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Manipulation of photoperiod perception advances gonadal growth but not laying date in the great tit

Lucia Salis, Samuel P. Caro, Roelof A. Hut, Louis Vernooij and Marcel E. Visser

In seasonal environments, organisms use biotic and abiotic cues to time various biological processes that are crucial for growth, survival and reproductive success. Photoperiod is the best-known cue used to regulate gonadal development, migration and moult of many animal species. In birds, the relationship between photoperiod and gonadal development is clearly established, but we have little understanding on whether photoperiod also regulates actual timing of egg laying under natural conditions. Elucidating the link between photoperiod and timing of breeding is however key to understand whether an evolutionary change in sensitivity to photoperiod is a possible mechanism through which organisms could adjust their seasonal timing in response to climate warming. Here, we investigated the causal relationship between photoperiod, gonadal growth and laying date in wild female great tits. We experimentally increased the photoperiod perceived by the birds in spring by clipping head feathers, and we subsequently monitored gonadal development in the lab and egg laying dates in the wild. We show that our manipulation increased the photoperiod perceived by the birds to a level that approximately corresponds to an advancement of ten calendar days. This increase in perceived photoperiod led to an acceleration of gonadal development, but not to an advancement of egg laying dates. Our results indicate that photoperiod sensitivity is not constraining the advancement of laying date under current environmental conditions and suggest that evolution of sensitivity to other supplementary cues is necessary to advance reproduction under global warming.

Keywords: adaptation, avian physiology, climate change, phenological mismatch, timing of reproduction

Introduction

Seasonal timing of various biological processes such as reproduction, migration or hibernation is crucial to the survival and reproductive success of an individual. To cope with future conditions, organisms often need to make physiological and developmental preparations well in advance to the actual event. In order to predict the appropriate
timing of these biological events, these organisms often use information from the environment, termed cues, which interact with their endogenous timing mechanisms (Visser et al. 2010, Helm et al. 2013, 2017). Both in birds and mammals, photoperiod is the primary cue used to initiate and regulate the physiological and behavioural cascades for most seasonal events. The annual variation in day length for example triggers the activation of the reproductive system that translates into hormonal changes, development of the gonads (usually, in birds, regressed outside the breeding period), courtship behaviours, pairing and ultimately, egg fertilizations (Lofts and Murton 1968, Murton and Westwood 1977, Wingfield and Kenagy 1991, Silverin 1994, Reparaz et al. 2014).

The underlying physiological and molecular mechanisms of the avian photoperiodic perception and responses have been extensively investigated (Farner 1985, Follett et al. 1985, Dawson et al. 2001, Goldman 2001, Ball and Balthazart 2002, Yoshimura et al. 2003, Sharp 2005, Yoshimura 2006, Ubuka et al. 2013, Dawson 2015). In most vertebrates, light is perceived by photoreceptors, located in different parts of the body. While in mammals, functional photoreceptors seem to be exclusively located in the eyes, in other vertebrates, light also penetrates the skull that is translucent, and directly stimulates photoreceptors located in the pineal gland and in the hypothalamus. In 1935 Benoit reported the first evidence of a critical role for extra-retinal photoreceptors in the regulation of seasonal reproduction in birds. Ducks exposed to long days developed their gonads even if they were blinded, but ducks covered with black caps on their head did not (Benoit 1935). Extra evidence for a decisive role of extra-retinal photoreceptors was collected by Menaker and colleagues (Menaker 1968, Menaker and Keatts 1968, Menaker et al. 1970, Underwood and Menaker 1970). For instance, sparrows with head feathers plucked grew their gonads whereas birds that were subcutaneously injected with India ink under their scalp did not show any reproductive response (Menaker et al. 1970). While in this last study it was an ‘all or nothing’ response, Bentley and colleagues show a quantitative response in European starlings exposed to different light intensities (Bentley et al. 1998). In Japanese quails, both pinealectomized and blinded individuals showed reproductive response to photic stimuli (Saylor and Wolfson 1968, Siopes and Wilson 1974). These studies show that, in birds at least, the extra-retinal photoreceptors located in the deep brain are sufficient to induce a complete reproductive response. More recent studies have described some of the precise mechanisms involved in photoreception, with hypothalamic photoreceptors characterized by neurons containing neuropsin (OPN5) as the functional photopigment (Nakane et al. 2014). OPN5 has been reported to be bistable and action spectra were measured (or predicted) peaking at 380 nm for both mouse and human, 415 nm for chicken and 419 nm for quail (Nakane et al. 2010, Yamashita et al. 2010, Kojima et al. 2011).

When an animal is exposed to the subjective stimulating photoperiod, a complex neuro-endocrine cascade is initiated, which orchestrates the activation of the reproductive system. This cascade involves thyroid hormones that regulate the secretion of the gonadotrophic releasing hormone-I (GnRH-I), a neuro-hormone considered as one of the most important stimulants of the reproductive axis in vertebrates (Sharp et al. 1990, Nakao et al. 2008, Hut 2011, Kriegsfeld et al. 2015, Hau et al. 2017). GnRH then stimulates the release of gonadotropins (FSH and LH) by the pituitary (Scanes 2000), which in turn stimulate the growth, and steroid production of the gonads. In most bird species, prolonged exposure to long days leads to a complete switch off of this hypothalamic–pituitary–gonadal (HPG) axis, resulting in a state of photorefractoriness where GnRH production and secretion decrease, gonads regress and moult occurs (Dawson et al. 2001). Pioneering work by Gwinner in the late 1970s and early 1980 showed the strong effect of photoperiod cycles on the HPG axis in starlings. Shortening the annual photoperiodic cycles to periods of eight months, or even shorter (up to 2.4 months cycles), induced repeated cycles of testicular growth and regression, and moult (Gwinner 1977, 1981, but see Dawson 2007).

The role that photoperiod exerts on the HPG axis has been widely investigated and led to a detailed comprehension of many of the mechanisms involved. These experiments were however mostly conducted in the lab, on males exclusively, and looked at gonadal size as the final proxy for breeding (Ball and Ketterson 2008, Zucker and Beery 2010, Caro 2012, Williams 2012, but see te Marvelde et al. 2012). This has resulted in a lack of understanding on the role that photoperiod and other cues play on the decision on when to effectively start breeding (i.e. copulate and produce offspring), which is the most important reproductive output in an ecological perspective (Visser et al. 2010). In particular, it remains unclear to what extend the development of the gonads is linked to egg laying dates in birds. While it is clear that the gonads need to be fully developed before eggs can be produced, few studies have explored gonad size together with lay date, even at the population level (Wingfield 1984, Pearson and Rohwer 1998, Caro et al. 2009), and the few that looked at this relationship at the individual level found no clear evidence (Schaper et al. 2012a, te Marvelde et al. 2012). Thus, although it has been shown that exposing captive wild birds to long photoperiod can advance egg laying date (Lambrechts et al. 1997), the interplay between photoperiod, gonadal size and timing of breeding remains obscure.

The relationship between photoperiod and laying date is likely modulated by supplementary cues such as food availability and temperature (Wingfield and Kenagy 1991). Photoperiod could serve as initial cue that generates a time window in which reproduction can potentially occur, and other cues would fine-tune when reproduction actually occurs. In our study species, the great tit Parus major, photoperiod plays a pivotal role in gonadal maturation and on the entire hypothalamic–pituitary–gonadal axis (Silverin et al. 1989, 1997, 1999), but temperature also directly affects timing of reproduction (Visser et al. 2009, Schaper et al. 2012b).
This effect of temperature on great tits’ timing could be adaptive since temperature also affects the phenology of the seasonal peak of abundance of the birds’ main prey (Visser et al. 2006). In warm years, caterpillar availability in spring is indeed earlier, as is the mean great tit population egg-laying date (Visser et al. 2003). However, if temperature can influence laying dates, it does not seem to influence the underlying neuro-endocrine mechanisms (Schaper et al. 2012b, Caro et al. 2013), which could constrain the necessary advancement of their timing of breeding, and by extension the restoration of the synchrony with their main prey, in response to climate change (Caro et al. 2013). An alternative mechanism through which great tits could advance their onset of reproduction, would be to change their sensitivity to photoperiod, rather than to temperature (Dawson 2005), like it has been shown in pitcher plant mosquitoes (Bradshaw and Holzapfel 2001).

The aim of this study was to investigate whether there is a causal relationship between photoperiod and laying date in wild great tits. We experimentally increased the photoperiod perceived by the birds during their pre-breeding season by clipping their head feathers. This manipulation (Fig. 1) increased the intensity of light perceived by the hypothalamic photoreceptors (Fig. 2) and by extension, the duration of daylight by an amount that represents an advancement of approximately ten calendar days (Fig. 3). We subsequently recorded the egg laying dates of the control and treated birds. In parallel, we measured gonadal growth in wild females kept under semi-natural conditions in outdoor aviaries, to investigate the underlying physiological effects of the clipping treatment.

**Material and methods**

All the experiments performed in this study were approved by the animal experimentation committee of the Royal Dutch Academy of Sciences (DEC-KNAW; protocol number NIOO12.05). All surgery was performed under Isoflurane anesthesia, and all efforts were made to minimize discomfort.

![Figure 1](image1.png)

Figure 1. Experimental treatments: (a) clipped; (b) control and (c) behavioural control. In clipped birds the head feathers were clipped; in the control group birds were only handled and in the behavioural control group head feathers were clipped and ink was injected subcutaneously.
the skull, to restore the light filtering properties of the intact, feathered skull. In 2013 and 2014 we also injected ink subcutaneously on the back in the clipped group. We made this latter injection to completely exclude the possibility that the ink injection has negative effects on the birds’ performances. Moreover in 2014, we did not include a behavioural control group as we had sufficient data for this group, and our main interest was in comparing the clipped and the control group. The sample size of each experimental group in the three different years is shown in Table 1.

Light measurements

To verify that clipping the head feathers increases the light intensity reaching the brain, we measured the light transmission through the skull in deceased birds. After surgical removal of the brain, the diffusor of the photospectrometer (JETI specbos 1211 with cosine diffusor mounted fiberoptic, Spectrapartners, Haarlem, the Netherlands) was placed under the skull and the transmitted absolute light spectrum was measured from 380 to 780 nm in 1 nm steps. All measurements were done outdoor, under clear sky natural light conditions, around noon. Each bird (n = 3) was measured three times: 1) with head feathers intact (control); 2) with the feathers clipped (clipped) and 3) with feathers clipped and ink injected subcutaneously (behavioural control). In each of the three different conditions, measurements were replicated three times and averaged.

Earlier studies by Menaker (1968), using blinded birds, showed that about 0.1 lux of light perceived via hypothalamic photoreceptors is sufficient to elicit testis growth and entrainment to the circadian rhythm in about 50% of the blinded birds (however see Fig. 1 in Dawson 2015). Our experimental manipulation induced a tenfold increase in the amount of light penetrating the skull (1 log unit; see Fig. 2), thus we assumed that the light intensity of 0.01 lux in clipped birds elicits the same effects on the underlying physiology as the effects of 0.1 lux in an intact bird. In other words, we expected clipped birds to be sensitive to light intensities that

Figure 2. Light transmissions and penetration curves for birds in the three treatments. (a) Light transmission in percentage of light intensity measured through the skull with feathers (control, red), no feathers (clipped, blue) or no feathers + inked (behavioural control, green). All three measures were collected subsequently on the same bird skull in three different bird skulls. (b) Solar photon flux spectrum with resultant photon flux through the skull in the three conditions (clipped, control, behavioural control). For comparison the relative absorbance curve of the putative avian photoperiodic photopigment neuropsin (OPN5, grey line) is also presented, with peak absorbance at 417 nm, as measured in quail and chicken (Nakane et al. 2010, Yamashita et al. 2010), using the opsin nomogram equations by Stavenga (Stavenga et al. 2000).

Table 1. Sample sizes of each experimental group in the field studies in 2012, 2013 and 2014. Number and percentage of birds in the experiment that were found back breeding later in the season are also indicated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Birds with laying date</th>
<th>% with laying date</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012 clipped</td>
<td>11</td>
<td>9</td>
<td>84%</td>
</tr>
<tr>
<td>2012 control</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2012 behavioural control</td>
<td>10</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2013 clipped</td>
<td>12</td>
<td>6</td>
<td>54%</td>
</tr>
<tr>
<td>2013 control</td>
<td>12</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2013 behavioural control</td>
<td>11</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2014 clipped</td>
<td>19</td>
<td>14</td>
<td>76%</td>
</tr>
<tr>
<td>2014 control</td>
<td>15</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>71</td>
<td>71%</td>
</tr>
</tbody>
</table>

Figure 3. Day length variation of different light intensity as proceeding during in the spring. The light intensity of 0.01 lux is the same as perceived in bird with clipped feathers and 0.1 lux in control bird with intact feathers. The perceived day length in birds with clipped feather is about 30 min longer, which corresponds to approximately 10 days in March and April.
would not elicit a response in intact birds. Earlier work by Daan and Aschoff (1975) shows that a light intensity of 0.1 lux is in between the nautical and civil twilights, while an intensity of 0.01 lux is close to the nautical twilight. Based on this knowledge, we calculated that in intact birds, the onset of light perception occurs between the civil and nautical twilight (when light intensity is 0.1 lux) while in clipped birds it occurs before the nautical twilight (when light intensity is 0.01 lux). Thus, we assumed that birds with clipped head feathers perceived a longer day length compared to intact birds throughout the duration of the experiment (Fig. 3).

Captive study and gonadal measurements

The captive study was performed in 2012 on birds caught in a mixed deciduous forest (Bennekom, The Netherlands). In early March, 16 female great tits were captured at night while sleeping in nest-boxes and brought to the laboratory where they were temporarily housed in individual cages. The next morning, they were unilaterally laparotomized under isoflurane anaesthesia by making a small incision between the last two ribs on the left side of the bird. Diameter of the largest ovarian follicle was measured (to the nearest 0.1 mm), using a scale engraved in the ocular of a binocular microscope. Follicle volume was calculated as: \[ V = \frac{4}{3}\pi a^3 \], where \( a \) is diameter/2. After the laparotomy, birds were returned to their individual cages for one more day to facilitate the recovering process. After full recovery, they were randomly allocated to the clipped or to the control group as described in the previous section. Each bird was also randomly allocated to one of two outdoor aviaries (i.e. there were birds from both treatments in each aviary), where they were exposed to natural photoperiod and temperature conditions. Food and water were provided ad libitum and at least one nest-box was available for each bird. In early April and early May, the diameter of the largest follicle was again measured, following the same procedures as in the first laparotomy. A few days after the third laparotomy, all birds were released in the wild at the place of capture.

Statistical analyses

To analyse the differences in light intensity transmitted through the skull of birds belonging to the three treatments we used a linear mixed model. Treatment, wavelength and their interaction (treatment \( \times \) wavelength) were fit as fixed effects and bird identity was fit as a random effect.

To analyse the effect of the clipping treatment on gonadal size in the captive study we used linear mixed models. The models included treatment, sampling date and their interaction (treatment \( \times \) date) as fixed effects and bird identity as a random effect. Date was fit as continuous variable in a first analysis and in a second analysis it was fit as a factor to analyse the effect of treatment in each month. All data on gonadal size were log transformed to account for exponential growth.

To analyse the effect of treatment on egg-laying date, we performed two analyses on the data obtained in the field study. In the first analysis, data from the three years were pooled and we performed a linear mixed-effects model analysis followed by a Kenward–Roger approximation (‘KRmodcomp’ function in the R package pbkrtest (Halekoh and Højsgaard 2014)) for testing the significance of fixed effects in mixed-effects models. The full model included treatment, experimental year and their interaction (treatment \( \times \) experimental year) as fixed effect and bird identity as a random effect. Bird identity was included in the model as some of the birds used in the previous year(s) as control were re-captured and used again in the following year(s). To perform this analysis, we only considered data from the clipped and the control groups as the data relative to the behavioural control group were available for two out of the three experimental years only. In the second analysis, we used linear models with treatment as fixed effect on three separated data-sets, one for each experimental year (2012, 2013 and 2014). In these analyses, all three treatments were considered when present.

All statistical analyses were performed with the R software (ver. 3.0.2).

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.jh9w0vt6d> (Salis et al. 2019).

Results

The measurements of the light transmitted through the skull showed that the clipping treatment significantly increased the light intensity throughout the spectrum measured (380–780 nm) (Fig. 2). The amount of light transmitted through the skull of clipped birds was significantly higher than the intensity in birds of the two control groups (Fig. 2, \( p < 0.001 \)).

In captivity, the treatments affected gonadal size as the interaction treatment \( \times \) date was significant (\( p = 0.016 \)). Thus, clipped birds grew their gonads faster than control birds (Fig. 4).

In the field, females from the clipped group did not breed earlier than control females (Fig. 5, Table 2; Kenward–Roger approximation test, \( p = 0.44 \)). Overall, egg-laying dates significantly differed only across experimental years (Table 2; \( p < 0.001 \)). When years are analysed separately, and thus when all three treatments are considered, we found no statistical differences between treatments, except in 2012, where females from the behavioural control group laid significantly later than the two other groups (Table 3; year 2012, \( p = 0.03 \)). However, this effect was not found in the following year (Table 3; year 2013, \( p = 0.24 \)). Overall, the egg-laying dates of clipped birds were never earlier than the control group (Table 3).
Among other cues involved in timing of reproduction, temperature has been shown to influence the onset of egg-laying in captive great tits, starlings and zebra finches (Meijer et al. 1999, Salvante et al. 2007, Visser et al. 2009, Schaper et al. 2012b, Caro et al. 2013). In general, higher or increasing temperatures trigger birds to lay earlier. Some experimental studies have also succeeded to modify egg-laying date via food availability (Nilsson 1994, Greico et al. 2002, Gienapp and Visser 2006). A meta-analysis study by Ruffino and colleagues suggests that food supplementation positively influences reproductive parameters among which timing of reproduction (Ruffino et al. 2014). Specifically to great tits, in the Hoge Veluwe females that experienced experimentally increased food availability in the previous year, laid their eggs earlier in the next year (Gienapp and Visser 2006). Similarly, food provisioning led to earlier egg laying in blue tits, however mortality of early-breeding females was higher, supporting the hypothesis that cost of reproduction earlier in the season might be too high compared to the fitness benefits gained via increased offspring’s fitness (Nilsson 1994, but see Greico et al. 2002).

In this study we assumed that differences in follicular development in the laboratory also occurred in free-living birds. As we could not laparotomize females in the field since they were almost impossible to catch multiple times without them deserting the nest, we cannot be certain that gonadal size was affected by the photoperiodic treatment also in the field. One way to measure both laying date and gonadal size on the same birds is be to keep the birds in pairs in aviaries, although, such study would not give us information about effects of photoperiodic manipulation on birds in the wild. Ovarian follicle growth measured in captivity may also seem relatively modest and late in the season compared to what could be expected in wild birds. However, with a difference of 0.6 mm between the control and treatment groups (1.2 mm vs 1.8 mm) (Fig. 4), clipped birds had follicles that were twice as big as control birds, and it must be noted that those females were housed in single-sex groups and not in pairs with males, which accelerates breeding in female (Hinde and Steel 1976,
Perfito et al. 2015). Furthermore, wild great tits housed in captivity breed on average three weeks later than in the wild (Visser et al 2009).

Regarding the lack of an advancement in egg laying date in the wild following our manipulations, besides the important role of supplemental cues, a possible explanation could be that birds sometimes sleep in a nest box and therefore they might have been not always exposed to the increased photoperiod as we assumed. However, also birds in captivity had access to nest boxes and there was a difference in the rate of gonadal growth. Furthermore we know from personal observations in our field sites that in the pre-breeding season (February/March) it is mainly the males and not the females who sleep in boxes at night and go out of the box before sunrise. To catch enough females for the experiment we had to check many nest-boxes as most of them contained males. Another reason for the lack of an observable significant effect could be due to the small sample size and the large variation in laying day which reduced the statistical power considerably in two out of the three experimental years (2012 = 0.12; 2013 = 0.58; 2014 = 0.19 post-hoc power analyses computed with G × power 3.1).

Figure 5. Mean (± SE) egg-laying date of clipped and control birds in the field study performed in 2012, 2013 and 2014. In 2012 the egg-laying date of the behavioural control group was significantly later than the control group. In the two other years, egg-laying dates did not differ among groups. Sample size of each group is indicated above.

Figure 5. Mean (± SE) egg-laying date of clipped and control birds in the field study performed in 2012, 2013 and 2014. In 2012 the egg-laying date of the behavioural control group was significantly later than the control group. In the two other years, egg-laying dates did not differ among groups. Sample size of each group is indicated above.

Table 2. Statistical analysis of the effect of the experimental treatments on egg-laying date in the field in 2012, 2013 and 2014. Results of the Kenward–Roger approximation are reported. F values (F), numerator of degrees of freedom (ndf), denominator degree of freedom (ddf) and p values are indicated. The data-set used here only includes the clipped and the control groups.

<table>
<thead>
<tr>
<th>Explanatory variable</th>
<th>F value</th>
<th>ndf</th>
<th>ddf</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment × year</td>
<td>0.58</td>
<td>2</td>
<td>49.0</td>
<td>0.57</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.61</td>
<td>1</td>
<td>51.1</td>
<td>0.44</td>
</tr>
<tr>
<td>Year</td>
<td>22.96</td>
<td>2</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

A key question for our study is at which light intensity birds get photostimulated. Work by Menaker (Menaker 1968) showed that about 0.1 lux of light, which corresponds to the light intensity at civil twilight (Daan and Aschoff 1975) induces testicular growth in sparrows. However, in a recent review, Dawson (2015) suggested that testicular growth in starlings kept under natural light conditions resembled more the testicular growth of birds kept under photoperiod as calculated from sunrise to sunset, than as calculated with the civil
photoperiod influences gonadal size of female great tits, but not their timing of laying (Schaper et al. 2012a, te Marvelde et al. 2012). Thus, given the results of this study, we expect that natural selection cannot act on photoperiod sensitivity alone as it seems to not regulate the timing of egg laying. Rather, a change in temperature sensitivity, or concomitant changes in sensitivities to both temperature and photoperiod (and possibly other cues), would be necessary for great tits to restore the phenological synchrony with their main prey. In that scenario, increasing sensitivity to photoperiod would allow an advance of the timing window during which reproduction is possible, and hence gonadal growth, while an increase in temperature sensitivity would allow an advance of the laying period within that new photoperiodic time-window. In the long run it will be necessary to fully understand the mechanisms underlying, and the environmental factors regulating, the phenological trait under study in order to predict the rate of adaptation. More studies in the wild are thus still needed to unravel the mechanisms underlying the fine-tuning regulation of egg laying.

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Competing interests – The authors declare no competing interests.

References


Table 3. Model results for the analysis of the effect of treatment on egg-laying date in the field in 2012, 2013 and 2014. The same analysis was performed on each experimental year separately. Egg-laying date indicate the day that the first egg of the clutch was laid in the wild. All estimates are relative to the control group. Treatment did not have any effect on egg-laying date with exception of year 2012 when the egg-laying date in the behavioural control group was significantly later than the control group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Estimate*</th>
<th>SE</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Clipped</td>
<td>−1.4</td>
<td>2.63</td>
<td>−0.53</td>
</tr>
<tr>
<td>Behavioural control</td>
<td>6.46</td>
<td>2.82</td>
<td>2.29</td>
<td>0.03</td>
</tr>
<tr>
<td>2013</td>
<td>Clipped</td>
<td>3.5</td>
<td>2.18</td>
<td>1.6</td>
</tr>
<tr>
<td>Behavioural control</td>
<td>2.5</td>
<td>2.05</td>
<td>1.22</td>
<td>0.24</td>
</tr>
<tr>
<td>2014</td>
<td>Clipped</td>
<td>−3.4</td>
<td>4.15</td>
<td>−0.83</td>
</tr>
</tbody>
</table>

* contrast to the control group.


