

# Relationship Among Phenolic Contents, Seed Predation, and Physical Seed Traits in *Mimosa bimucronata* Plants

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**Abstract** Phenolic contents were compared between *Mimosa bimucronata* seeds from infested and non-infested fruits to assess induced defense response. By measuring leg length of the bruchid beetle *Acanthoscelides schrankiae*, we verified whether phenolic contents affected bruchid body size. In addition, the relationship between physical seed traits and phenolic contents was examined. Results showed that seeds from infested fruits had significantly greater phenolic contents than seeds from non-infested fruits, which suggested induced defense. Body size variation in *A. schrankiae* was marginally nonsignificant according to phenolic contents among plants (negative trend), indicating that phenols may interfere directly with bruchid performance. Seeds that were more irregularly shaped had significantly greater phenolic contents than those that were more uniform. Therefore, the most perfectly spherical seeds may be more vulnerable to seed predation, and our results suggest that the production of phenolic compounds was increased in infested fruits, which in turn may affect *A. schrankiae* development.

**Keywords** *Acanthoscelides schrankiae* · Induced defense · Insect body size · Phenolic compounds · Physical seed traits · Seed predation

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Seed predation can be an important interspecific process regulating plant community structure and composition (Janzen 1971) because it acts as a selective force that affects plant abundance, distribution, and evolution (Harper et al. 1970). Among the most important seed predators are the bruchid beetles (Janzen 1971; Southgate 1979). Given that all bruchid larvae feed exclusively in seeds (Southgate 1979), their adult body size, potential fecundity, and longevity are determined by resources obtained during larval development, even though these aspects also depend on whether they continue to feed as adults (Timms 1998; Gianoli et al. 2007). Thus, seed quality—mainly the amount of nutrients and concentrations of chemical defenses—strongly influences bruchid behavior, infestation, survival, life-history traits, and fitness (e.g., Fox et al. 1994; Thiery et al. 1994; Fox and Mousseau 1996; van Huis and de Rooy 1998; Or and Ward 2004). However, little is known about how seed quality affects bruchid beetle development within a single plant species under field conditions.

Although plant quality is crucial to insect development and abundance, defenses against herbivory and/or seed predation are usually costly to the plant, and the intensity of such defenses can be related to plant quality and vulnerability to insect attacks (Fowler and Lawton 1985; Fineblum and Rausher 1995; Rohner and Ward 1997; Or and Ward 2004; Schoonhoven et al. 2005; Hu et al. 2008). When the production of defenses is positively correlated with insect attack, this strategy is known as an induced defense (Schultz and Baldwin 1982; Karban and Baldwin 1997). Such induced defenses, however, have rarely been examined in relation to seed predators (Karbon and Baldwin 1997; Or and Ward 2004). Therefore, to comprehend the extent to which these beetles can induce defenses on their host plants, data from natural populations are needed.

Variations in seed morphology traits are very common within and among plant species. They may affect seed vigor

(physiological seed quality), seed persistence in the soil, and seedling growth, establishment, and survival (Thompson and Grime 1979; Bekker et al. 1998; Illipronti Jr. et al. 1999; Funes et al. 1999; Moles et al. 2000; Moles and Westoby 2004, 2006; Klug-Pumpel and Scharfetter-Lehrl 2008). To categorize seeds according to their size, shape, and color, computer-imaging analysis increasingly has been employed as an advanced tool rather than traditional methods (Dell'Aquila 2007; Dana and Ivo 2008). Such physical traits can also be determinant for the success of seed-eating insects, including bruchid beetles (Szentesi and Jermy 1995; Yang et al. 2006).

Some female bruchid beetles display size-discriminative behavior, i.e., preferring large seeds during the egg-laying period (Thanthianga and Mitchell 1990; Cope and Fox 2003; Yang et al. 2006). Because larvae are sedentary, larger seeds may provide more food and nutrients for development, thereby contributing to greater predator fitness (Hu et al. 1995; Yang et al. 2006). However, not only seed size may be directly related to bruchid infestation. For leguminous plant species, Szentesi and Jermy (1995) have found that the more closely the shape of seeds approximates a sphere or the larger the seed volume, the higher the probability of bruchid infestation.

Although physical seed traits may influence bruchid oviposition behavior, little is known about how seed size and shape are related to biochemical seed changes, which may also have relevant implications for seed-discriminative behavior. For example, phenolic compounds can be feeding deterrents to many insects (Jolivet 1998; Schoonhoven et al. 2005); therefore, if variations in physical seed traits are directly related to concentrations of those phenolics, the choice of ovipositioning on seeds with specific physical traits, i.e., “a sign” of lesser amounts of defensive compounds, would confer an advantage to bruchid offspring. Given that seed morphological heterogeneity is related to features such as color, size, and shape (Matilla et al. 2005; Dell'Aquila 2007), significant variation in these physical traits would be expected in plants that produce heterogeneous seeds, including many weeds and pioneering plants, in which heterogeneity is considered a strategy for coping with variability in environmental conditions.

*Mimosa bimucronata* (DC.) Kuntze (Fabaceae: Mimosoideae) is considered a pioneering plant as well as a weed that produces seeds with morphological variation. Likewise, bruchid beetles attack its seeds. Therefore, this insect–plant system is highly appropriate for answering our proposed questions. The objectives of this study were to: (1) determine whether the concentration of phenolic compounds in seeds from infested fruits differs from that from non-infested fruits, thereby suggesting an induced defense response; (2) examine whether bruchid body size (usually a reliable predictor of insect performance) variation within a plant population can

be explained by variations in phenolic contents among plants; and (3) investigate the relationship between physical seed traits and phenolic contents. The first two questions are important for understanding whether phenols are determinant defensive compounds for this plant–bruchid system, whereas the third has relevant implications for those systems where bruchid beetles attack post-dispersed seeds on which eggs are directly laid.

## Materials and Methods

### Study System

*M. bimucronata*, a perennial tree native to Brazil, Paraguay, Argentina, and Uruguay (Burkart 1959), reaches 10 m tall and has intense branch ramification. It is an important agent for restoration of degraded sites, even though this plant is also considered a weed that grows in dense populations in pastures (Lorenzi 2000). This species grows predominantly in damp lowlands, floodplains, and on the margins of rivers and lakes (Lorenzi 2000). Its seeds (located in craspedium fruits) are attacked in the pre-dispersal phase by the beetle *Acanthoscelides schrankiae* Horn (Coleoptera: Bruchidae) (Silva et al. 2007). This insect is known to feed in seeds from 12 plant species, including *M. bimucronata* (ten *Mimosa*, one *Acacia*, and one *Schrankia*) (Nápoles 2002; Silva et al. 2007). This bruchid species occurs in the Bahamas, Ecuador, the USA, Mexico, the Dominican Republic, Venezuela, and Brazil (Jesus Romero Nápoles, personal communication). Little is known about this beetle, and published information is limited (Johnson 1983, 1990; Silva et al. 2007; Tomaz et al. 2007).

The production peaks for immature and mature *M. bimucronata* fruits occur in March and in April and May, respectively. Only a few mature fruits remain on plants until the end of the year. Most *A. schrankiae* eggs are deposited on unripe pods during February, although a few can be seen on fruits until July and August. More than 70% of fruits present only one egg per seed, and one larva usually feeds in an individual seed (Silva et al. 2007).

### Study Sites

These *Mimosa* trees comprised two natural populations within Botucatu municipality in the state of São Paulo, Brazil. Our two study areas—Lageado ( $\cong 6,700 \text{ m}^2$ ) and Rubião ( $\cong 10,000 \text{ m}^2$ )—were located at the Faculdade de Ciências Agrárias (Unesp/Botucatu Campus) (22°50'52" S; 48°25'46" W) and near the Rubião Júnior District (22°53'07" S; 48°29'23" W), respectively. They are 8,794 m apart along a straight line. The Lageado and Rubião populations grow along a small river and a lake, respectively, and plants

are surrounded mainly by grasses. Because the *Mimosa* trees in each population showed an aggregated distribution, the total number of plants was not precisely determined. Their intense ramification of large thorny branches made it difficult to penetrate the stands, so that an exact estimation of plant densities was impractical. Nevertheless, we observed no more than 40 plants at each site. Most of the examined plants were at the edges of their populations; ten (Rubião) and 15 (Lageado) were randomly selected for this study.

#### Assessment of Phenolic Contents and Bruchid Body Size

For assessment of phenolics and bruchid body sizes, fruits were collected only from Rubião. Between February and August 2006, 40 mature fruits per plant were harvested every 15 days ( $n=280$  fruits). In the laboratory (28°C under 12-h light), they were placed in individual cylindrical plastic containers (1,000 ml) covered with small pieces of *voile* fabric, and the emergence of *A. schrankiae* was recorded periodically. All emerging insects ( $n=130$ ) were identified then fixed in 70% ethanol. The tibia and femur in the second pair of legs (right side) were measured with a stereomicroscope (Nikon SMZ 800) coupled to a digital camera. After each leg was photographed at standard amplification, we recorded tibia and femur lengths (mm) by MetaVue software (version 6.3r4) for image analysis. Bruchid body sizes were estimated by summing tibia plus femur lengths from each beetle (leg length), and the mean value of insect size per plant was then calculated.

After collection, fruits were separated into infested and non-infested groups and carefully dissected to extract undamaged seeds. To assess any differences in the allocation of chemical defenses between seeds from both types of fruits, their levels of phenols (i.e., hydrolysable tannins, which are esters of gallic acid) were measured. All seeds were oven-dried (105°C for 24 h) and milled to fine powder. Phenols were extracted from 100 mg of milled seeds for each fruit type and were quantified by the Folin and Ciocalteu (1927) method. Because undamaged seeds had been previously separated, each plant provided two samples: seeds from infested and non-infested fruits. The mean of three replicates per sample was used for biochemical and statistical analysis. Values for phenolic contents (mg gallic acid/g dry weight) were calculated per plant for each fruit type.

#### Assessment of Physical Seed Traits and Phenolic Contents

To determine physical seed traits, fruits were randomly collected in June 2006 only from Lageado (500 fruits from 15 plants). They were dissected in the laboratory to extract the undamaged seeds. All seeds were organized into a single sample, from which 1,000 were taken at random and

photographed individually at standard amplification with a stereomicroscope (Nikon SMZ 800) coupled to a digital camera (Fig. 1a). Afterward, each seed was placed in a small transparent plastic capsule labeled for that particular sample. Using MetaVue software (version 6.3r4) for image analysis as well as methods adopted by Illipronti Jr. et al. (1997), we performed the following measurements and recorded shape descriptors for each seed:

**Area:** based on the color tool from the software, the area of each photographed seed was automatically calculated ( $\text{cm}^2$ ) (Fig. 1b). This “Shrink” tool has high precision that eliminates image blur, which is otherwise problematic mainly in border areas.

**Perimeter:** the length of the seed contour was provided by “Shrink” simultaneously with our area values.

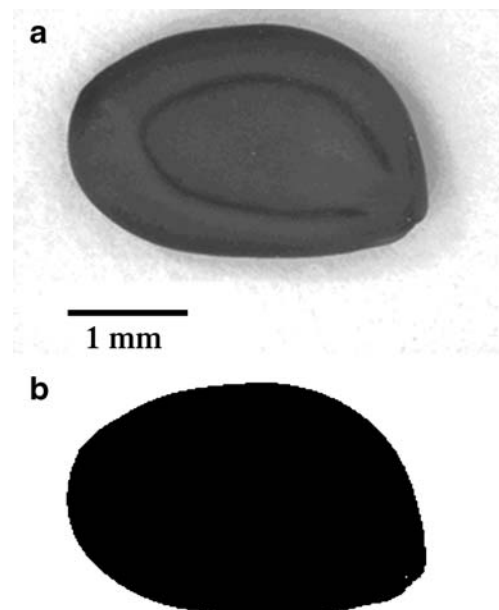
**Seed length:** a line was traced from the embryo axis to the opposite side (major axis).

**Seed width:** a line was measured that crossed perpendicularly at the exact midpoint of the length line (minor axis).

**Shape factor:** shape was calculated as the perimeter squared divided by  $4 \times \Pi \times \text{Area}$ , given that it is a classical shape feature. The minimum value (=1.0) represented a spherical shape (see Illipronti Jr et al. 1997).

**Eccentricity:** seed length was divided by width.

**Sphericity:** the ratio was determined between minimum radius (distance between the center of mass and the nearest point on the seed contour) and maximum radius (distance between the center of mass and the farthest point on the seed contour). A value of 1.0 signified an exact circle, with values ranging from 0.0 to 1.0.



**Fig. 1** Photograph of a *M. bimucronata* seed (a) and corresponding image after “shrinking” (b) for area and perimeter measurements

**Table 1** Results from a linear mixed-effects model with plants as the random effect. Fruit type (infested and non-infested) was the fixed effect (compared treatments). Fruit type was nested within plants, and phenolic contents (mg gallic acid/g dry weight) of *M. bimucronata* seeds was the response variable

Fixed effect	Value	Standard error	df	t	P
(Intercept)	30.53	1.86	49	16.40	<0.0001
Fruit types <sup>a</sup>	34.35	1.68	49	20.42	<0.0001

<sup>a</sup> Value=difference between means from infested and non-infested fruits

While these 4,000 measurements were being performed, the seeds were stored in an incubator at 16°C (18 h of white light). Because *M. bimucronata* seeds are very flat (Fig. 1a), their volumes were not recorded in this study. For exploratory analyses, the mean, minimum, and maximum values, plus variances and standard deviations, were calculated for all physical traits. Correlation analyses (Zar 1999) were run between traits. From the original 1,000 seeds, 100 were discarded from this analysis because they showed some cracks on their integuments. The remaining seeds were separated into two equal portions for between-group comparisons (mean values) for each trait. This sorting into groups was performed many times at random until significant differences between groups, considering all physical traits, were identified by *t*-tests (Zar 1999). Separations were based on shape characteristics: group 1—seeds with a more regular shape, approximating a circle (low eccentricity and shape factor; high sphericity); and group 2—more irregularly shaped seeds (high eccentricity and shape factor; low sphericity). These grouped seeds were then oven-dried, and

phenols from 300-mg powdered samples were quantified as described above. Three replicates per group were used for biochemical and statistical analysis.

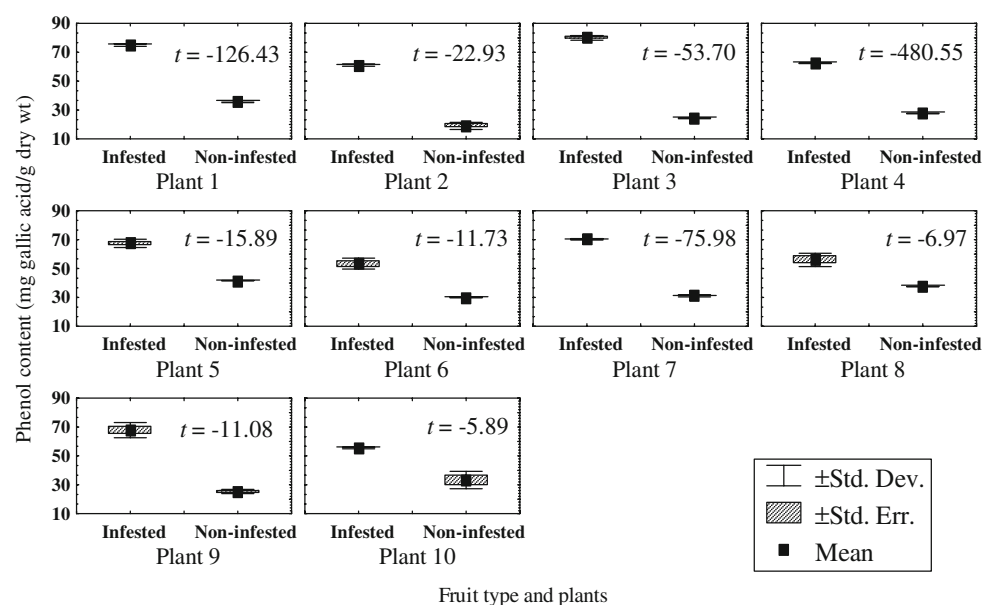
### Statistical Analysis

To assess differences in phenolic contents between fruit types, we used a linear mixed-effects model (Crawley 2007) considering plants as the random effect and fruit type as the fixed effect because our data collection followed a hierarchical design. Replicates were nested within fruit types, which in turn were nested within plants. Mean values for contents in seeds from infested and non-infested fruits were also compared within each plant by *t*-tests. To verify whether the level of phenolics affects bruchid body size, mean values of leg length (dependent variable) were regressed (Zar 1999) against mean content values for seeds from infested fruits (independent variable); each plant represented one paired data point (leg length and phenols). Although ten plants were randomly selected within Rubião, only eight were used in regressions because those with <5 insects were not considered. For physical seed traits, mean values of phenolic contents were compared between both seed groups by the *t*-test.

### Results

The concentration of phenolics differed significantly between infested and non-infested fruits (Table 1), with seeds from infested fruits having significantly more phenols (Fig. 2). This difference was demonstrated for all plants. Variations in

**Fig. 2** Comparison of mean phenolic contents between seeds from infested and non-infested fruits. All paired comparisons were statistically significant by *t*-tests ( $P < 0.05$ )



**Table 2** Mean, maximum, minimum, variance, and standard deviation for physical traits of *M. bimucronata* seeds ( $N=900$ )

Seed traits	Mean	Maximum	Minimum	Variance/ Standard deviation
Area (mm <sup>2</sup> )	11.144	16.738	5.518	2.985/1.728
Perimeter (mm)	13.471	20.916	9.203	1.739/1.319
Length (mm)	4.502	5.553	2.838	0.174/0.418
Width (mm)	3.414	4.321	2.102	0.137/0.370
Shape factor <sup>a</sup>	1.312	2.768	1.101	0.034/0.185
Eccentricity <sup>a</sup>	1.327	1.768	1.009	0.017/0.131
Sphericity <sup>a</sup>	0.581	0.897	0.176	0.013/0.116

<sup>a</sup> Dimensionless

beetle body sizes were marginally nonsignificant as a function of phenolic contents among plants ( $r^2=0.496$ ;  $F(1;6)=5.906$ ;  $b=-0.003$ ;  $P=0.051$ ). This suggested that these secondary compounds interfered directly with bruchid performance because the regression coefficient ( $b$ ) was negative.

The mean, maximum, minimum, variance, and standard deviation observed for physical traits of *M. bimucronata* seeds are shown in Table 2. Seed area and sphericity presented the greatest and smallest variance and standard deviation, respectively (Table 2). The correlation coefficients for physical seed traits showed high values for area, perimeter, width, and length (Table 3). The shape factor was significantly correlated with eccentricity and sphericity, showing, respectively, a positive and a negative relationship with these traits (Table 3). However, some correlations provided nonsignificant results because sphericity was not significantly correlated with perimeter and length, and shape factor was not correlated with length (Table 3). It is interesting that the shape factor was negatively correlated with seed area, meaning that smaller

seeds also had more irregular shapes (Table 3). The significance of a few small correlation coefficients ( $-0.109$ ,  $-0.179$ ,  $-0.075$ , and  $0.084$ ) occurred due to the large data series ( $N=900$ ). When seeds were randomly separated into two groups, significant results were found for all physical traits (Table 4). However, because these separations were performed many times, we presented only the single combination that produced significant results (Table 4). Our comparison of phenolic contents led to highly significant differences, and seeds from group 2 (more irregularly shaped) had higher levels of phenolics (Fig. 3).

### Discussion

Based on our results, we suggest that phenols are important defensive compounds against the bruchid *A. schrankiae* because variations in its body size (c.f., leg length) were negatively related to the level of phenolic contents among *M. bimucronata* plants, albeit following only a marginally nonsignificant trend ( $P=0.051$ ). Furthermore, we found that seeds from infested fruits had more phenolic contents than those from non-infested fruits, perhaps as a response to seed predation. It is important to note that differences in phenolic contents between seeds from infested and non-infested fruits were significant for all studied plants, suggesting that this is an induced defense (Karban and Baldwin 1997). However, further exploration is needed to determine whether greater production of phenolic compounds by *M. bimucronata* can affect bruchids in such a manner that many would not complete their growth within seeds, thereby reducing infestation rates significantly.

Most bruchid beetles are associated with one, two, or a few host plants, probably because the difficulties of dealing

**Table 3** Results from correlation analyses between pairs of physical traits for *M. bimucronata* seeds ( $N=900$ ), expressed as correlation coefficients ( $r$ )

Seed traits	Perimeter	Length	Width	Shape factor	Eccentricity	Sphericity
Area	0.737****	0.895****	0.816****	-0.109***	-0.075*	0.247****
Perimeter		0.727****	0.543****	0.586****	0.084**	0.044
Length			0.578****	0.0054	0.296****	0.0113
Width				-0.179****	-0.601****	0.520****
Shape factor					0.214****	-0.231****
Eccentricity						-0.622****

\* $0.01 \leq P < 0.05$ , significant result;

\*\* $0.001 \leq P < 0.01$ , significant result;

\*\*\* $0.0001 \leq P < 0.001$ , significant result;

\*\*\*\* $P < 0.0001$ , significant results

**Table 4** Results from *t*-tests comparing mean values between both seed groups within each physical trait

Source of variation	<i>df</i>	Mean values ( $\pm$ SD)		<i>t</i>	<i>P</i>
		Group 1	Group 2		
Eccentricity	898	1.30 ( $\pm$ 0.10)	1.35 ( $\pm$ 0.15)	-6.76	<0.0001
Shape factor	898	1.18 ( $\pm$ 0.03)	1.45 ( $\pm$ 0.20)	-28.62	<0.0001
Sphericity	898	0.61 ( $\pm$ 0.10)	0.55 ( $\pm$ 0.12)	9.00	<0.0001

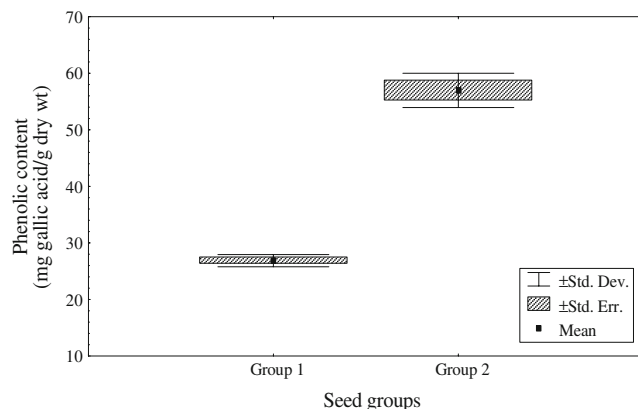
with several types of defensive compounds have favored the evolution of specialization in this insect group (Janzen 1980; Johnson 1990; Hulme and Benkman 2002). However, even though specialization has brought advantages in host use by these beetles, some secondary chemical compounds, such as condensed, polyphenolic tannins, may exert a negative influence on bruchid development (Boughdad et al. 1986). The extent of such effects may directly depend on the extent of plant-bruchid adaptation, which is probably related to their co-evolution. Thus, a more negative impact by plants on bruchid performance should be expected when there is a recent history of host-plant utilization. Therefore, it is possible that the bruchid preference for *M. bimucronata* has arisen recently (in an evolutionary context) because our results suggest that high levels of phenolic compounds on *M. bimucronata* seeds do affect *A. schrankiae* development.

Both physical and chemical seed traits may influence seed predation and oviposition choice. For example, some studies have shown that bruchid females prefer laying eggs on larger seeds (Thanthianga and Mitchell 1990; Yang and Horng 2002; Cope and Fox 2003; Yang et al. 2006), and seeds of that size usually provide more nutrients and food for progeny (Hu et al. 1995; Yang et al. 2006). However, large seeds may also require greater investments in defenses (physical and chemical) (Grubb et al. 1998), which may account for some nonsignificant relationships between seed dimensions and granivore preference (Janzen 1969; Kollmann

et al. 1998). Szentesi and Jermy (1995) observed that when seed shape approached a sphere, there was a higher probability of seed infestation by bruchids, a pattern that was also true for seed volume. Here, we showed that more irregularly shaped seeds (group 2) had significantly more phenol levels. Because the shape factor and seed area were negatively correlated (Table 3), irregular seeds were also smaller than those that more closely approximated a circle. Therefore, we can conclude that large *M. bimucronata* seeds have lower phenolic contents. These results are important because they demonstrate that physical and chemical traits are directly related, thereby offering the possibility that some seed eaters, e.g., bruchids, may prefer to feed and lay eggs on seeds with specific physical traits in order to reduce larval consumption of defensive secondary compounds.

Many bruchid species oviposit on unripe pods, and the larva finishes its development before the seed ripens (Szentesi and Jermy 1995). In these cases, pod traits (chemical and physical) would be more critical than seed traits. *A. schrankiae* females oviposit on unripe pods (Silva et al. 2007), and most adults emerge from fruits when seeds are fully ripe. Therefore, differences in physical seed traits are not associated with the “decision-making” process by those particular beetles. Nevertheless, our findings have important applications in other plant-bruchid systems, where predation occurs in the post-dispersal phase. In such systems, if females distribute their eggs according to physical seed traits, the relationship between seed secondary compounds and those traits should be thoroughly investigated if we are to obtain a more complete explanation of insect ability to discriminate among such traits. In addition, previous experiences by bruchids should be considered when analyzing their ability to discriminate (Yang et al. 2006).

In the broad sense, features of plant shape can serve as useful tools for explaining patterns of insect herbivory. For example, fluctuating asymmetry (FA), a possible indicator of developmental instability (Møller 1995; Zvereva et al. 1997; Hagen et al. 2003), leads to a positive relationship between the abundance of herbivores and degree of leaf asymmetry, whereas the concentrations of secondary compounds, such as tannins, may differ according to asymmetry levels (Cornelissen et al. 2003; Cornelissen and Stiling 2005). FA might also be a useful indicator of

**Fig. 3** Comparison of mean phenolic contents between seed groups. Significant difference was observed by *t* test ( $t=-16.21$ ;  $df=4$ ;  $P<0.0001$ )

environmental stress (Parsons 1990; Tracy et al. 2003; Puerta-Piñero et al. 2008). Here, we concluded that the most perfectly spherical seeds may be worse at avoiding seed predation because of their lower phenol concentrations. In addition, induced defense was suggested because phenolic compounds, found at higher levels in infested fruits, probably affect bruchid body size. If stress is positively correlated with the production of more irregular seeds containing more secondary compounds, then plants in a poor physiological state would be better defended against seed predators. Corroborating this hypothesis (although not evaluating seed-shape effects), Or and Ward (2004) investigated whether seeds from water-stressed *Acacia* plants were less able to produce secondary defense compounds to reduce herbivory. There, concentrations of pipelicolic acid were higher in physiologically impoverished plants, which probably diminished seed predation. The plant response was itself related to an induced defense that may have augmented fitness. Therefore, mainly for post-dispersal seed predation systems, we encourage future studies aimed at testing the impact of different levels of plant stress on variations in physical and chemical seed traits. This in turn would influence bruchid performance and the oviposition preference.

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