The effects of trail following on the distance between opposite sex and the effect of its benefit on the evolution of trail following in Pacific abalone *Haliotis discus hannai*

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Background. Aggregation of animals affects their fertilization rate in species that utilize external fertilization. However, the process of aggregation has not been widely examined in many species or using theoretical models. The Pacific abalone *Haliotis discus hannai*, which is an externally fertilizing gastropod species, shows aggregation in the wild. This study was conducted to evaluate whether mucus trail following shortens the distance between members of the opposite sex. We also examined whether the fertilization rate increased by mucus trail following is an evolutionary driving force in mucus following behavior. **Methods.** Whether *H. discus hannai* follows the trail mucus of another individual was tested using y-maze. The distance between members of the opposite sex of the abalones that followed the trail mucus was compared to that of abalones that did not follow the trail mucus using an individual-based model consistent with the behavior of *H. discus hannai.* Finally, to examine whether mucus trail following evolved to shorten the distance between members of the opposite sex , simple population genetic models of a diploid population undergoing nonoverlapping, discrete generations was constructed. **Results.** *Haliotis discus hannai* chose the arm with trail mucus more frequently than the arm without trail mucus, regardless of the sex of the abalone that secreted the mucus and reproductive season. In the model, the distance between the opposite sex was shortened by the mucus trail compared to without mucus trail following; however, the difference in distance between opposite sex members was only several centimeters. Additionally, simple population genetic models indicated that the shortening effect of the distance between the opposite sex members was a weak evolutionary driving force in mucus trail following. **Conclusions.** These results suggest that behavior of mucus trail following evolved as a mechanism to increase the fertilization rate; however, the increased fertilization rate was weak evolutionally driving force in mucus trail following .

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Abstract

Background. Aggregation of animals affects their fertilization rate in species that utilize external fertilization. However, the process of aggregation has not been widely examined in many species or using theoretical models. The Pacific abalone *Haliotis discus hannai*, which is an externally fertilizing gastropod species, shows aggregation in the wild. This study was conducted to evaluate whether mucus trail following shortens the distance between members of the opposite sex. We also examined whether the fertilization rate increased by mucus trail following is an evolutionary driving force in mucus following behavior.

Methods. Whether *H. discus hannai* follows the trail mucus of another individual was tested using y-maze. The distance between members of the opposite sex of the abalones that followed the trail mucus was compared to that of abalones that did not follow the trail mucus using an individual-based model consistent with the behavior of *H. discus hannai.* Finally, to examine whether mucus trail following evolved to shorten the distance between members of the opposite sex, simple population genetic models of a diploid population undergoing nonoverlapping, discrete generations was constructed.

Results. *Haliotis discus hannai* chose the arm with trail mucus more frequently than the arm without trail mucus, regardless of the sex of the abalone that secreted the mucus and reproductive season. In the model, the distance between the opposite sex was shortened by the mucus trail compared to without mucus trail following; however, the difference in distance between opposite sex members was only several centimeters. Additionally, simple population genetic models indicated that the shortening effect of the distance between the opposite sex members was a weak evolutionary driving force in mucus trail following.

Conclusions. These results suggest that behavior of mucus trail following evolved as a mechanism to increase the fertilization rate; however, the increased fertilization rate was weak evolutionally driving force in mucus trail following.

Introduction

Studies of the Allee effect in external fertilization species is important for population management because decreasing the density of adults decreases the fertilization rate (Berec, Angulo & Courchamp, 2007). Previous studies indicated that water flow (Babcock & Keesing, 1999), spawning synchrony (Calabrese & Fagan, 2004), and the distance between opposite sex (Babcock & Keesing, 1999; Levitan, Sewell & Chia, 2016) affect egg fertilization. Although the distance between individuals of the opposite sex decreases naturally with increasing population density, distance can also decrease because of aggregation, defined as the result of animals remaining close together. Models including aggregation showed higher rates of fertilization rate compared to models that did not consider aggregation at the same population density (Claereboudt, 1999; Lundquist & Botsford, 2004; Zhang, 2008). The process of aggregation has not been widely examined in many species or by using theoretical models. It is important to understand the cues used by individuals to recognize individuals of the opposite sex. For instance, when individuals visually recognize other individuals, the encounter rate is low at low population densities; if individuals recognize each other though olfaction, they can search for the opposite sex from a distant location (Jumper, & Baird, 1991). Although a model has been developed that considers the distance over which individuals can recognize opposite sex (Baird & Jumper, 1995; Coates & Hovel, 2014), the cue used by animals for recognition remains unclear. Gastropod species follow mucus trails likely to encounter the opposite sex, as they prefer to follow mucus from the same species (Nakashima, 1995) and opposite sex (Erlandsson & Kostylev, 1995; Johannesson et al., 2010; Ng et al., 2011). However, whether the encounter rate of opposite sex is increased by mucus trail following has not been tested in any species.

Thus, the encounter rate of the opposite sex should be compared between individuals that follow a mucus trail and those that do not.

In *Haliotis* species, a gastropod species that uses external fertilization, the effect of the distance between members of the opposite sex and aggregation on the fertilization rate have been investigated as important factors in population management. It is thought that male sperm release and female egg release are not completely synchronized in *Haliotis* species: based on spawning behavior in aquaculture systems, males release sperm over several hours while females release eggs intermittently during male sperm release (Uki & Kikuchi, 1983). Similar asynchronous spawning behavior between males and females was also observed in wild population of *H. kamtschatkana* (Breen & Sloan, 1988). Because spawning of *Haliotis* species occurred even in the absence of the opposite sex in a tank (Uki & Kikuchi, 1983) and field (Breen & Adkins, 1980), these species may spawn in response to environmental changes rather than upon recognition of the opposite sex and spawn in synchronization with males and females. Aggregation during spawning behavior was observed in *Haliotis* species (Breen & Adkins, 1980; Stekoll & Shirley, 1993), possibly as a mechanism to shorten the distance between members of the opposite sex and increase the fertilization rate during asynchronous spawning.

In *H. discus hannai*, although spawning may occur under stormy condition (Sasaki & Shepherd, 2001), aggregation is observed even in the absence of spawning（see supplemental Fig. S1）. This aggregation behavior may shorten the distance between members of the opposite sex and increase the fertilization rate of asynchronous spawning in *H. discus hannai*. It is probable that these aggregations are formed by mucus following because they can not reach the origin of smell using water-borne chemicals (Uchida et al., 2010). This study was conducted to examine the relationship between mucus trail following and distance between the opposite sex. First, whether *H. discus hannai* follows the mucus trail of another individual was tested in a tank. Next, whether mucus trail following can shorten the distance between members of the opposite sex was tested. The distance between opposite sex abalones who do not follow trail mucus may be shortened by abalones who follow trail mucus when both locomotion types coexist; thus, shortening of the distance between members of the opposite sex may not be the evolutionary driving force of mucus trail following. Therefore, whether abalones that follow trail mucus produce more offspring than those that do not was tested using an individual-based model.

Materials & Methods

Study species

Haliotis discus species is a marine gastropod mollusk in the family *Haliotidae*. Japanese *H. discus* species consists of *H. discus hannai* (Ezo abalone) and *H. discus discu.* This species inhabits our study site in Iwate prefecture in Japan (Hara & Sekino, 2005).

Following the mucus trail of another individual

To evaluate whether *H. discus hannai* follows a mucus trail, binary choice tests were conducted using a y-maze on December 11–28, 2015, August 17–20, 2016, and June 26–July 17, 2017. The y-maze consisted of 50-cm stem and 50-cm arms (width, 9 cm; height, 5 cm). The details of the experimental abalones are described in Supplemental data S1. Experiments were conducted as follows.

Step 1: To attach the trail mucus to the surface of the y-maze, one arm of the y-maze was closed using a plastic plate where individuals (marker) were allowed to move (right arm: n = 38, left arm: n = 37). The marker individual often began creeping within 1 h and reached the end of arms of the y-maze. After reaching the end of arm, the marker was removed, and the closed arm was opened. If marker individuals remained at the starting point for over a day, they were replaced with a new marker individual.

Step 2: The tested individual (follower) was released at the starting points and the direction of creeping was observed. Step 2 was started at 18:00 because *H. discus hannai* are active after sunset. Followers often reached the end of the arms within several minutes. If a follower remained at a star point for over 30 min, the direction of creeping was observed using a video camera until 6:00.

The effects of marker-tracker combinations and season on the mucus trail following rates were tested using a generalized liner model (binomial distribution) with likelihood ratio tests using the “Anova” function in the car package R v3.5.2. The marker-tracker combinations and season did not affect the mucus trail following rates (see Results). Additionally, statistics type II error may have occurred because of the small sample number. Therefore, whether abalones tend to creep in arms with mucus trail of another abalone was determined using a binomial test without dividing the abalone into experimental groups.

Mucus trail following shortens the distance between members of the opposite sex

To examine whether mucus trail following shortens the distance between members of the opposite sex, an individual-based model consistent with the behavior of *H. discus hannai* was constructed (see also supplemental Fig. S2 and supplemental file S1) using NetLogo 5.3.1. (https://ccl.northwestern.edu/netlogo/). The script are available on GitHub with file name; supplemental file S1 (https://github.com/YMatsumoto5536/PeerJ-netlogo). In this model, one model female abalone that followed the mucus (mutant females), one model female abalone that did not follow the mucus (wild females), and model male abalone that did not follow the mucus (wild males) were generated in the field. The distance between wild males of the mutant females and wild females were compared; this comparison revealed whether mucus trail following is effective for shortening the distance between opposite sex members from the aspect of female benefit, however, did not consider the distance between wild males and mutant females is shortened by mucus trail following of mutant females.

Space and time of individual based models: The size of the patch was 10 × 10 cm and the field in the models was constructed from 30 ×10 patches (i.e., 3 m^2 field size). Model abalones were generated in the field. The field was a closed system; when model abalones reached a boundary, they turned 180° in a random direction and began moving. Each tick corresponds to one day.

Rules of model abalones behaviors: Model abalones moved simultaneously according to the following procedure. The behaviors of mutant females were based on movement distance in the tank (see Supplemental Fig. S4) and the mucus trail following rate. The mucus trail following rate, irrespective of sex combination and season (binomial distribution: trail number = 75, number of arms with mucus = 60, estimated following rate = 0.8, Table 1), was applied in the model because the effects of the sex combination of mucus on the following rate were small. All types of abalones left the mucus on the patches while moving (see details Fig. 1). The distance moved was determined for each individual/day by random number following the exponential distribution (mean = 48.76 patches). Before the mutant females moved by 1 patch (10 cm), they checked the presence of mucus at the patches in front of them. If mucus was present within the search range, mutant female moved to one of the patches with mucus according to the mucus trail following rate. This behavior was repeated until reaching the determined locomotion distance. Although the wild female and wild male moved the same distance as the mutant female, the direction of creeping was random. Some studies have suggested that the trail mucus of gastropods is degraded by bacteria within a day (Herndl & Peduzzi, 1989; Peduzzi & Herndl, 1991). Therefore, the functional period of the mucus was set to 1 day in this study. After moving for 30 days in the model, the nearest neighbor distance of each female was measured. The difference between the distance of mutant females and wild females was compared using the generalized liner mixed model (Gaussian distribution) using the “lmer” function in the lmer package R v3.5.2. To conduct paired comparison within simulations, simulation number was treated as a random-intercept in the model. The effects of locomotion type on the nearest neighbor distance was tested with likelihood ratio tests using the “Anova” function in the car package.

Whether mucus trail following evolved to shorten the distance between opposite sex members

To examine whether the abalone evolved the ability to follow mucus trails mucus to shorten the distance between members of the opposite sex, we constructed simple population genetic models for a diploid population undergoing nonoverlapping, discrete generations. However, *H. discus hannai* undergoes repeated spawning for several years. In this model, mutant abalone that followed the mucus trail and wild abalone that did not follow the mucus trail were evaluated for one autosomal locus with two alleles, Dominant (D) and recessive (d). Individuals with “DD” and “Dd” followed the mucus trail, while individuals with “dd” did not. The population size was set to 10,000 because beneficial traits are removed from population by random genetic drift processes when the population size N is small; however, the random genetic drift effect could not be completely excluded. Therefore, in addition to the model in which individuals with DD and Dd followed the mucus trail (mucus trail following model), a control model in which individuals with DD and Dd did not follow the mucus was constructed to eliminate the effects of random genetic drift. At the start of simulation, 9990 model abalones with dd and 10 model abalones with Dd were generated. Model abalones were generated at male to female ratio of 1:1. The field in the models was constructed from 1000 ×1000 patches (i.e., 10,000 m^2 field size). The behavioral rules of mutant type and wild-type were the same as in the model described above. After 30 days in the model, the nearest neighbor distance between members of the opposite sex were measured. The generation of fertilized eggs by each female based on the nearest neighbor distance between males was determined by the following equation: Fertilization = $88.31^{-0.32 \times distance}$, as described by Babcock and Keesing (1999) (Babcock & Keesing, 1999). The rate of mutant type and wild-type in the new generation (n + 1) was determined based on the ratio of the number eggs of each type of the previous generation (n). This cycle was repeated for 100 generations and the number of each allele was counted. The details of the simulation are described in Supplemental data S3 and model are available on GitHub with file name;Supplemental file S2 and S3 (https://github.com/YMatsumoto5536/PeerJ-netlogo).

ResultsThe

Following the mucus trail of another individual

Mucus trail following rates in each experiment are shown in Table. 1. The combination of marker and follower combinations (likelihood-ratio test, $df = 4$, $\chi^2 = 2.68$, $p = 0.61$), season ($df = 1$, $\chi^2 = 0.026$, $p = 0.87$), and their interaction term ($df = 3$, $\chi^2 = 0.36$, $p = 0.95$) did not affect the mucus trail following rate. The abalones chose the arm with a mucus trail more frequently than the arm without a mucus trail when the abalone were not divided into experimental groups (binomial test, $p = 1.588e\text{-}07$).

Mucus trail following shortened the distance between opposite sex members

The distance between wild males and mutant females was shorter than that between wild males and wild females ($df =1$, $F = 6.90$, $p = 0.009$); however, the estimation value of the difference in distance was only 2.4 (±SD = 0.8) cm.

Whether mucus trail following evolved to shorten the distance between opposite sex members

In the mucus trail following model, the number of D alleles (Fig. 1a, see also supplemental data S4) increased with the generation at a rate of 34.6% (346/1000 simulations). In the control model, D alleles (Fig. 1b, see also supplemental data S5) also increased within the population at a rate of 27.3% (273/1000 simulations). The proportion of the simulation in which the D allele increased within the population significantly differed between the mucus trail following model and control model (2-sample test for equality of proportions without continuity correction, $\chi^2 = 12.47$, $df = 1$, $p = 0.000414$); however, the 95% confidence interval of the proportion difference was 3.26–11.3%.

Discussion

Following the mucus trail of another individual

Our experiments strongly suggested that the mucus trail of another individual affected the decision regarding the creeping direction of *H. discus hannai.* The mucus trail of *H. discus hannai* attracted their larvae (Roberts, 2001); thus, it is not surprising that adult individuals also reacted to the mucus trail. Individuals touched the bottom of the tanks using their tentacles during creeping; thus, the tentacles may function as a sensory organ. The tentacles of *H. asinina* react to extracted protein from mucus trails *in vitro* (Kuanpradit et al., 2012). This result in *H. asinina* supports that *H. discus hannai* also searched for the mucus trails using their tentacles. In this study, *H. discus hannai* followed the mucus irrespective of the marker sex and season. Selective following of mucus trail from the opposite sex would indicate that this behavior evolved to shorten the distance between members of the opposite sex as in other species (Ng et al., 2011). In this study, because the focal abalone did not allow for the selection of mucus from different sexes simultaneously, we can not deny the possibility that *H. discus hannai* could choose the mucus from opposite sex. While, sex-related differences in mucus components have not been detected in *H. asinina*, a *Haliotis* species. Therefore, the preference for mucus from the opposite sex may not occur in *H. discus hannai* even if the focal abalone can choose the mucus from different sexes simultaneously. Although the preference for mucus from the opposite sex in *H. discus hannai* was not detected in this study, mucus trail following may shorten the distance between opposite sex members more efficiently than creeping without mucus trail following.

Mucus trail following shortens the distance between members of the opposite sex

Although some previous studies suggested that mucus trail following increases the encounter rate of the opposite sex members (Ng et al., 2013), no studies have shown definitive results. Previous studies only investigated the rate of mucus trail following rather than the function of this behavior. This study shows that the distance between opposite sex members was shortened by mucus trail following compared to creeping without mucus trail following using an individual-based model. However, the distance between members of the opposite sex of mutant females was longer than that of wild females in 4907 of 10,000 simulations. Additionally, the distance between opposite sex members of wild males was shortened by following of mutant females, indicating that the fertilization of wild males can increase without mucus trail following. Therefore, the benefit of increasing the fertilization rate may be weak as an evolutionary driving force of the trail mucus following, although the difference in the distance between opposite sex members was significant.

Locomotion patterns may have affected the distance between males and females by more than mucus trail following in our model. The random number generated from the probability distribution of the locomotion distance in *H. discus hannai* consisted of lévy flight (Supplemental Fig. S4), which is an effective searching behavior when the target is sparsely distributed (Sims et al., 2012). The locomotion pattern in another *Haliotis* species was found to involve lévy flight in the wild (Strain, Johnson & Thomson, 2013); therefore, the locomotion pattern of *H. discus hannai* in the wild may also involve lévy flight. This study focuses on mucus trail following as a method of shortening the distance between members of the opposite sex; however, other behaviors such as locomotion patterns should also be investigated in the future.

Whether mucus trail following evolved to shorten the distance between members of the opposite sex

In some cases, the D allele increased within the population in the mucus trail following model (346/1000 simulations) and its proportion was higher than that in the control model (273/1000 simulations). These results indicate that increasing the fertilization rate is the evolutionary driving force in the mucus trail following in our model, although the benefits were small. The d allele was not perfectly removed from all simulations although the number of d alleles decreased within the population (Fig. 1a). This is because the d allele was present in Dd; thus, individuals with dd were removed from population because of their low fertilization rate, while Dd individuals were not removed from the population and showed a high fertilization rate as mutant abalone. This study indicates that the distance between members of the opposite sex was shortened (i.e., increasing fertilization rate) by mucus trail following and that the alleles expressed during mucus trail following increased within the population in an individual-based model.

The D allele disappeared within the population in 654 of 1000 simulations at the start of simulations. This may be because the wild-type abalone, which did not follow the mucus trail, can shorten the distance between members of the opposite sex when the mutant type followed the mucus trail of the wild-type. Thus, the few D alleles were removed from the population quickly the occasion when the shortening effect was not effective. This suggests that other benefits of mucus trial following may be required to begin increasing the D allele. For example, mucus trail following decreased the energy cost for adhesion and locomotion; gastropods species require energy to secrete a mucus trail to adhere and creep on the substrate (Davies & Hawkins, 1998). However, an individual following a mucus trail can decrease the amount of the mucus being secreted (Davies & Blackwell, 2007; Hutchinson et al., 2007). *Haliotis discus hannai* may follow mucus to save the energy required for secreting mucus rather than to increase the fertilization rate, as the mucus trail was followed and mucus was secreted even in the non-reproductive season. The evolutionary driving force of mucus trail following in *H. discus hannai* may be clarified in studies considering the benefits of this behavior.

Conclusions

This study indicates that trail mucus affects the direction of creeping in *H. discus hannai*. Additionally, the possibility that mucus trail following evolved as a mechanism to increase the fertilization rate was indicated by the individual-based model. This study also indicates that increasing the fertilization rate is not only evolutionary driving force of mucus trail following because the D allele, which is expressed during mucus trail following, were removed from the populations in 654 of 1000 simulations. The findings showing that *H. discus hannai* follow the mucus trail regardless of the reproductive season and that both sexes secreted mucus also supported the presence of an evolutionary driving force other than increasing the fertilization rate.

Estimating the aggregation and nearest neighbor distance is important for the population management of *Haliotis species* (Button, 2008) because these factors affect egg fertilization rate (Babcock & Keesing, 1999). The aggregation size in the wild can be reproduced by using a process in which individuals follow the mucus trail of each other rather than a process in which individuals remain in a preferred spot on a rock in *Nodilittorina unifasciata* (Stafford, Davies & Williams, 2007). An individual based model constructed based on the mucus trail following may be helpful for predicting the aggregation of *H. discus hannai* if mucus following is more beneficial than choosing a preferred location based on environmental characteristics. The location preference based on environmental characteristics and its benefits should be tested in the wild to apply our individual-based model to population management.

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Figure 1 (on next page)

Figure 1

(A) Change in frequency of allele D in the mucus model and (B) Change in frequency of allele D in the control model. (C) The comparison of the number of case that allele D increase and the case that allele D lost in the mucus model. (D)The comparison of the number of case that allele D increase and the case that allele D lost in the control model.

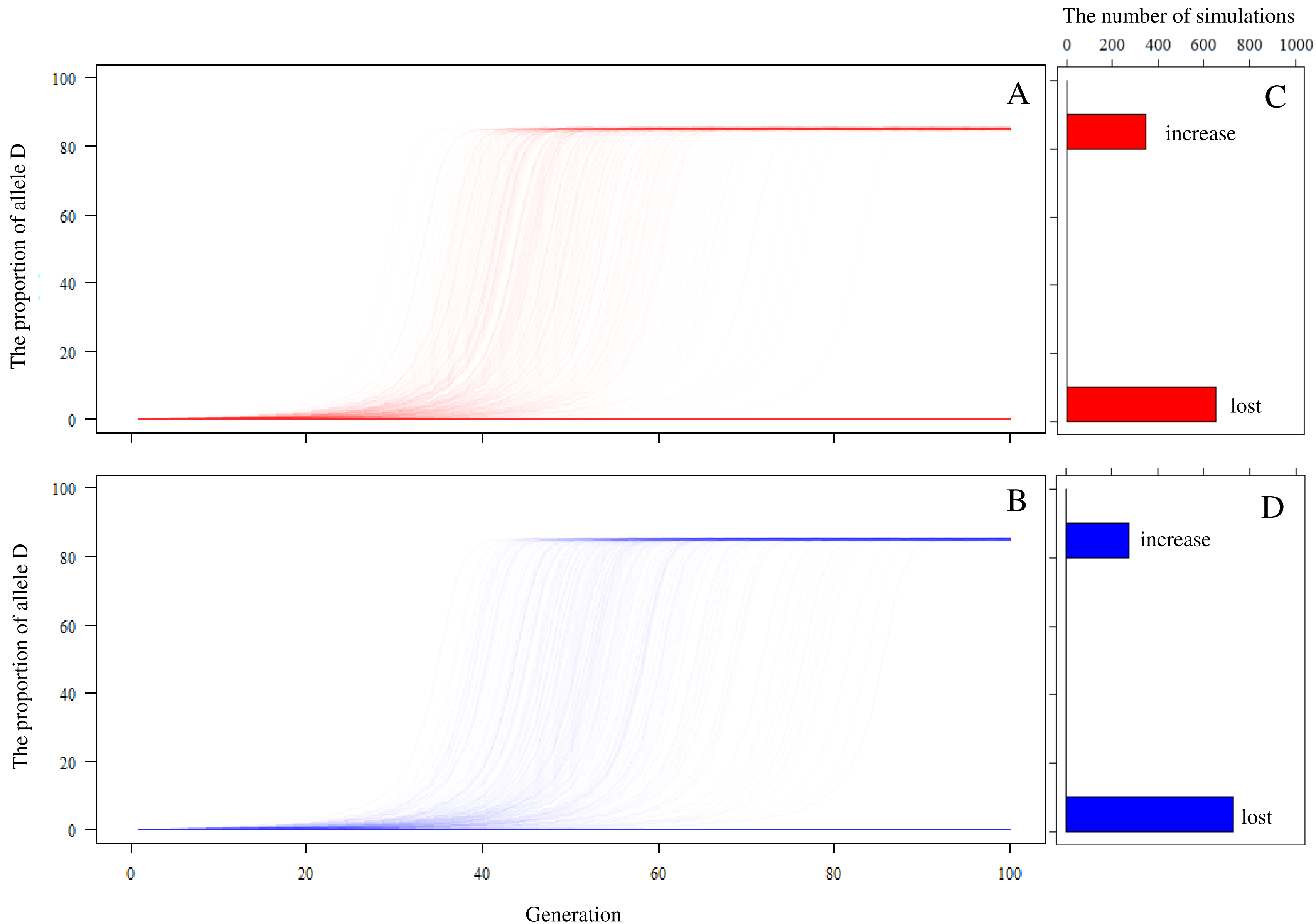
The number of simulations
0
200
400
600
800
1000
100
80
60
40
20
0
A
C
increase
lost
The proportion of allele D
B
D
increase
lost
0
20
40
60
80
100
Generation

Table 1(on next page)

Table. 1

The number of abalones choosing arm with mucus or without mucus.

Experimental season	marker	follower	with mucus	without mucus	estimate	95 % confidence interval
Reprocutive season	male	male	5	1	1	0.36-1.00
	female	female	8	1	0.89	0.52-1.00
	female	male	9	1	0.8	0.55-0.99
	male	female	8	3	0.72	0.44-0.95
Non-reproductive season	male	male	8	4	0.67	0.35-0.91
	female	female	7	2	0.67	0.4-0.97
	female	male	8	1	0.88	0.52-1.00
	male	female	7	2	0.88	0.39-0.97
total			60	15	0.8	0.69-0.88