

1 **A physiological marker for quantifying differential reproductive investment**
2 **between the sexes in Yellow-legged gulls (*Larus michahellis*)**

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4 **Francisco Ramírez^{a,*}**, Keith A. Hobson^b, Owen S. Wangensteen^a, Meritxell
5 Genovart^c, Ginés Viscor^d, Carola Sanpera^a, Lluís Jover^e

6

7 ^a Dept. de Biologia Animal, Universitat de Barcelona. Avda. Diagonal 645, Barcelona
8 08028, Spain

9 ^b Environment Canada, 11 Innovation Blvd., Saskatoon, SK, S7N 3H5, Canada

10 ^c Institut Mediterrani d'Estudis Avançats (CSIC-UIB), Miquel Marquès 21, 07190
11 Esporles, Mallorca, Spain

12 ^d Dept. de Fisiologia (Biologia) - Universitat de Barcelona. Avda. Diagonal 645,
13 Barcelona 08028 – Spain

14 ^e Dept. de Salut Pública, Universitat de Barcelona. Casanovas 143, Barcelona 08036,
15 Spain

16

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18 *Corresponding author.

19 Phone: +3493-4021041

20 Fax: +3493-4035740

21 ramirez@ub.edu

22

23 Coauthors e-mail:

24 Keith A. Hobson: Keith.Hobson@EC.GC.CA

25 Owen S. Wangensteen: owenwangensteen@arrakis.es

- 26 Meritxell Genovart: m.genovart@uib.es
- 27 Ginés Viscor: gviscor@ub.edu
- 28 Carola Sanpera: csanpera@ub.edu
- 29 Lluís Jover: lluis_jover@ub.edu
- 30
- 31 Individual contribution to the article:
- 32 Work design: F.R., K.A.H., L.J.
- 33 Field work and laboratory analyses: F.R., O.S.W., M.G.
- 34 Manuscript elaboration: F.R., K.A.H., M.G., G.V., C.S., L.J.

35 **Abstract**

36 Asymmetry between males and females in the energy they invest initially in
37 reproduction has resulted in the evolution of differing reproductive strategies (caring
38 females vs. competitive males). However, parental care in many birds is shared by both
39 sexes suggesting that male energy expenditure in agonistic behaviors and courtship
40 feeding might compensate female costs of clutch production. Here, we tested the
41 hypothesis that initial investment in reproduction by both sexes in the Yellow-legged
42 Gull (*Larus michahellis*), a species with biparental care, is similar from a physiological
43 perspective. In this income breeder, female and male reproductive investment during
44 early breeding can be ultimately related to muscular activity (local foraging effort
45 required for clutch production in females and courtship feeding and agonistic behaviors
46 in the case of males). Thus, we evaluated sex-specific patterns of creatine kinase (CK,
47 IU/L) levels in plasma, an indicator of physical effort associated with muscular activity
48 dependent behaviors, through incubation as a reflection of the physiological response
49 of both sexes to the reproductive investment they made up to clutch completion. Raw
50 levels of CK were related to plasma levels of total proteins (TP, g/dL) to account for
51 the differential physiological state of individuals when sampled (i.e. differential
52 dehydration). Female costs of clutch production were associated with post-laying
53 levels of CK/TP. We grouped females according to their relative investment in clutch
54 production: <15.8%, 15.8 to 17.3% and >17.3% of Field Metabolic Rate; which
55 showed increasing values of CK/TP (24.6, 53.1 and 66.0IU/g, respectively). Moreover,
56 we found similar CK/TP trends throughout incubation for both sexes (CK/TP=50.2-
57 [3.3 × days from laying]) suggesting similar physiological responses to reproductive
58 effort and, therefore, analogous sex-specific initial investment. Thus, male investment
59 in agonistic behaviors and courtship feeding apparently equaled female investment in

60 clutch production. The use of CK measurements is revealed as a useful approach to
61 investigating overall reproductive investment in reproduction for both sexes, providing
62 further insights into our comprehension of reproductive strategies in seabirds.

63

64 **Key words:** Creatine kinase, clutch production, muscular activity, reproductive
65 strategies, sex allocation, total plasma protein.

66 **1. Introduction**

67 Energy invested early in the reproductive cycle is an important component of the
68 reproductive strategy followed by each sex (Patterson et al., 1980). According to sex
69 allocation theory (Trivers, 1972), the initial asymmetry in the sex-specific energy
70 invested in gametogenesis results in differing reproductive strategies between the sexes.
71 Because of high energy invested by females in gametogenesis, they usually invest
72 heavily in parental care since it is more profitable to continue with, rather than abandon,
73 a costly activity (Dawkins and Carlisle, 1976). In contrast, low energy requirements for
74 gametogenesis in males make competitiveness and extra-pair fertilizations a more
75 profitable strategy for them (but see Kokko and Jennions, 2008). Sex-specific
76 differences in initial reproductive investment are particularly evident in birds because of
77 female costs of clutch production. Under this scenario, biparental care typical of many
78 bird species (Lack, 1968) contrasts with classical sex allocation theory and suggests that
79 male energy expenditure before clutch completion in behaviors such as male-male
80 competition for mates, mate guarding or courtship feeding, might compensate female
81 costs of clutch production (Gladstone, 1979; Burger, 1981; Giudici et al., 2010). As
82 proposed by Baylis (1981), theory related to sex allocation strategies should be framed
83 in terms of total energy costs associated with a mating event, rather than the production
84 of individual gametes.

85

86 Male contribution to parental care is particularly important in the case of seabirds
87 (Furness and Monaghan, 1987). Although qualitative sexual differences in reproductive
88 investment patterns have been reported among gulls (*Laridae*), it is generally accepted
89 that parental care (including incubation and chick rearing) does not quantitatively differ
90 between the sexes (e.g. Butler and Janes-Butler, 1983; Burger, 1984; Stenhouse et al.,

91 2004). On the other hand, gulls have been described as income breeders, i.e. they
92 mainly adjust their food intake concurrently with breeding, without reliance on stores
93 (e.g. Hiom et al., 1991; Pons, 1992; Oro et al., 1995, 1996; Hobson et al., 1997;
94 Hobson, 2006; Saino et al., 2010). Thus, female reproductive investment early in the
95 reproductive cycle should be closely related to local foraging effort in order to supply
96 the requirements of clutch production. In contrast, male reproductive investment during
97 this period should be mainly devoted to courtship feeding and agonistic behaviors
98 directed to compete for mates or to defense of their mates and nests (Burger, 1984, and
99 references therein). Despite the qualitative differences in sex roles, both male and
100 female reproductive investments are ultimately related to muscular activity, although
101 parents remaining motionless on their nests are also serving an important role of
102 protecting their nesting territories (see Burger, 1984). In this regard, exercise-induced
103 muscle damage is characterized by the release of muscle-specific proteins such as
104 creatine kinase (CK) to plasma (Knuth and Chaplin, 1994; Smith et al., 2004) and
105 measurements of CK levels have been previously used as an indicator of physical effort
106 associated with muscular activity dependent behaviors such as migration or foraging
107 (Guglielmo et al., 2001; Navarro et al., 2008).

108

109 Here, we investigated patterns of CK in plasma through incubation in a seabird species
110 with biparental care, the Yellow-legged Gull (*Larus michahellis*, Naumann 1840) as a
111 reflection of the physiological response of individuals to the reproductive investment
112 made up to clutch completion. As incubation constitutes a rest phase that allows
113 individuals to recover their physiological state after the high energy demanding periods
114 of pre-laying and laying (Alonso-Alvarez et al., 2002), recovery patterns in their CK
115 levels should be expected. We first evaluated the relationship between costs associated

116 with clutch production, as estimated from Field Metabolic Rate (FMR), and CK patterns
117 in females. If clutch production and local foraging effort are closely related processes,
118 as we expect for income breeders, we predicted that females producing larger eggs
119 would have higher CK levels due to greater foraging efforts. Secondly, we tested the
120 hypothesis of comparable sex-specific investment before clutch completion by
121 comparing CK trends for both sexes throughout incubation. We predicted that similar
122 reproductive investment would result in similar CK recovery patterns.

123

124 **2. Materials and methods**

125 *2.1. Model species and field methods*

126 The Yellow-legged Gull was selected as an appropriate model species for this study
127 since they are iteroparous, long-lived and sexual maturity is not reached until the 4th
128 summer. These traits make this species particularly susceptible to trade-offs between
129 current breeding and future reproductive output according to life-history theory
130 (Williams, 1966) and, therefore, to selective pressures responsible for the differential
131 allocation in reproduction between the sexes.

132

133 In gulls, females typically show modal clutches truncated to three eggs (Reid, 1987),
134 although different clutch sizes can be also observed within population (e.g. two-egg
135 clutches represent about 20% of overall clutches, Bosch et al., 2000). Incubation, which
136 is shared by both sexes (Burger, 1984; Alonso-Alvarez et al., 2002), is gradually
137 established as successive eggs are added to the clutch (Parsons, 1972). Due to the
138 impossibility of sampling non-incubating birds and with the aim of standardizing the
139 inclusion of individuals in the study (due to differing clutch sizes), bird sampling was
140 performed after clutch completion. In particular, during peak laying of the breeding

141 season of 2008 (from 2-13 April), nests of Yellow-legged gulls breeding at the
142 Peninsula de la Banya (40°40'N, 0°45'E; within the Ebro Delta Natural Park, Spain)
143 were tagged when the first egg was laid, and inspected daily until clutch completion,
144 with the aim of determining laying order, final clutch size and end of the laying period.
145 Fresh, newly-laid eggs (< 24h after laying) were collected and replaced by dummy eggs,
146 kept refrigerated and transported to the laboratory where they were frozen until
147 processing. From those nests with a modal clutch of three eggs, 53 adults were caught
148 during incubation using traps set at nests. Adults were captured early in the morning to
149 avoid variation in the individuals' physiological state throughout the day and the time
150 elapsed between capture and sampling was similar for all individuals (about 15 minutes)
151 in order to reduce the variability in levels of analyzed biochemical parameters
152 associated to sampling procedure. Adult mass was measured using a dynamometer to
153 the nearest mg. Two mls of blood were taken from the tarsal vein; 1.5 ml was placed in
154 a vial with EDTA to later extract the plasma for the biochemical parameter
155 determinations and the rest was preserved in a neutral vial for molecular identification
156 of sex. All samples were frozen at -23 °C until sample analysis.

157

158 ***2.2. Laboratory procedures and data processing***

159 Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the
160 CHD genes (Ellegren, 1996; Griffiths et al., 1998). To extract DNA, blood was boiled in
161 100mM NaOH for 10 minutes at 100°C before being added to the PCR reaction. PCR
162 protocols were modified from Fridolfsson and Ellegren (1999) (see Genovart et al.,
163 2003) using the primer set 2550F-2718R, and PCR products were visualized on a 3%
164 agarose gel stained with ethidium bromide scoring a single band in males and two bands
165 in females.

166

167 Plasma biochemical analyses of total protein (TP) and CK were determined using a
168 Cobas 6000 automated analyzer (Roche Diagnostics, Mannheim, Germany).
169 Manufacturer's guidelines were followed for all methods. TP (g/dL) was analyzed by
170 biuret assay (Weichselbaum, 1946), whereas CK (IU/L) was determined by kinetic
171 methods following recommendations of IFCC (Bergmeyer et al., 1986; Schumann et al.,
172 2002). Two individuals, one male and one female, were excluded from subsequent
173 analyses since they showed CK values more than four times higher than male and
174 female average concentrations. Occasional dehydration of animals, due to long foraging
175 trips or exhausting exercise, is usually associated with hemoconcentration, leading to
176 overestimates of plasma concentration. To reduce the variability of CK values due to
177 undesirable factors such as the differential physiological state of individuals when
178 sampled (i.e. differential dehydration), raw levels of this parameter were related to TP,
179 which is widely accepted as a more conservative and well regulated parameter sensitive
180 to dehydration status. In this way, we obtained standardized levels of CK related to
181 grams of total plasma proteins (CK/TP) which were analyzed throughout.

182

183 To evaluate the influence of clutch production on female CK values, costs associated
184 with clutch synthesis were estimated following Ruiz et al. (2000). Eggs were initially
185 boiled to allow separation of different egg components (i.e. albumen and yolk). Each
186 component was then freeze dried to constant mass and yolks were subjected to a lipid
187 extraction with several rinses of 2:1 chloroform-methanol. In each step, sub-samples
188 were weighed in order to ascertain protein and lipid content of each egg component.
189 Clutch energy content (hereafter energy value) was then estimated by using appropriate
190 conversion factors accounting for 39.75 kJ/g for lipids and 23.64 kJ/g for proteins

191 (Ricklefs, 1974). Estimated energy values were refined by adjusting them to account for
192 energy costs derived from absorption, metabolism and deposition of rebuilt materials.
193 Average costs for lipids and proteins in birds were considered to be about 22% and 45%
194 of estimated energy values, respectively (Blaxter, 1989). In this way, we obtained
195 estimates of total amount of energy invested during egg synthesis (hereafter energy
196 investment). As a measure of female costs of clutch production we calculated the ratio
197 between energy investment in clutch synthesis and FMR (kJ/d) during clutch
198 production. Following Ruiz et al. (2000), we assumed modal clutch (three eggs)
199 production to last 16 days (10 days for the rapid yolk deposition, 2 days for the
200 deposition of albumen, membranes and shells and 2 days for the follicle triggering
201 interval). FMR was considered to be 3.29 times the Basal Metabolic Rate (BMR,
202 Birtfriesen et al., 1989) and BMR was estimated from the relationship $BMR =$
203 $2.1857 \cdot \text{mass}(\text{g})^{0.7347}$ (Furness and Monaghan, 1987).

204

205 *2.3. Statistical analyses*

206 Linear models (LMs) were used to evaluate the relationship between female costs of
207 clutch production and their CK/TP values. The number of days elapsed between clutch
208 completion and capture of individuals (hereafter elapsed time) as well as the relevant
209 interactions were included in this model to take into account any temporal trend in this
210 parameter through the incubation period. LM was also applied to evaluate temporal
211 trends of CK/TP throughout incubation. Here, the effect of elapsed time on the
212 biochemical parameter was established. Sex and relevant interactions involving this
213 factor were also included in these models in order to detect and quantify differences in
214 temporal trends according gender. Statistical analyses were done using SPSS 15.0
215 (SPSS Inc., Chicago, USA).

216

217 **3. Results**

218 Of the 53 adults analyzed, 35 individuals were females and 18 were males. Biochemical
219 parameters for both sexes are summarized in Table 1. When evaluating the influence of
220 female costs of clutch synthesis on CK levels, the model indicated a significant effect
221 on the CK/TP temporal trend ($F_{1,30}=5.03$, $p=0.03$ for the interaction between costs of
222 clutch production and elapsed time). In order to explore this interaction, females were
223 grouped according to tertiles of female costs of clutch production: low costs (<15.8% of
224 FMR), high costs (>17.3%) and medium costs (15.8 to 17.3%). Higher costs of clutch
225 production resulted in higher initial levels of CK/TP and a more pronounced decrease in
226 these values along the incubation period (Fig. 1).

227

228 The absence of the interaction sex*elapsed time ($F_{1,47}=0.001$, $p=0.98$) indicated CK/TP
229 slopes did not significantly differ between sexes. This pattern consisted of a gradual
230 decrease of CK/TP values through the incubation period ($F_{1,48}=44.75$, $p<0.001$, 95% CI
231 for the estimated slope=-4.3 to -2.3). Moreover, we did not detect a significant effect of
232 sex, suggesting similar average values of CK/TP for both sexes ($F_{1,48}=0.124$, $p=0.73$,
233 95% CI for the estimated differences between males and females=-8.79 to 6.17; Fig. 2).

234

235 **4. Discussion**

236 Previous studies aimed at quantifying reproductive investment and, therefore,
237 reproductive costs have been focused largely on trade-offs between investment in
238 incubation or chick rearing and parental survival and fecundity (Navarro and Gonzalez-
239 Solis, 2007, and references therein). However, to fully understand the evolution of
240 reproductive strategies followed by each sex, all reproductive costs, including clutch

241 production in the case of females and agonistic behaviors and courtship feeding in the
242 case of males, need to be considered (Gladstone, 1979; Baylis, 1981; Burger, 1981;
243 Nager et al., 2001). The initial asymmetry in reproductive investment between the sexes
244 proposed by classical sex allocation theory (Trivers, 1972) has been previously assumed
245 but never measured. This is due, in part, to the fact that traditional approaches would
246 require a unique measure that integrates all the energy demanding processes related to
247 reproduction. In this regard, reported results suggested that CK activity in plasma is a
248 reliable indicator of the physiological responses to increased muscular activity to which
249 males and females are subjected to during this period (i.e. initial reproductive
250 investment) and provide, therefore, a new and excellent method to examine a central
251 component of the reproductive strategy followed by each sex. In particular, this
252 physiological assay has revealed as a reliable proxy to female investment in clutch
253 production and suggests that initial reproductive investment of males, including
254 agonistic behaviors and courtship-feeding, apparently counterbalance female investment
255 in clutch synthesis.

256

257 ***4.1. Female investment in clutch production***

258 As expected for an income breeder, the relationship between female costs of clutch
259 production and plasma CK values agrees with an initial reproductive investment by
260 females that is closely related to local foraging effort. Females that invested heavily in
261 clutch synthesis corresponded to those with higher muscular damage (i.e. higher CK
262 levels) after clutch completion, indicating greater muscular activity probably due to
263 greater foraging efforts. In other words, greater foraging efforts, as indirectly indicated
264 by higher levels of CK in plasma, allowed females to produce larger and more
265 energetically expensive eggs, probably enhancing egg hatchability and chick survival

266 and, therefore, females' current reproductive success (Parsons, 1970; Ruiz et al., 1998;
267 Christians, 2002). However, the life-history theory (Williams 1966) predicts that
268 increasing current reproductive efforts may reduce females' future reproductive output
269 by reducing their fecundity or survival until future breeding seasons.

270

271 Whenever initial female reproductive investment is closely related to local foraging
272 effort, reproductive effort before clutch completion will depend on females' physical
273 condition and foraging efficiency: the fittest and most experienced females will require
274 lower efforts to deal with clutch production. Indeed, several female intrinsic traits such
275 as body condition, age or experience have been related to the amount of resources
276 allocated to egg synthesis (as indicated by egg size, see Christians 2002). Such female
277 intrinsic traits may also influence their physiological response to reproductive
278 investment: the fittest females would show smaller physiological responses to similar
279 reproductive requirements (see Guglielmo et al., 2001 for the relationship between
280 birds' trained state and plasma CK activity). Rather than direct estimations of resources
281 allocated to egg synthesis (e.g. egg size or mass), this physiological assay is therefore
282 proposed as a better and more realistic approach to investigate among-female relative
283 reproductive investment before clutch completion.

284

285 ***4.2. Sex-specific reproductive investment***

286 Methodological limitations to sampling gulls (see Material and methods) forced us to
287 investigate the recovery patterns of CK in plasma through incubation and so we
288 speculated about sex-specific physiological responses to the reproductive investment
289 they were subjected to up to clutch completion. In this regard, similar recovery trends of
290 CK for both sexes suggested similar recovery patterns of muscular damage and,

291 therefore, comparable reproductive investments before clutch completion. Male
292 investment, including agonistic behaviors and courtship feeding during pre-laying and
293 laying, apparently counterbalance female investment in clutch production.

294

295 Although speculative, our interpretation is in agreement with Burger (1984) who
296 reported higher frequencies of agonistic behaviors for males of Herring Gull (*Larus*
297 *argentatus*) before clutch completion, whereas both sexes contributed about equally to
298 incubation, brooding and feeding. Additionally, results of a physiological approach for
299 Cory's shearwaters (*Calonectris diomedea*), another seabird species with biparental
300 care, revealed that males were in poorer condition than females before clutch
301 completion probably due to reproductive investment of males in agonistic behaviors and
302 courtship feeding (Giudici et al., 2010). In these iteroparous and long-lived species,
303 parents are particularly limited in how they can increase effort in current reproductive
304 investment in order to moderate the impact on future reproduction attempts (Williams,
305 1966; Croxall, 1982; Chaurand and Weimerskirch, 1994; Tveraa et al., 1997; Velando
306 and Alonso-Alvarez, 2003; Navarro and Gonzalez-Solis, 2007). In this context,
307 selective pressures directed at making investment in reproduction by both sexes equal
308 should be expected.

309

310 **5. Conclusions**

311 Despite qualitative differences in sex roles of Yellow-legged gulls, CK levels in plasma
312 are shown to be a reliable proxy to the overall energy investment that both sexes are
313 subjected to up to clutch completion. This allowed quantitative comparisons of sex-
314 specific reproductive investment during pre-laying and laying and suggested that
315 agonistic behaviors and courtship feeding in the case of males may compensate for

316 females' costs of clutch production. This physiological approach is expected to assist in
317 understanding the selective pressures that have modulated sex roles in reproduction
318 since provide information about a central component of the reproductive strategy
319 followed by each sex.

320

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453 **TABLES**

454 Table 1.- Levels (Median, Minimum and Maximum) of biochemical parameters (CK and TP) measured in incubating Yellow-legged gulls.
 455 The female reproductive effort in gametogenesis, i.e. the energy investment to clutch production relative to the Field Metabolic Rate
 456 (FMR) during the clutch production period (16 days), have been also summarized.

		Female				Male			
		n	Median	Min	Max	n	Median	Min	Max
Biochemical parameters									
< 4 days	CK (IU/L)	7	1590.6	916.7	2827.8	4	1408.8	1265.3	1670.8
	TP (g/dL)	8	3.3	2.8	4.0	5	3.1	2.7	3.7
5-9 days	CK (IU/L)	12	716.5	176.2	1626.9	10	657.8	195.9	1302.7
	TP (g/dL)	12	3.4	2.7	4.7	10	3.3	2.6	4.0
>10 days	CK (IU/L)	15	363.3	196.2	669.6	3	624.2	520.8	781.7
	TP (g/dL)	15	3.3	2.7	4.3	3	3.2	2.5	3.3
Gametogenesis									
	Energy investment (kJ)	35	2793.7	2249.2	3296.4				
	FMR (kJ·d ⁻¹)	35	1051.7	949.5	1142.0				
	Reproductive effort (%kJ)	35	16.6	14.1	20.7				

457

FIGURES

Figure 1.- CK/TP patterns through incubation (Elapsed time: number of days elapsed between clutch completion and capture of individuals) for females grouped according to three differential costs of clutch production (energy investment in clutch synthesis respect Field Metabolic Rate FMR, see Material and Methods): low costs (<15.8% of FMR), high costs (>17.3%) and medium costs (15.8 to 17.3%)

Figure 2.- Temporal trends of CK/TP through incubation (Elapsed time: number of days elapsed between clutch completion and capture of individuals) for each sex. Solid line represents regression line predicted by the model (see Material and Methods and Results).

Figure 1.

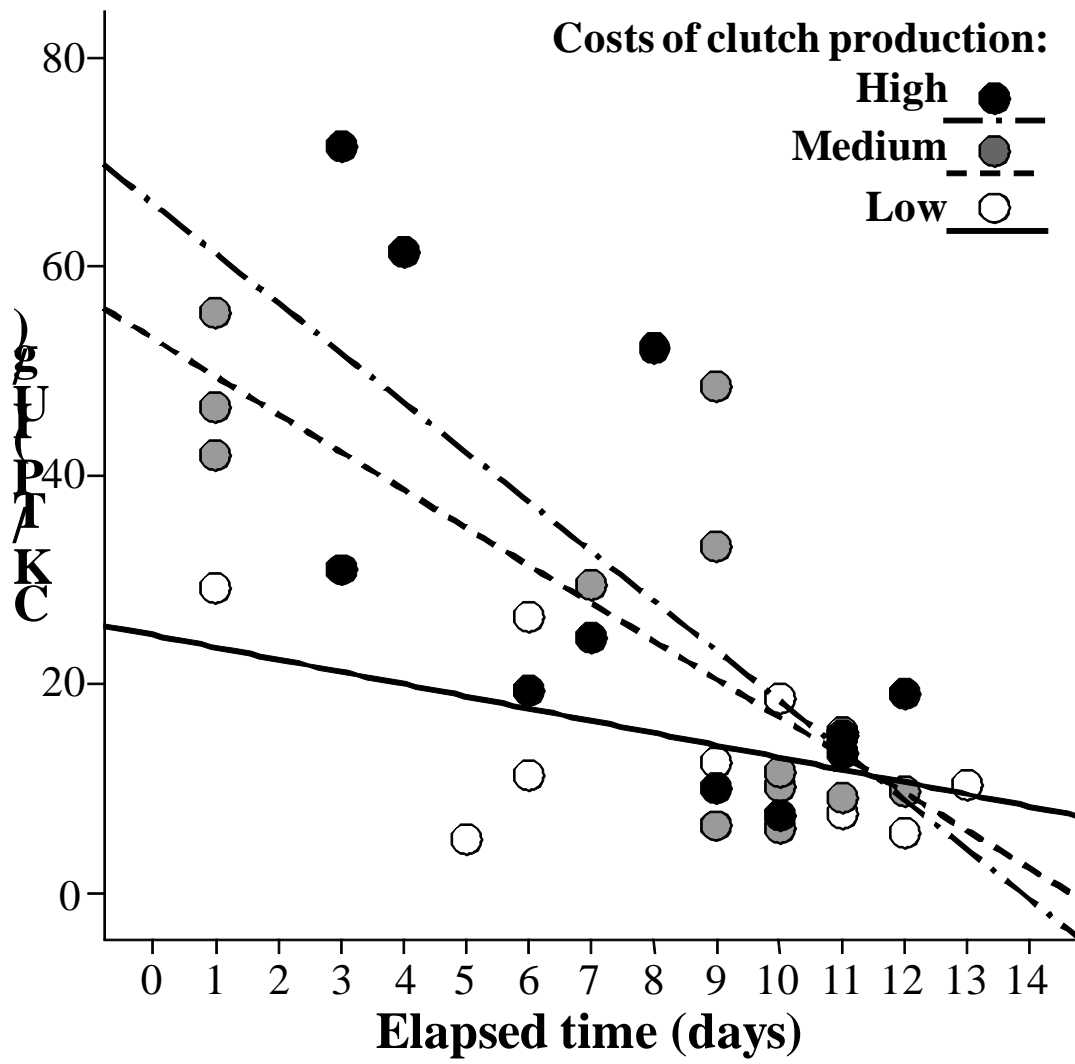


Figure 2.

