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RNase reverses segment sequence in the anterior of a beetle egg (*Callosobruchus maculatus*, Coleoptera)

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Introduction

In insects, abnormalities in segment sequencing are used to explore the normal regulation of body pattern development along the head-to-tail axis. Partial reversal of segment sequence is among such abnormalities. One type of partial reversal involves the replacement of head and thoracic segments with abdominal segments oriented in mirror image symmetry to the original posterior segments (double abdomen). The converse arrangement occurs in double cephalons. In *Drosophila* the mutations *bicaudal* (Bull 1966) and *bicoid* (Frohnhöfer et al. 1986) produce double abdomen-like arrangements while *dicephalic* produces double heads (Lohs-Schardin 1982). In *Chironomus samoensis* only double abdomen occurs as a mutation (Kalthoff and Albetieha 1986). No double abdomen mutation is known in *Chironomus riparia*, but transcript expression profiling of AP bisected early embryos has revealed a second gene – *panish* – with the same function as *bicoid* in *Drosophila* (Klomp et al. 2015).

Both types of reversal have also been obtained by other experimental means. In the Dipteran *Chironomus* production of double abdomens and double cephalons is correlated with the rearrangement of ooplasm produced by centrifugation of the egg (Yajima 1960). Moreover, in *Chironomus* (Yajima 1964) and *Smittia* (Kalthoff 1971, 1983) UV irradiation of the anterior egg half at the absorption maximum of pyrimidines and purines (253.7 nm) produced double abdomens. UV exposure of the posterior produced double cephalons only in *Chironomus* (Yajima 1964). Development of double abdomens after puncture of the anterior egg of *Smittia* was correlated with the occurrence of an extraovate suggesting the inactivation or elimination of an active component (Schmidt et al. 1975). Puncture of the anterior in RNase produced double abdomens (Kandler-Singer et al. 1976). Together, these results offered the first indication of a role for cytoplasmic polynucleotides in insect segment pattern formation. In sum, experimentally, both types of reversal have been obtained in the Dipterans.

In Coleoptera only double abdomens have been induced by temporary constriction of eggs of three species: the bean beetles *Callosobruchus maculatus* and *Bruchidius obtectus* and the potato beetle *Leptinotarsa decemlineata* (van der Meer 1984, 1985). The pea beetle *Callosobruchus maculatus* is a global pest of stored legumes. Temporary separation of the yolk mass before nuclei enter the anterior egg half produces a decaying anterior yolk mass (van der

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Meer 1979: Fig1). This is often accompanied by partial reversal of segment sequence in the posterior egg half provided it is exposed to the decaying anterior fragment. No reversal occurred in the posterior fragment when the anterior egg fragment developed into an anterior half of the embryo (van der Meer 1984). This suggests that reversal in the posterior fragment of the egg is not due to a lack of contact with a developing anterior half, but to a factor leaking from the decaying anterior egg fragment into the posterior one. Since anterior puncture of *Smittia* eggs submerged in RNase caused double abdomens (Kandler-Singer et al. 1976), I test here the hypothesis that in *Callosobruchus* RNase leaking from the decaying anterior egg fragment induced double abdomens in the posterior one.

One purpose of these experiments is to establish that localized maternal RNA at the anterior pole controls head-to-tail polarity in *Callosobruchus* embryos. They were carried out during postdoctoral research between 1978 and 1982. Given the renewed interest in this subject, these results are made available now. Another purpose is to increase the diversity of species for comparative studies of the genetic regulation of anterior-posterior segment pattern development. This regulation has been elucidated in detail for *Drosophila* (Jaeger 2011), but it is not canonical for insects. A surprising diversity of anterior-posterior regulatory mechanisms is being uncovered not only between insect Orders (Sander 1994; Patel 1994; Patel et al. 1994; Lynch 2012; Lynch 2014), but also within the Order of the Diptera (Stauber et al. 2002; Klomp et al. 2015; Lemke et al. 2008; Lemke and Schmidt-Ott 2009; Lemke et al. 2010). This raises the question whether the same diversity of regulatory mechanisms exists within other insect Orders.

The large Order of beetles (Coleoptera) is particularly interesting for comparative embryological study because it includes long, intermediate and short germ types of embryonic development. In long germ development the posterior segment pattern is specified nearly simultaneously with the other segments. Thus, maternal control encompasses the entire segment pattern. By contrast, in short germ development the posterior segments are specified sequentially. Moreover, in many insects with short germ development, the germ rudiment is specified in the posterior region of the egg, far away from the anterior pole. Thus, maternal control covers extra-embryonic, cephalic and sometimes thoracic domains, but excludes abdominal segments which are produced later within the “growth zone”.

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It is not known whether the difference in Coleoptera between sequential segment specification in short germ rudiments and simultaneous segment specification in long germ rudiments is correlated with differences in the maternally provided transcription factors involved in these different kinds of specification. Maternally located mRNA transcripts have been identified in the anterior of *Tribolium* eggs (Fu et al. 2012; Schopfmeier et al. 2009; Schmitt-Engel et al. 2012) as well as in the posterior (Schmitt-Engel et al. 2012). It is conceivable that the size of the germ rudiment relative to egg size is correlated with properties of the *maternal* components of segment specification. Therefore, it would be of interest to compare the maternal contributions to the genetic regulation of segment specification within the Coleoptera between the short germ species *Tribolium* and the long germ species *Callosobruchus* (Lynch et al. 2012).

Coleoptera are also suitable for comparative embryological study because genetic regulation of anterior-posterior segment patterning in the short germ embryo of *Tribolium* has been explored to some extent. So far, early maternal and zygotic contributions to establishing the expression domains of zygotic gap, pair-rule and segment polarity genes have been found for the anterior (Wolff et al. 1998; Bucher et al. 2005; van der Zee et al. 2005; Schröder 2003; Schoppmeier and Schroeder 2005; Kotkamp et al. 2009; Schoppmeier et al. 2009; Fu et al. 2012; Schmitt-Engel et al. 2012), the posterior (Copf et al. 2004; Schmitt-Engel et al. 2012; Lynch et al. 2012) as well as for the termini (Schopmeier and Schroder 2005). Pair-rule and segment polarity genes have been identified in two other Coleoptera, *Dermestes* and *Callosobruchus* (Patel et al. 1994). Further study of *Callosobruchus* is promising for a more comprehensive comparison with *Tribolium*.

So far, maternal contributions to segment patterning in *Callosobruchus* have not been explored. Here I provide evidence that RNase activity in the anterior egg can induce double abdomen development suggesting a role for maternal messengers. Reversal frequency depended on developmental stage, enzyme concentration and temperature regimen. The reversal was specific for RNase activity and for treatment of the anterior egg pole prior to arrival of nuclei. This sets the stage for using transcript expression profiling (Klomp et al. 2015) of bisected *Callosobruchus* eggs to identify the gene or genes that produce the axis determinants. I also introduce a way of mass collecting eggs.

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Materials and Methods

Adults

Pea beetles (*Callosobruchus maculatus* Fabr., syn. *Bruchus quadrimaculatus* Fabr.) were reared, eggs collected and larval cuticles prepared and analysed according to van der Meer (1979). To avoid development of allergy against pea beetle dust, the incubator in which beetles are reared was placed outside of the working space. Oviposition and egg collection were done at 70 % relative humidity in a hospital baby incubator on a cart. The incubator was fitted with an exhaust fan to create negative internal pressure and an exhaust hose to the outside of the building. Humidity was maintained via an intake from a heated water bath.

Mass collection of eggs on methylcellulose-coated beans

Large numbers of eggs were collected in minutes by letting beetles lay eggs on brown beans coated with methylcellulose. Methylcellulose is a water-soluble carbohydrate polymer produced by Dow Chemical under the brand name Methocel. Grade A indicates methylcellulose products, grade K hydroxypropyl methylcelluloses. Grade A15 or K35 are suitable for making a thin film on beans because their low molecular weight allows for making a low-viscosity solution (Dow Chemical, n.d.).

A 0.5% solution of methocel was prepared by adding 15 ^{0}C water to methocel powder very slowly and in that sequence while agitating with a magnetic stirrer until the powder has dissolved. A pouch of flexible nylon mesh containing beans is lowered into a beaker with methocel solution and stirred gently for one hour. The pouch with beans was transferred into another beaker with water. The beans were dried on a screen at room temperature or at 40 ^{0}C overnight making sure the beans do not stick to each other. In a dry atmosphere the film is not sticky, but it may become sticky in a humid atmosphere or when touched with fingers.

Eggs were collected according to van der Meer (1979), but on methylcellulose coated beans. Egg-covered beans were placed in a pouch of flexible nylon mesh and the pouch was placed in a beaker with water. The water was kept at 15 ^{0}C to slow development and because methylcellulose is more soluble at lower temperature. The pouch was gently moved up and down

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for 30 minutes. Most eggs detached in 5 minutes. Typically, 98% of detached eggs (n= 44) developed into caudal plate stage embryos and 85% of detached eggs (n= 195) developed into normal larvae.

Staging of development

Developmental stages were determined by inspection of serially sectioned eggs. Eggs were fixed on the bean in a mixture of 80% ethanol, 40% formaldehyde and undiluted acetic acid in a ratio of 17:1:2 by volume for 20 - 30 minutes at 60 ^{0}C and allowed to cool. Eggs were removed from the bean with a razor blade. The chorion was removed from the egg with sharp tungsten needles followed by fixation overnight in the same mixture at room temperature. Eggs can be stored indefinitely in 70% ethanol. Before further processing eggs were washed in 80% ethanol. To spot an egg during and after embedding in paraplast, eggs were stained for 30 to 60 minutes by adding a few drops of thionine to the container with eggs in 80% ethanol (dissolve 1 g. thionine in 10 ml. 100% ethanol at room temperature and add distilled water up to 100 ml.). Eggs were dehydrated via ethanol and xylol and embedded by gradually adding paraplast (Sherwood 60 ^{0}C) to the xylol. Eggs were oriented in the liquid paraplast with a hair loop under a dissection microscope. Serial sections – transverse or sagittal – were stained on glycerine coated slides. The glycerine keeps the yolk attached to the slide. Sections were stained with Geidies' modification of azan-novum (Schulze and Graupner 1960).

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Table 1: Dependence of developmental stages on temperature (^{0}C).

Stage description	Abbreviation	70% relative humidity			distilled water		
		30	25	22	30	25	22
meiosis I - anaphase	M I	0.25	0.5	1-3.5	–	–	4
meiosis II - fusion pronuclei	M II	1	1.5 - 2.5	4	–	–	–
2 nuclei	NM 2	2	3 - 4	5 - 6	–	4	8
4 nuclei	NM 4	–	4	6 - 7	–	–	–
4-8 nuclei	NM 4-8	3	–	–	–	–	–
8-16 nuclei	NM 8-16	–	5 - 6	8	–	–	–
16-32 nuclei	NM 16-32	4	–	8	4	–	–
32-64 nuclei	NM 32-64	5	6 - 7	9 - 10	–	–	–
64-128 nuclei	NM 64-128	–	8	10 - 11	–	–	12
128-256 nuclei	NM 128-256	6	–	11 - 12	–	8	–
young syncytial blastoderm	SBy	6	9.5-10.5	12 - 13	8	12	–
mid-syncytial blastoderm 1	SBm1	8	–	16	–	–	24
mid-syncytial blastoderm 2	SBm2	10	12	20	–	16	–
old syncytial blastoderm	SBo	12	–	24	–	–	–
young cellular blastoderm	CBy	13	16	–	–	–	–
old cellular blastoderm	CBo	14	18 - 20	–	–	20	–

Development times are in hours $\pm$ 15 minutes. Development slows down when eggs develop in water or oil. SBy: nuclei have just arrived in periplasm, no transverse cell membranes, pole cells formed; SBm1: transverse cell membranes begin to form; Sbm2 & SBo: transverse cell membranes completed; CBy: tangential cell membranes completed; CBo: early ventral plate.

Chemicals

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RNase A (type III-A from bovine pancreas, EC 3.1.27.5), oxidized RNase A (type XII-AO), RNase S (from bovine pancreas, Grade XII-S), S-peptide (Grade XII-PE), and S-protein (Grade XII-PR) were obtained from Sigma (St. Louis, Missouri), DNase I (from bovine pancreas, deoxyribonucleate 5'-oligonucleotidohydrolase EC 3.1.4.5, Miles Laboratories, Elkhart, Indiana) and Proteinase K (from *Tritirachium album*, EC 3.4.21.64, Boehringer Mannheim, 15686, Indianapolis, Indiana). Oxidized RNase A was obtained by performic acid treatment of RNase A (Richards and Vithayathil 1959). Subtilisin treatment splits RNase A into S-protein and S-peptide that remain attached. This complex is referred to as RNase S and has full RNase activity. S-protein has some residual activity, but S-peptide is inactive (Richards and Vithayathil 1959). All solutions were prepared in distilled water to facilitate diffusion down a concentration gradient into the egg (Kandler-Singer and Kalthoff 1976). DNase I was dissolved immediately before use because solutions containing 100 μg/ml in dilute buffer are stable in the range of pH 4.0 to 9.0 only for a week or longer at about 5°C (Kunitz 1950). RNase S' (1 μg/ml) was reconstituted by mixing equimolar concentrations of S-peptide (0.84 μg/ml) and S-protein (0.16 μg/ml) (Kandler-Singer and Kalthoff 1976).

Puncturing

Eggs were punctured with a sharp glass needle while submersed in various RNases and control substances. To penetrate the thick chorion it is crucial to use a closed glass needle with sufficient wall thickness. I used type 7740 borosilicate glass with inner filament, outer diameter 1.2 mm, inner diameter 0.6 mm, wall thickness 0.3 mm from Karl Hilgenberg Glaswarenfabrik, Strauchgraben 2, 34323 Malsfeld, Hessen, Germany, www.hilgenberg-gmbh.de. Needles with a diameter tapering to 10 μm were pulled with a Kopf pipette puller.

Eggs were fastened by inserting them with the posterior end into a thin line of soft silicon rubber (Terostat-55 from Henkel Teroson GmbH, Postbox 105 620, Hans-Bunte Strasse 4, 69046 Heidelberg 1, Germany, www.henkel-adhesives.de or www.teroson.de) deposited on a microscope slide. The acetic acid released during hardening of the silicon rubber was rinsed off before puncture.

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Classification and calculation of results

'Dead' embryos includes all embryos that did not produce a larva with a differentiated cuticle. 'Normal' embryos comprises embryos that produced larvae with a normal segment pattern including one or two with missing anterior segments due to puncturing damage. Percentages are calculated as a fraction of punctured eggs.

Results

Controls

Nuclear migration stage eggs were punctured at the anterior pole while submerged in distilled water (pH 6) and solutions of DNase I, oxidized RNase A, proteinase K, S-protein and S-peptide (Table 2). No control treatment produced reversal except S-protein (8% reversal at 5 µg/ml and 9% at 10.0 µg/ml) in accordance with its residual RNase activity (Richards & Vithayathil, 1959: Fig. 3). RNase S' which is reconstituted from S-protein and S-peptide in equimolar amounts produced 11% reversal at 5.0 µg/ml and 24% at 10.0 µg/ml. (Table 4, Fig1a) confirming that the combination of S-protein and S-peptide has higher RNase activity than S-protein alone. These results indicate that partial reversal of segment sequence is not due to the effect of solvent (distilled water), enzyme activity not specific for RNA (DNase I, proteinase K) or of protein (oxidized RNase A, S-peptide). I conclude that partial reversal of segment sequence is specific for RNase activity.

Fig1 Double abdomen and normal first instar larvae of *Callosobruchus maculatus*. Ventral views of (a) double abdomen with reversed second abdomen pointing left (phase contrast), (b) normal larva. Comparison shows that in (a) the head segments and the first two thoracic segments have been replaced by a reversed sequence of T3 through A8 which joins the non-reversed abdomen located to the right of the dashed symmetry line (Sp/p). Abbrev.: head segments: Lbr: labrum, antennae (not visible), Md: mandibulae, Mx: maxillae, Lb: labium; thorax: 3 segments T1, T2, and T3, each with a pair of legs (L); abdomen: A1– A11, St: stigma, Sp: spine of segment A1 (reproduced with permission by Taylor & Francis Ltd. www.taylorandfrancis.com from van der Meer 1985 Fig1)

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Table 2: Controls

treatment	concentration. ug/ml	nuclear migration stage	punctured n	dead n	dead %	normal n	normal %	reversal n	reversal %
distilled	n/a	16-32	408	87	21	320	78	0	
water	n/a	64	310	99	32	209	67	0	
DNase I	100	16-32	125	39	31	85	68	0	
oxidized	5	16-32	64	9	14	–		0	
RNase A	10	16-32	71	11	16	–		0	
proteinase K	5	16-32	63	35	56	28	44	0	
	10	16-32	70	49	70	21	30	0	
	100	16-32	67	67	100	0	0	0	
S-protein	5	16-32	141	57	40	69	49	11	8
	10	16-32	142	57	40	72	51	13	9
S-peptide	10	16-32	143	69	48	72	50	0	

Stage dependence of reversal of segment sequence

To determine whether the frequency of partial reversal of segment sequence depends on stage of development, eggs were punctured at the anterior pole while submerged in 0.5 µg/ml RNase A. There is a well-defined dependence on stage of development with a maximum reversal yield of 14% of punctured eggs at nuclear migration stages between 16 and 64 nuclei (Table 3).

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Table 3: Influence of stage of development on the frequency of reversal after anterior puncture in 0.5 μg/ml RNase A.

stage (hr at 30 ^{0}C)		punctured n	dead n	dead %	normal n	normal %	reversal n	reversal %
NM2	(2hr)	171	61	36	103	60	7	4
NM4-8	(3hr)	236	94	40	125	52	17	7
NM16-32	(4hr)	184	46	25	112	61	26	14
NM64	(5hr)	183	59	32	99	54	25	14
SBy	(6hr)	166	42	25	115	69	9	5
SBm	(7hr)	188	51	27	137	73	0	0

Concentration dependence of reversal of segment sequence

In order to increase the yield of double abdomens I determined the effect of enzyme concentration after anterior puncture at the stage that yielded a maximum frequency of partial reversal of segment sequence (NM16-32, Table 3). There is a well-defined concentration-dependence with a maximum yield of 22% double abdomens at 2.0 μg/ml RNase A and 17% at 1.0 μg/ml of RNase S (Table 4).

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Table 4: Influence of enzyme concentration on reversal yield after anterior puncture in RNase A and RNase S at NM16-32

	RNase A							RNase S						
µg/ml	punctured	dead		normal		reversal		punctured	dead		normal		reversal	
	n	n	%	n	%	n	%	n	n	%	n	%	n	%
0.2	152	20	13	132	87	0	0	–	–	–	–	–	–	–
0.5	387	112	29	229	59	46	12	139	83	60	56	40	0	0
0.8	132	77	58	37	28	18	14	143	83	58	58	41	2	1
1.0	283	134	47	114	40	35	12	312	68	22	190	61	54	17
2.0	143	55	38	57	40	31	22	143	71	50	67	47	5	3
5.0	138	120	87	9	7	9	7	136	88	65	29	21	19	14
8.0	134	128	96	4	3	2	2	140	108	77	15	11	17	12
10.0	65	1	2	64	98	0	0	70	61	87	5	7	4	6

RNase S' is reconstituted from S-protein and S-peptide. It is included as a standard for comparison against the control test with S-protein and S-peptide (Table 2). I also determined dependence of yield on the concentration of RNase S' to see whether a higher yield could be obtained (Table 5). RNase S' produced 24% double abdomens at 10.0 µg/ml compared to RNase A which produced 22% at 2.0 µg/ml.

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Table 5: Reversal yield after anterior puncture
in RNase S' at NM16-32

μg/ml	punctured	dead		normal		reversal	
	n	n	%	n	%	n	%
1.0	136	24	18	110	81	2	2
2.0	131	87	66	29	22	15	12
5.0	195	127	65	46	24	22	11
10.0	131	42	32	57	44	32	24

Influence of puncture site on reversal of segment sequence

Eggs were punctured during NM 16-32 while submerged in 0.5 μg/ml of RNase A (23-24 ^{0}C). Lateral puncture was on the left at 50% of egg length, midway between the flat dorsal side and the domed ventral side of the egg. Ventral and dorsal puncture was at 50% of egg length half way between the sides of the egg.

Table 6: Influence of puncture site on reversal
of segment sequence in 0.5 μg/ml RNase A.

site	punctured	dead		normal		reversal	
	n	n	%	n	%	n	%
posterior	156	19	12	137	88	0	0
lateral	127	33	26	94	74	0	0
ventral	119	74	62	45	38	0	0
dorsal	120	119	99	1	1	0	0
anterior	387	112	29	229	59	46	12

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The survival rate after mid-dorsal puncture was extremely low. This cannot be due to the loss of the maternal pronucleus in the maturation island which is on the dorsal side because these eggs were punctured during early nuclear migration.

Is target RNA associated with protein ?

Maternal messengers in *Drosophila* are anchored in the anterior and posterior by proteins. If this applies to *Callosobruchus* target RNA, then removal of the anchors from the anterior before RNase treatment might increase the frequency of reversal. Eggs in NM 16-32 stage were punctured at the anterior while submerged in 1.0 μg/ml proteinase K followed by a second puncture in 0.5 μg/ml in RNase A (23-24 ^{0}C). A concentration of 1.0 μg/ml. was chosen because at 5.0 μg/ml proteinase K alone, 46% of treated eggs survive (Table 2). To prevent degradation of RNase A by proteinase K after transfer of eggs into RNase A I used separate puncture chambers for proteinase K and RNase A, rinsed eggs punctured in proteinase K with fresh distilled water followed by a rinse in 0.5 μg/ml of RNase A, washed forceps used for transfer of slide-mounted eggs between puncture chambers with detergent followed by distilled water, transferred eggs to a second chamber and punctured them a second time in the same location while submerged in 0.5 μg/ml of RNase A.

Of 63 eggs pre-punctured in 1.0 μg/ml proteinase K followed by puncture in 0.5 μg/ml RNase, 36 survived (57%) compared to 46% after puncture in 5.0 μg/ml proteinase K alone. Of the double-punctured eggs, 33 developed normally (52%) and 3 showed reversal of segment sequence (5%). Compared with the 12% of double abdomens after puncture in 0.5 μg/ml RNase A alone at NM 16-32 (Table 4), pretreatment with a proteinase lowered rather than raised the yield of double abdomens. Thus, I found no evidence that pre-treatment with proteinase K removes putative anchor proteins from maternal messengers located in the anterior of the egg.

Effect of temperature on frequency of reversal of segment sequence

The correlation between reduced RNase activity in protein S and lower reversal frequency (Table

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2) supports the conclusion that reversal is the specific result of the level of RNase activity. To further support that correlation, nuclear migration stage eggs with 64 nuclei were submitted to three temperature regimens during and after anterior puncture in 10.0 µg/ml RNase S' (Table 7). At this concentration NM 16-32 eggs punctured at room temperature (23-24 ^{0}C) yield 24% reversal (Table 5). Following oviposition eggs were kept for 5 hrs at 30 ^{0}C, that is up to NM 64. Puncturing lasted from 5 to 6 hrs at either 15 or 30 ^{0}C followed by incubation at 30 or 15 ^{0}C. Control eggs underwent the same temperature regimens as the punctured eggs, but were not attached to adhesive or punctured.

Table 7: Influence of temperature during and after puncture in 10 µg/ml RNase S' starting at NM 64.

temperature in ^{0}C	punctured	dead		normal		reversal	
before–during–after	n	n	%	n	%	n	%
30 — 30 — 30	138	46	33	50	36	42	30
control (distilled water)	310	99	32	211	68	0	0
control (no puncture)	–	–	–	–	–	–	–
30 — 15 — 30	140	136	97	0	0	4	3
control (no puncture)	138	3	2	135	98	–	–
30 — 15 — 15	123	121	98	2	2	0	0
control (no puncture)	128	40	31	88	69	–	–

At 30-30-30 ^{0}C, RNase redirects slightly less than half of the eggs developing normally after puncture in distilled water (68%) to partially reversing their segment sequence (30%) without affecting the survival rate which is 67% and 68%, respectively (Table 7: top). From there

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the reversal frequency declines with temperature to 24% at 23-24 ^{0}C (Table 5) to 3% at 30-15-30 ^{0}C and to zero when 15 ^{0}C is maintained after puncture (Table 7).

The decline in reversal frequency from 30% under the 30-30-30 ^{0}C regimen to 3% under the 30-15-30 ^{0}C regimen to 0% under the 30-15-15 ^{0}C regimen could be due to an effect of temperature alone on survival frequency. To investigate this possibility I determined survival for development at different temperatures starting at maturation and without puncture in RNase. In 10 ^{0}C 100 % survived to the caudal plate stage declining to 31% at larval stage (n = 45). At 15 ^{0}C 98% reached the caudal plate stage and 55% the larval stage (n = 42). This compared to 69% normal larvae under the 30-15-15 ^{0}C regimen (Table 7). At 23-24 ^{0}C 98% reach the caudal plate stage (n = 44) and 85% continue into the larval stage (n = 195). The causal plate stage looks normal in the dissecting microscope (Fig1a) indicating that low temperature does not block segment specification or morphogenesis, but affects larval development. This would affect the frequency of both normal larvae and double abdomens because both frequencies are determined at the larval stage. Therefore, the decline in reversal frequency can be attributed to the effect of low temperature on RNase activity.

In sum, reversal yield depends on developmental stage, enzyme concentration and temperature. A maximum of 30% of eggs treated at 30 ^{0}C with 10.0 μg/ml RNase S reconstituted from S-protein and S-peptide developed double abdomens.

Discussion

The purpose of these experiments was to show that localized maternal RNA at the egg poles controls anterior-to-posterior polarity in *Callosobruchus* embryos. Introduction of RNase into the anterior of the egg of *Callosobruchus* replaces anterior segments with posterior ones in mirror image symmetry to the original posterior segment pattern (double abdomen). This effect is specific for the anterior egg pole because puncture at other sites did not produce double abdomens. So far there is no evidence for a posterior control centre because posterior puncture in RNase did not produce partial reversal of segment sequence of the kind known as double cephalon (Table 6).

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Reversal is specific for RNase activity because inactivated RNase, proteinase and DNase did not produce double abdomens. Thus reversal is not due to an effect on protein, but on RNA. The strongest evidence for the specificity of the effect is that at the same concentration (10 µg/ml) and temperature (23-24 ^{0}C) the frequency of reversal was correlated with the degree of RNase S' activity. S-peptide has no residual RNase activity and produced no reversal. S-protein has residual RNase activity and produced 9% reversal. RNase S' which is reconstituted from S-protein and S-peptide produced 24% reversal. These observations indicate that the partial reversal of segment sequence is due specifically to the enzymatic destruction of RNA in the anterior of the egg.

The decline of reversal frequency from 30% under the 30-30-30 ^{0}C regimen to 3% under the 30-15-30 ^{0}C regimen to 0% under the 30-15-15 ^{0}C regimen (Table 7) supports the conclusion that reversal is due to enzyme activity since it depends on temperature. One would expect that the decline of reversal frequency due to reduced enzyme activity at lower temperature would be compensated for by a rise in normal development. But the frequency of normal development is 2% or less at lower temperature (Table 7: 30-15-30 ^{0}C and 30-15-15 ^{0}C). I speculate that this may be the compounded effect of low temperature on RNase activity and on larval development.

The reversal of segment pattern specification occurs before nuclei have arrived at the surface of the egg to begin forming a syncytial blastoderm. No nuclei have arrived at the anterior during the stages at which maximum reversal frequencies are obtained by anterior puncture in RNase (NM 16-32, NM 64). This suggests an early specification of segment pattern using maternally produced cytoplasmic messengers deposited at the anterior end of the egg and acting as a source of morphogen gradients in the egg. This result sets the stage for identifying the gene or genes regulating segment specification by transcript expression profiling (Klomp et al. 2015) of bisected *Callosobruchus* eggs.

The author declares that he has no conflict of interest.

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