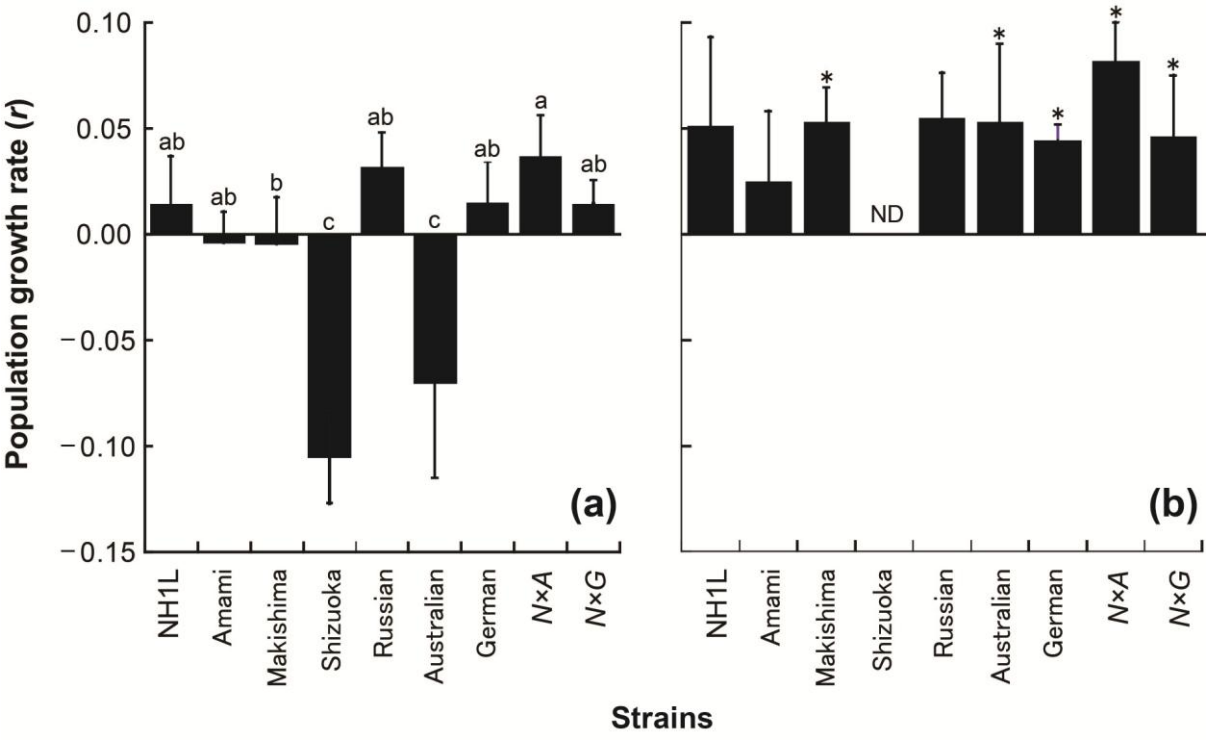
Population growth rate (r)
0.10
0.05
0.00
−0.05
−0.10
−0.15
ab
ab
b
c
ab
c
ab
a
ab
(a)
ND
*
(b)
NH1L
Amami
Makishima
Shizuoka
Russian
Australian
German
N×A
N×G
Strains

Fig. 1

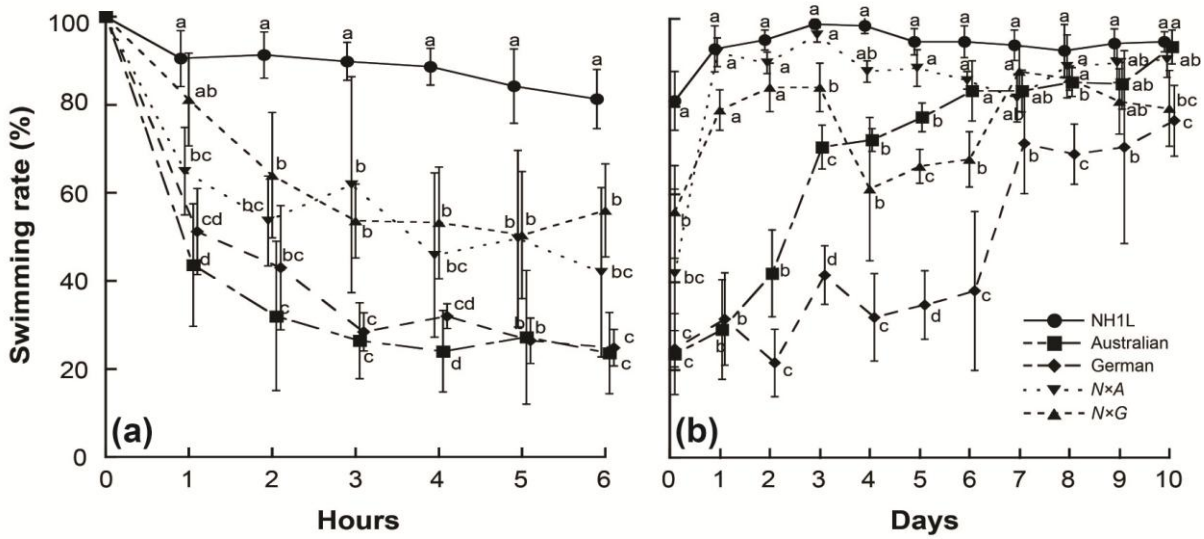

Fig. 2